NF-κB Activity Induced by Inflammatory Cytokines under Transplantation Environment Impairs Pancreatic Islet Survival and Intraportal Transplantation Outcome

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To my family

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II Abbreviations

A20	zinc finger protein A20 (also known as TNFAIP3)
AES	amino-terminal enhancer of split
BCL2	B-cell lymphoma protein 2
BCL-xL	also known as BCL2- like 1
BLI	bioluminescence imaging
CAT	catalase
CBP	cyclic-AMP-response element (CREB)-binding protein
c-FLIP,	cellular FLICE inhibitory protein
COX	cyclooxygenase
CXCL	chemokine (C-X-C motif) ligand
DR	death receptor
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
FasL	Fas ligand
GPx	glutathione peroxidase
GSIS	glucose-stimulated insulin secretion
ERK	extracellular signal-regulated kinase
HAT	histone acetyltransferase
HBO	hyperbaric oxygen
HDAC	histone deacetylases
HIF-1α	hypoxia-inducible factor-1α
IBMIR	instant blood mediated inflammatory reaction
IEQ	islet equivalents
ICAM1	intracellular adhesion molecule 1
IDDM	insulin-dependent diabetes mellitus
IFN	interferon
IHC	immunohistochemistry
IκB	inhibitor of NF-κB
IKK	IκB kinase
IL	interleukin

iNOS	inducible nitric oxide synthase
IP10	chemokine (C-X-C motif) ligand 10
LOX	lipoxygenases
MAPK	mitogen-activated protein kinase
MCP1	monocyte chemoattractant protein 1 (also known as CCL2)
MIP2	macrophage inflammatory protein 2
MMP	matrix metalloproteinase
MnSOD	manganese superoxide dismutase (also known as SOD2)
MSK1	mitogen- and stress-activated protein kinase-1
NADPH	nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear Factor κB
NLS	nuclear localization signal
PI	propidium iodide
RHD	Rel-homology domain
ROS	reactive oxygen species
Ser	serine
SMRT	silencing mediator for retinoic acid and thyroid hormone receptor
STZ	streptozocin
T1D	Type 1 Diabetes
TAD	transactivation domain
TF	tissue fact
Trx	thioredoxin
TNF	tumor necrosis factor
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling
VCAM1	vascular cell adhesion molecule 1
VEGF	vascular endothelial growth factor
XIAP	X-linked inhibitor of apoptosis protein

III Summary

Intraportal islet transplantation suffers from low efficiency caused by substantial islet mass loss after transplantation. We hypothesized that this process is induced by an environment of inflammatory cytokines and lack of oxygen under dynamic control of the transcription factor NF-κB. Here we show that cytokine-induced NF-κB activation in islet cells was compromised by hypoxia in a time-dependent manner which resulted in a diversely modulated NF-κB target gene expression profile. Ex vivo islet cell death analysis showed that proinflammatory cytokines alone did not cause evident cell death in pancreatic islet within 24 hours, while the combination of cytokines with hypoxia resulted in a strong induction of cell death that could be blocked dose-dependently by a selective IKK-β inhibitor. Gene expression analysis revealed that cytokine-induced NF-κB activity promoted anti-apoptotic genes under normoxia whereas it repressed these genes under hypoxia, indicating that the pro-apoptotic role of NF-κB activity under hypoxia had resulted from its repression of anti-apoptotic genes. Functional studies were carried out using an intraportal transplantation model where porcine islets were infused to the liver of diabetic NMRI nu/nu mice. Immunohistochemistry study showed that NF-κB activation in the transplanted islets was detectable shortly after transplantation, however, its extent and intensity decreased by time. Systemic NF-κB inhibition by IKKβ inhibitor administration in transplanted animals significantly prolonged islet graft survival and improved islet transplantation outcome. Immunohistochemical studies showed that systemic NF-κB inhibition at current dosage had no obvious impact on islet embolism-induced liver tissue necrosis or neutrophilic granulocyte migration/infiltration in the transplanted islets, indicating that the primary target of NF-κB inhibition is the transplanted islet. Collectively, these findings indicate that while NF-κB has an anti-apoptotic role under normoxia, hypoxic transplantation environment compromises its activity and transforms it to a pro-apoptotic transcription factor in pancreatic islets. We conclude that cell signaling therapy by NF-κB inhibition represents a suitable strategy to improve islet transplantation efficiency.

IV Zusammenfassung

Die intraportale Inseltransplantation ist beeinträchtigt durch geringe klinische Wirksamkeit, da es zu einem schnellen Verlust der transplantierten Zellen kommt. Als wesentlichen Mechanismus hierfür wurde der Transkriptionsfaktor NF-κB vermutet, der für die Steuerung des Zellzyklus und insbesondere für die Apoptose von Betazellen verantwortlich ist. Als erstes fanden wir eine zeitabhängige Einschränkung der zytokin-induzierten NF-κB-Aktivierung in Inselzellen durch Sauerstoffmangel. Im Ergebnis wird das durch NF-κB aktivierte Genmuster durch Hypoxie erheblich verändert. Die Kombination aus proinflammatorischen Zytokinen und Sauerstoffmangel verursachte vermehrten Zelltod in isolierten Langerhansschen Inseln. Das Absterben der Zellen konnte durch die Anwesenheit von IKK-β Inhibitor konzentrationsabhängig gebremst werden. Zytokine in normaler Sauerstoffumgebung induzierten keinen Zelltod innerhalb einer Beobachtungszeit von 24 Stunden. Die zytokininduzierte Aktivierung von NF-κB führte zur vermehrten Expression der anti-apoptischen Gene BCL-xL, c-FLIP and Survivin. Im Sauerstoffmangel wurde dieser Vorgang blockiert und es dominierten apoptotische Gene. Deutliche Hinweise einer nahezu sofortigen NF-κB Aktivierung fanden sich immunhistochemisch in Schweineinseln, die diabetischen Nacktmäusen intraportal infundiert worden waren. Diese Aktivierung war nur kurz nach Transplantation festzustellen, aber ihre gezielte Inhibition durch den IKK-β Antagonisten führte zu einer signifikanten Verbesserung des langfristigen Transplantationserfolges. Es konnten keine Effekte der IKK-b Hemmung auf andere Zellen (Leber, neutrophile Granulozyten) außer Inselzellen festgestellt werden. Zusammenfassend fand sich eine anti-apoptotische Funktion von NF-κB bei normalem Sauerstoffdruck, aber eine pro-apoptotische Wirkung im Sauerstoffmangel. Weitere Untersuchungen müssen zeigen, ob die gezielte und kurzfristige Hemmung von NF-κB eine neue klinisch anwendbare Therapieform zur Vermeidung von frühem Transplantatverlust wird.

1. Introduction

1.1 Type 1 Diabetes

1.1.1 The islets of Langerhans

The islets of Langerhans are the micro-organs that resume the endocrine pancreas physiology. They are the cell clusters scattered throughout the pancreas. The human pancreas contains about one million islets corresponding to 1-2% of the total pancreas mass, each composed of about two to three thousands endocrine cells (1). The islets consist of 5 major endocrine cell types (2). *1)* β-cells, constituting 60-80 % of the islet cells, which are responsible for pulsatile insulin release. *2)* α-cells, constitute 10-20 % of the islet cells and produce glucagon, which promotes glycogen breakdown in the liver and thereby increases glucose concentrations. *3)* δ-cells, constitute 3-10 % of the islet cells and secrete somatostatin, which inhibits the release of other hormones including insulin and glucagon. *4)* PP-cells, constituting 0,5-1% of the islets cells and secreting pancreatic polypeptide, which has been shown to stimulate gastric secretion and *5)* Epsilon cells, constituting 0,5-1 % of the islets cells and producing the hormone ghrelin, regulating appetite and promoting the release of growth hormone. Besides the endocrine cells, endothelial cells, neurons and immune cells (such as dendritic cells) also reside in the islets to a lesser extent.

1.1.2 Type 1 Diabetes

Type 1 diabetes (T1D) was previously called insulin-dependent diabetes mellitus (IDDM) or juvenile-onset diabetes. T1D is an autoimmune disease where insulin producing β-cells in the islets of Langerhans are mistakenly destroyed by the immune system. Both genetic risk factors and environmental triggers have been implicated in the pathogenesis of T1D. It usually strikes in childhood, adolescence, or young adulthood, and lasts a lifetime. T1D currently afflicts approximately 5 million individuals in the world and about 300,000 subjects in Germany (3). The incidence of type 1 diabetes in children younger than 15 years is increasing. Prevalence of T1D under age 15 years is predicted to rise from 94,000 in Europe in 2005, to rise by 70% to 160,000 in 2020 (4).

1.1.3 Treatments for T1D

The therapy of choice for type 1 diabetes is tight regulation of glucose with exogeneous insulin administration which has remained the standard of care for T1D for nearly 90 years. While

exogenous insulin therapy improves the quality of life and decreases the risk of diabetic complications, hypoglycemic episodes remain a limiting and potentially dangerous side effect of intensive exogenous insulin therapy. This factor is especially problematic in about 10% of diabetic patients who demonstrate poor glucose control or who suffer from hypoglycemic unawareness (5).

1.2 Islet transplantation as a potential cure for T1D

For those T1D patients threatened with insufficient glycemic control or hypoglycemic unawareness, β-cell replacement, either by whole organ pancreas transplantation or by allogeneic islet transplantation, could restore accurate and prompt glucose regulation. Although whole organ pancreas transplantation is now considered the best option and can offer a sustained normoglycemic state in these patients, it is associated with the risks of any major surgical procedures and higher surgical morbidity. On the contrary, islet transplantation is a minimally invasive method of achieving insulin independence in which islets can be perfused percutaneously into the liver via the portal vein in local anesthesia (6). In addition, it does not require management of the exocrine secretions of the pancreas, avoids the possible complications related to enzymes production by the exocrine cells, as experienced in whole organ pancreas transplantation (7). However, islet transplantation is associated with less optimal outcomes and might require retransplantations (5).

1.2.1 The history

The success of islet transplantation has set an excellent model of bench to bedside translational research, from a hypothesis to clinical application (8).

The first attempt of islet transplant as a treatment for diabetes could be considered that of Dr. Watson-Williams in 1894, when he transplanted a mixture from sheep pancreas under the skin of a young patient with end-stage diabetes. The patient died a few days later due to diabetic coma. However, significant progress has been achieved in the field of islet transplantation ever since then (9; 10).

After numerous animal experimental investigations, in 1990, the first successful series of human islet transplantations was reported by the Pittsburgh group: prolonged insulin independence was achieved with a steroid-free immunosuppressive regimen based on the agent FK506 (11). This was the first unequivocal evidence of long-term reversal of diabetes after human islet transplantation, with insulin independence lasting up to five years (1). Six years later, in 1996, by including the use of endotoxin-free reagents and improved peritransplant recipient treatment, the Giessen group headed by Dr. Bretzel and Dr. Hering was able to achieve 100% initial graft

function and a 40% insulin independence at 1 year after transplantation (12). In 1999 in Edmonton, a landmark for clinical islet transplantation was initiated by Shapiro et al. who reported insulin independence in seven out of seven consecutive T1D patients treated with islet transplantation using a glucocorticoid-free immunosuppressive regimen (13). Since this exciting report, clinical islet transplantation activity has dramatically increased. In the most experienced centers, approximately 80% of patients treated with islet transplantation could achieve insulin independence within the first year after transplantation (14).

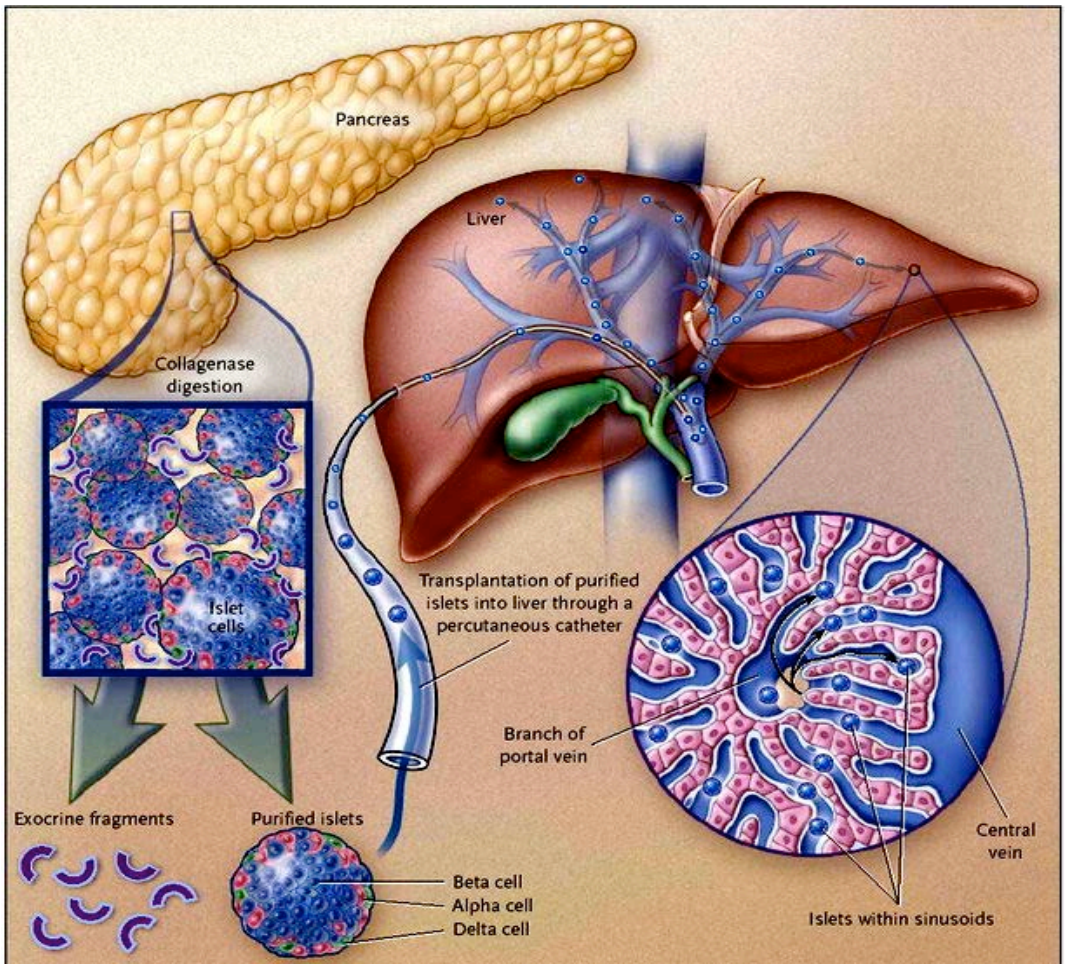

Figure 1-1. The process of clinical islet transplantation. The donor pancreas is digested with collagenase to free the islets from surrounding exocrine tissue. The freed islets, containing mostly beta and alpha cells, are purified by density–gradient centrifugation to remove remaining exocrine cellular debris. The purified islets are infused by gravity into a catheter that has been placed percutaneously through the liver into the portal vein, whence they travel to the liver sinusoids. Adapted from *Robertson 2004* (6).

Islet transplantation can offer numerous benefits to the patients, including stabilized glucose metabolism, restoration of symptom awareness, sustained decrease in severe hypoglycemic episodes, and reduction of hemoglobin A1C levels, which together confer an improved life quality of the recipients. Even when the graft function declined and the patient needs external insulin

administration again, long-term functioning islet graft could be detected for a long time after transplantation by C-peptide levels. In addition, there is evidence of a possibly slower process of diabetic complications (15).

1.2.2 Factors influencing the survival of the transplanted islet

Currently, islet transplantation remains an experimental procedure which is on a crossroad. Its application is limited only to the most severe diabetic patients who suffer from hypoglycemic unawareness. Several factors, such as limited supply of donor pancreases, complication of the procedure related to long-term immunosuppression, could be responsible for the limitation. However, a key factor limiting its widespread application is the requirement of 10,000 islet equivalents (IEQ)/kg in most recipients, generally derived from two or more cadaveric donors (16). On the other hand, there are strong evidences that more than 50% of the transplanted islets are destroyed in the immediate post transplant period (10; 17). Both non-immune and innate immunological factors have been indicated in the early graft loss or primary non-function of the transplanted islets (18-20).

1.2.2.1 Hypoxia

Pancreatic islets have evolved to survive best in highly oxygenated blood and in nutrient rich surroundings. While they only constitute 1-2% of the total pancreatic volume, the islets receive up to 10% of the whole pancreatic blood flow at basal level and could further increase their blood flow up to 15% of the total pancreatic blood flow in response to glucose stimulation (21). The highly enriched blood flow in the islets is supported by a dense vascular network. It has been reported that each β-cell within the islets is surrounded by at least one capillary endothelial cell, which facilitates proper glucose sensing and the distribution of secreted hormones (22). Accordingly, the tissue oxygen tension in pancreatic islets has been recorded as approximately 40 mmHg which is in close equilibrium to that of venous blood (23). Taking together, these reports reflect a high demand of oxygen for islet metabolism and function.

Unfortunately, pancreatic islets prepared for transplantation suffer from hypoxia in each step of the procedure, starting from the very beginning of the procurement and preservation of the donor pancreas. The following isolation process, which itself is a big stress, destroys all the microvasculature connection and neural regulation in the isolated islets and subsequently make them depend on diffusion for oxygen and nutrient supply during the culture and early post-transplantation stage. In the case of intraportal islet transplantation, the situation is deteriorated by the occurrence of thrombus formation surrounding the islets and increasing the oxygen diffusion

distance from red blood cells (24). In contrast to its native residence environment in the pancreas where blood flow is sufficient and highly oxygenated (pO2 of 40 mmHg), it has been observed that the pO2 of the portal circulation is typically 10–15 mmHg (16) and the intraportally transplanted islets only possess a pO2 of 8–10 mmHg (25). Moreover, even after the conclusion of islet revascularization which has been supposed to finish within 2 to 4 weeks after transplantation, the low oxygenation of intraportally transplanted islets still prevails due to insufficient and poor revascularization at the transplantation site (25; 26).

On the other hand, pancreatic islets are extraordinarily sensitive to hypoxic injuries, probably due to the very low expression of antioxidant enzymes including cytoplasmic Cu/Zn superoxide dismutase (SOD), the mitochondrial Mn SOD, catalase (CAT) and glutathione peroxidase (GPx), essentially all the major cellular antioxidant enzymes (27). Therefore, it is not surprising to find that substantial early graft loss occurs after transplantation which is largely ascribed to hypoxia causing islet cell death in both apoptosis and necrosis forms (28-30). Accordingly, the experimental procedures to improve islet graft oxygen availability or to improve islet revascularization process have been demonstrated to be beneficial for the transplanted islets. Hyperbaric oxygen (HBO) therapy in the peri-transplantation stage has been shown to improve the survival and function of islet grafts (29; 31; 32). Increasing the antioxidant ability of the transplanted islets, by means of overexpression of the oxidant metallothionein (33), through the use of the catalytic antioxidant probe AEOL10150 (34), or by administration of pyruvate (35) has been shown to reduce islet vulnerability and improve islet transplantation outcome. Improving graft revascularization, either by elevating VEGF production (36) or by inhibition of the angiostatic factor thrombospondin-1 (37), has been demonstrated to improve islet graft oxygenation and function. Collectively, these data indicate that oxygen availability is a key factor in determining the survival of the transplanted islets and the outcome of islet transplantation.

1.2.2.2 The IBMIR

Immediately after intraportal transplantation, the islets are exposed to a cascade of inflammatory reactions, namely "instant blood mediated inflammatory reaction", IBMIR.

The IBMIR is a thrombotic/inflammatory reaction which happens immediately when isolated islets get in direct contact with the blood. It is characterized by the activation of coagulation and complement system, platelet aggregation, and infiltration of the islet grafts by neutrophilic granulocytes and monocytes (24; 38). Tissue factor (TF), a 47 kD transmembrane glycoprotein that acts both as a receptor and as a cofactor for the cleavage of factor VII to VIIa and for the activity of factor VIIa in the TF (extrinsic) pathway of coagulation, was found to be synthesized

and secreted by pancreatic islets and its blockage abrogated IBMIR in a loop tube system (24). Therefore, TF is supposed to be the trigger of IBMIR (24; 39). Following the stressful isolation process, human islets have been shown to release tissue factor which in turn upregulates thrombin, leading to platelet activation and binding to the surface of the islets and the subsequent formation of a fibrin capsule around the islet and disruption of the islet morphology (40). Accordingly, interference with TF with a low-molecular weight factor VIIa (FVIIa) inhibitor (Ro69) or with a monoclonal anti-TF antibody (CNTO859) eliminated the IBMIR in the tube system (39) and enhanced islet engraftment in a mouse model (41).

Although the exact mechanisms of IBMIR remain to be fully elucidated, it has been described as one of the most important causes of early islet loss after transplantation (38; 42-44). The infiltrating inflammatory cells are directly cytotoxic to the islet graft as neutrophil degranulation can lead to enzymatic digestion of target cells, and both neutrophils and macrophages are major phagocytes (45). Importantly, these cells can generate superoxidase to form reactive oxygen species (ROS) that can directly damage islets which are exquisitely susceptible to oxidative stress (46). Furthermore, these cells produce inflammatory cytokines such as interleukin-1 (IL-1), tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ) which deteriorate islet survival by triggering cellular inflammatory responses.

1.2.2.3 Proinflammatory cytokines

Cytokines are small signaling proteins that are produced from one or more of the different leucocytes: lymphocytes, monocytes, dendritic cells, macrophages and granulocytes. Also other cell types have been shown to secrete cytokines, epithelial cells produce IL-1 and endothelial cells can express IL-1 and IL-6 in addition to a number of chemokines. They are the major tools by which the different immune cells communicate. There are a large number of cytokines with a broad array of actions, among which TNF-α, IL-1 and the IFNγ are proinflammatory ones that have been proposed as possible mediators of pancreatic β-cell destruction (47).

In the early post-transplantation stage, the main sources of proinflammatory cytokines are the infiltrating immune cells attracted by IBMIR. These cytokines activate death programs in β-cells by upregulating Fas expression and activation of caspases (48-50). On the other hand, these cytokines induce in parallel the anti-apoptosis machineries in β-cells, among which the transcription factor NF-kappaB (NF- κB) signaling pathway was demonstrated to be key regulator (51).

Interestingly, the ischemic liver tissue has been implicated as another major source of inflammatory cytokines following intraportal islet transplantation in a recent study (52). It is

demonstrated that the transplantation of islets resulted in increased production of the inflammatory cytokines IL-1β and TNF-α in the liver tissue at both mRNA and protein levels, which could be prevented by ischemic-preconditioning that is associated with significantly improved function of intraportally transplanted islet grafts. This study highlights the important roles of both the hypoxic portal vein environment and the inflammatory cytokines in the early destruction of the transplanted pancreatic islet.

1.2.2.4 Other factors

Among those transplanted islets that survived from the early destruction by hypoxia and IBMIR, a progressive decline of function has been observed. Data from The Collaborative Islet Transplant Registry (CITR), collected across clinical islet programs, show that while 70% of patients achieve insulin independence within the first year, the percentage of patients who remain insulin-independent is close to 35% by the third year. At 9 years, even among the original Edmonton patients who achieved 100% insulin independence in the first year, only 10% or less maintain successful graft function. (5; 53).

The progressively declining islet graft function indicates that there are other factors which influence the long-term survival and function of transplanted islets. The exact cause for progressive islet dysfunction is not completely defined yet. However, it reflects most likely a multifactor process, including not only acute and chronic allo-rejection, but also the metabolic exhaustion of islet graft, the recurrence of autoimmunity and the chronic toxicity of immunosuppressive drugs exerting diabetogenic and antiproliferative effects to the islets (16; 54).

1.3 NF- κB signaling pathway

NF- κB was identified as a nuclear protein binding to enhancer elements of the κ light chain gene in 1986 (55). In mammals, the NF-κB family is composed of five members: RelA (p65), RelB, Rel (c-Rel), NF-κB1 (p50 and its precursor p105) and NF-κB2 (p52 and its precursor p100) (56). These proteins form homodimeric and heterodimeric complexes to regulate gene expressions. Today, NF- κB is known as an inducible transcription factor relevant for the regulation of inflammation, immunity, cell death, proliferation and developmental processes (57).

1.3.1 NF- κB activation pathways

Three major pathways leading to the activation of NF- κB have been identified so far, including the canonical (classical), alternative (non-canonical) and atypical pathways (Figure 1-2).

The canonical or classical NF- κB pathway can be activated by proinflammatory cytokines, T cell costimulation, Toll-like receptors and products of bacterial or viral origin. The canonical NF-κB pathway mainly leads to the generation of p65-p50 dimers and plays a major role in innate immunity and inflammation. IKKβ plays a central role in this pathway to phosphorylate IκBα at Ser 32 and Ser 36, allowing its ubiquitination and subsequent degradation by the 26S proteasome (58). This leads to an unmasking of the p65 nuclear localization signal (NLS) and allows NF-κB nuclear entry, as well as DNA-binding, which can then activate target gene transcription.

The alternative NF- κB pathway is activated by a subset of NF-κB inducers, including lymphotoxin-β, BAFF and CD40 ligand. This pathway occurs predominantly in B cells, where the p52/RelB heterodimer is the major NF-κB dimer (59). As alternative NF- κB signaling primarily causes the release of p52/RelB heterodimers, only certain defined subsets of NF- κB target genes are activated (60). The alternative NF-κB pathway is NEMO-independent and relies solely on the sequential activation of NIK and IKKα (61).

The atypical NF- κB cascade is activated in response to atypical stimuli, such as DNA damage, oxidative stress, doxorubicin or UV-light, which trigger a slow and weak activation of NF-κB (62; 63). In contrast to the canonical or alternative ones, the atypical NF-κB signaling pathway is only partially elucidated.

It must be noted, however, that there is a basal NF-κB transcriptional activity which might be regulated by the constitutive shuttling between cytoplasm and nucleus of NF-κB, IκB, and the IKKs-with a predominant cytoplasmic location in resting cells (64).

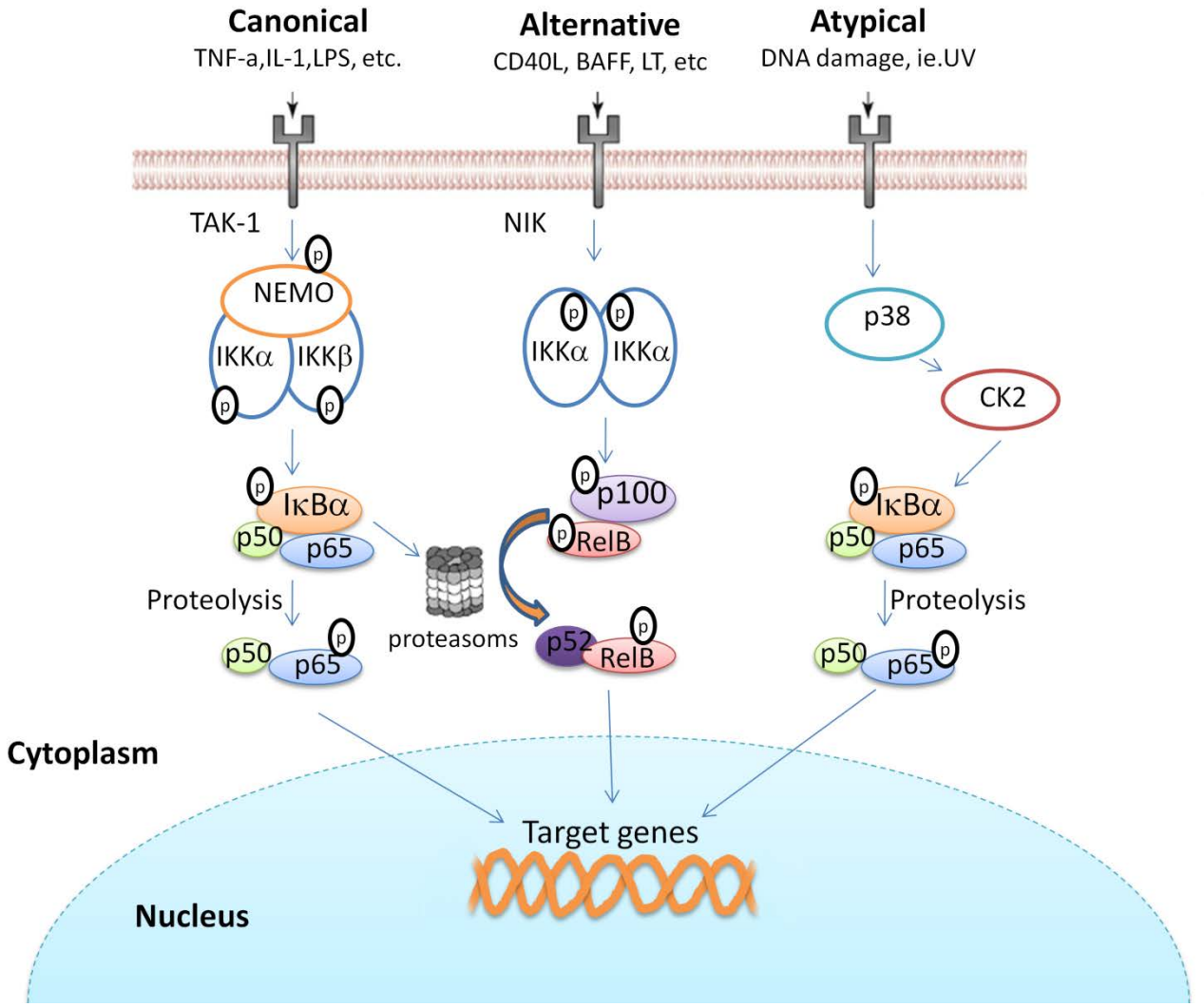

Figure 1-2: Signaling pathways leading to the activation of NF- κB. *(A)* The canonical pathway is activated by a large number of agonists, such as TNF, IL-1 and LPS, through the binding of various cytokine receptors or TLRs and stimulate the IκB kinase (IKK) complex (IKKα-IKKβ-NEMO) to phosphorylate IκBα and promote its degradation via the proteasome pathway. Then, the heterodimer p50-p65 is released and migrates to the nucleus where it binds to specific κB sites and activates a variety of NF- κB target genes. *(B)* Alternative NF-κB activation pathway. NIK and IKKα are sequentially activated, which then induces the processing of the inhibitory protein p100. p100 proteolysis releases p52 which forms heterodimers with RelB. This pathway is NEMO independent and relies on IKKα homodimers. *(C)* The atypical pathway is triggered by atypical stimuli, such as DNA damage. Signals proceed via a p38 MAP kinase and CK2 to phosphorylate and degrade IκBα via an IKK-independent pathway. Subsequently, freed NF- κB moves into the nucleus to activate its target genes. Note that genotoxic stress can lead to IKK activation via a SUMO/ATM-dependent pathway (not shown). Modified from *Viatour et.al 2005* (65).

1.3.2 NF- κB target genes

NF- κB is a central player in the regulation of both inflammatory response and cell survival. It carries out its roles by regulating the numerous genes involved in many cellular processes, including cell division, cell survival, differentiation, immunity and inflammation. Once activated, the freed NF- κB interacts with nuclear import machinery and translocates to the nucleus, where it binds to the promoter of its target genes to initiate transcription. The active NF- κB transcription factor promotes the expression of over 150 target genes involving in different cellular process including inflammatory response, cell life or death decision, angiogenesis and transcription regulation (Figure 1-3).

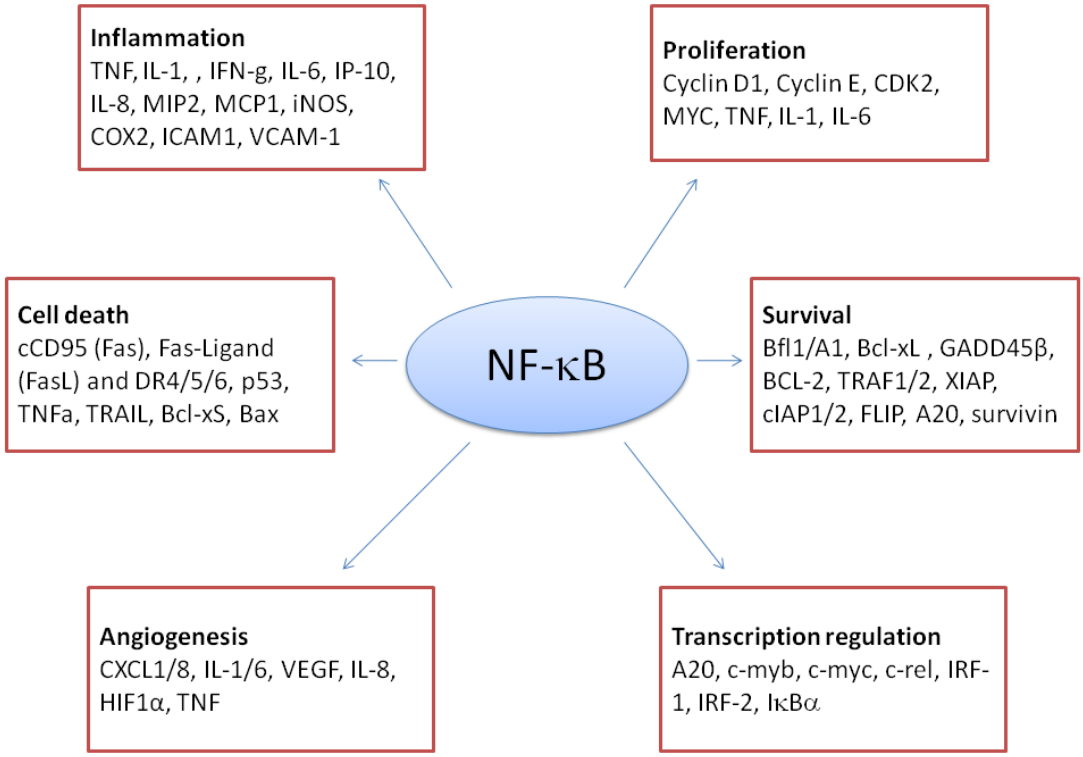

Figure 1-3 NF- κB target genes. NF-κB activation affects the transcription of genes involved in cell life or death decision, angiogenesis, inflammation and other transcription factors. Adapted and modified from *Pahl 1999* (66), *Dutta 2006* (67) and *Baud 2009* (68).

While NF- κB is typically regarded as a potent activator of gene expression, it can be turned to an active repressor of gene expression under certain circumstances. It has been demonstrated that, under UV-C or other DNA damaging agents' treatment, NF- κB not merely lost its ability to induce transcription, but rather acquired a dominant transcriptional repressor function for the anti-

apoptotic genes, an effect that was particularly clear when UV or doxorubicin treatment was combined with TNF treatment (69). How RelA is converted from gene activator to gene repressor remains to be fully elicited, however, post-translational modifications of RelA have been described that alter its transcriptional properties, the most important ones being phosphorylation and acetylation. Instead of being phosphorylated and subsequently interact with its coactivator proteins the cyclic-AMP-response element (CREB)-binding protein (CBP)/p300 and become a activator of gene transcription, RelA/p65 is associated with the corepressors histone deacetylases (HDACs) via altered post-translational modifications under these circumstances and turn itself into a repressor of anti-apoptotic genes to promote cell death (Figure 1-4) (63; 69; 70).

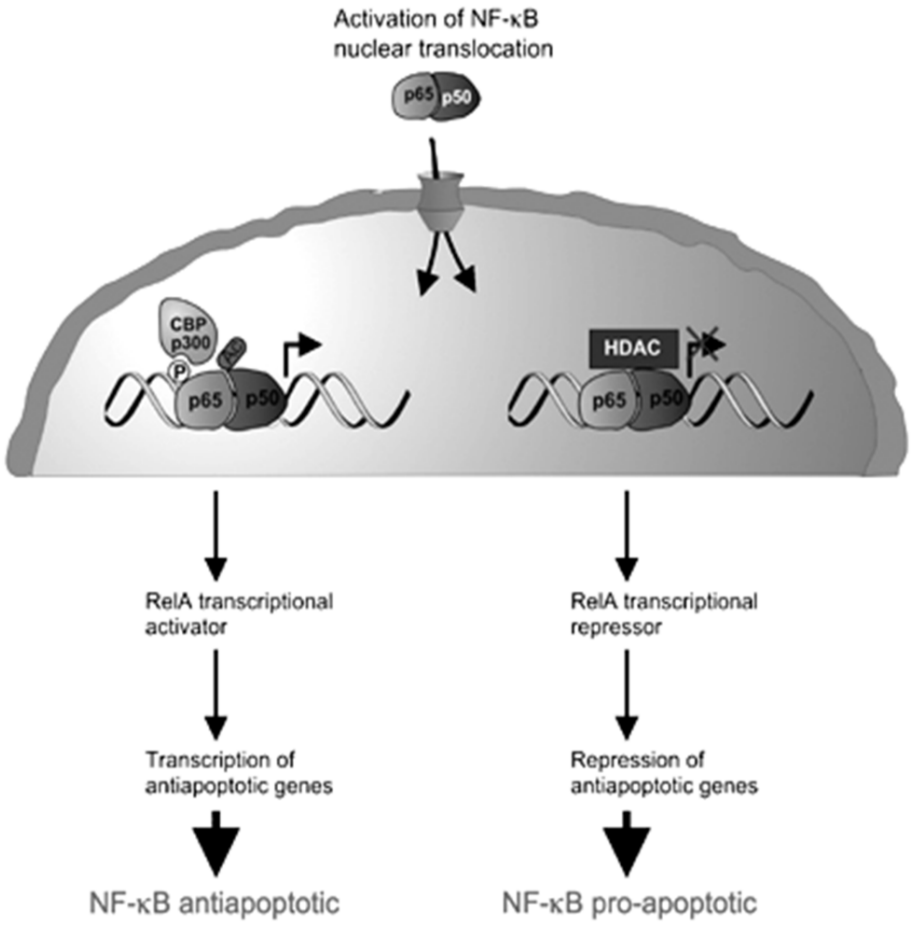

Figure 1-4 Life and death decisions by NF-κB/RelA. Upon nuclear translocation, the transcriptional potential of NF-κB could be modulated by post-translational modifications of its subunits. Following the recruitment of its coactivator proteins such as CBP/p300 and subsequent RelA acetylation, NF- κB activate the transcription of antiapoptotic gene expression and promotes cell survival. However, under certain circumstances NF- κB can repress expression of certain target genes by its interaction with corepressors (histone deacetylases, HDAC). Repression of antiapoptotic target genes converts NF- κB in a proapoptotic factor. Adapted from *Janssens 2006* (63).

1.3.3 NF- κB in pancreatic islets

In contrast to the well-established anti-apoptotic role in most cell types, the role of NF- κB in islet cells has been controversial as both pro- and anti-apoptotic functions were reported.

In vitro blockade of NF-κB has been shown to be protective for islet cells. Infection of human islets with an adenoviral vector encoding a non-phosphorylatable, non-degradable variant of IκBα protected the islets from IL-1 induced function impairment, nitric oxide production and Fas-mediated Apoptosis (71). Similarly, infection of rat β-cells with a nondegradable mutant form of IκBα inhibited cytokine-stimulated nuclear translocation and DNA-binding of NF-κB, prevented cytokine-induced gene expression of inducible nitric oxide synthase (iNOS) and Fas, and finally improved β-cell survival after IL-1β plus IFN-γ treatment, mostly through inhibition of the apoptotic pathway (72). In a recent study, islets expressing a degradation-resistant IκB (DeltaNIkappaBalpha) were resistant to the deleterious effects of IL-1β and IFN-γ *in vitro*; and conditional and specific NF-κB blockade in β-cells *in vivo* resulted in nearly complete protection against multiple low-dose streptozocin-induced diabetes in mice (51). Collectively, these findings are in agreement with a pro-apoptotic role of NF-κB in β-cells.

On the other hand, an anti-apoptotic function of NF-κB has also been reported. It has been demonstrated that NF-κB activation in β-cells leads to induction of the anti-apoptotic gene A20 which results in the protection from TNF-induced cell death (73). Interestingly, β-cells expressing a nondegradable form of IκBα have been shown to be more susceptible to TNF-α plus IFN-γ but more resistant to IL-1β plus IFN-γ, and that inhibition of β-cell NF-κB accelerated the development of autoimmune diabetes in NOD mice, indicating that the dominant effect of NF-κB is prevention of TNF-induced apoptosis under conditions that resemble autoimmune type 1 diabetes (74). Recently, overexpression of the NF-κB subunit c-Rel in human islet has been reported to provide a protection against cell death induced by cytokine mix (TNF-α plus IL-1β), streptozocin (STZ) or H_2O_2 (75).

Coming to islet transplantation, however, only two published observations addressed directly the role of NF-κB on islet graft survival in animal models. Using mice with targeted disruption of the c-Rel gene (c-Rel-/-) as recipients of H-2 mismatched islet allografts, Yang *et al.* found superior islet graft survival and decreased intra-islet infiltration and intra-graft expression of cytotoxic attack molecules perforin and granzyme B in the c-Rel-/- recipients (76). Grey *et al.* demonstrated that overexpression of A20, a zinc finger protein that inhibits NF-κB activation, in the transplanted islets preserve functional islet cell mass and protect islet graft from apoptosis in a

syngenic islet transplantation model (77). Both reports enlighten a protective role of NF-κB blockage either in the recipients or the transplanted islets, suggesting that systemic NF-κB inhibition in the transplanted animals may have an additive protective effect on islet engraftment.

Collectively, there are some major drawbacks of these investigations when the clinical islet transplantation comes to the main concern:

1) Most of the *in vitro* investigations were carried out under normal oxygen tension conditions, whereas the transplanted islets are subjected to a markedly decreased oxygen tension environment irrespective of the transplantation site (25).
2) Almost all the investigations utilized gene manipulation techniques which are not applicable for clinical application.
3) Using gene manipulation techniques to investigate the role of NF-κB could have potentially unwanted side effects. For instance, the frequently used nondegradable super-repressor form of IκB, IκB superrepressor (IκB-SR), has been shown to affect non-NF-κB pathways such as nuclear import/export (78).

Nevertheless, as different conditions have been applied in each report where different roles of NF-κB were concluded, these reports highlight the complexity of NF-κB's activity in pancreatic islets depending on its activation context. As pancreatic islets are transplanted into the portal vein system where they suffer from the uniquely combined stress of hypoxia and proinflammatory microenvironment, the role of NF-κB in intraportal transplanted islet graft survival and function remains to be elicited.

2. Aim of the study

The general aim of this thesis was to study the role of NF-κB in pancreatic islet survival and function in the context of intraportal islet transplantation. In particular, we sought to study its roles in pancreatic islets under combined hypoxia and cytokines stress *in vitro* and in intraportal transplantation *in vivo*.

Thus, the specific aims of this thesis include:

1. To evaluate the impact of hypoxia on NF- κB signaling and target gene expression.
2. To evaluate the role of cytokine-induced NF- κB activity in islet survival under normoxic and hypoxic conditions.
3. To evaluate the effect of systemic NF- κB inhibition on islet graft survival and function after intraportal transplantation.

3. Materials and Methods

3.1 Materials

3.1.1 Chemicals

Product	Manufacturer
Acetic acid	Roth
Agarose	Invitrogen
Ammonium persulfate (APS)	Bio-Rad
Aprotinin	Sigma
Boric acid	Fluka
BMS-345541	Sigma
Bovine serum albumin (BSA)	Sigma
Calcium chloride ($CaCl_2$)	Sigma
L-Cysteine	Sigma
DMEM 41966 Medium	Invitrogen Gibro
Dimethyl sulfoxide (DMSO)	Fluka
1,4-Dithiothreitol (DTT)	Biocheringer
ECL Western Blotting Substrate	Thermo Scientific Pierce
EGTA	Fluka
Ethanol	Merck
Ethylendiaminetetraacetic acid (EDTA)	Fluka
Fetal calf serum (FCS)	bio west
Ficoll 400	Serva
Formaldehyde	Roth
X-gal	AppliChem
Glucose	Sigma
Glycerol	Acros Organics
β-Glycerophosphate	Acros Organics
Glycine	Roth
Guanidine-HCl	Roth
HEPES	Sigma
Hoechst 33342	Sigma

Hydrochloric acid 30% (HCl)	Merck
Imidazole	Fluka
IPTG	AppliChem
Isopropanol	Merck
Leupeptin	Sigma
Lithium chloride (LiCl)	Merck
Magnesium chloride ($MgCl_2$)	Merck
Magnesium sulfate ($MgSO_4$)	Merck
β-Mercaptoethanol	Fluka
Methanol	Merck
L-Methionine	Sigma
Nonidet P40	Roche
Phenylmethanesulfonyl fluoride (PMSF)	Fluka
Potassium chloride (KCl)	Fluka
Potassium dihydrogen phosphate (KH_2PO_4)	Fluka
di-Potassium hydrogen phosphate (K_2HPO_4)	Fluka
Tissue-Tek O.C.T. Compound	Sakura
Potassium hydroxide (KOH)	Merck
Sc-514	Merck
Skim milk powder	Merck
Sodium azide (NaN_3)	Fluka
Sodium chloride (NaCl)	Roth
Sodium dodecyl sulfate (SDS)	Bio-Rad
Sodium fluoride (NaF)	Roth
Sodium dihydrogen phophate (NaH_2PO_4)	Acros organics
di-Sodium hydrogen phosphate (Na_2HPO_4)	Merck
Sodium hydroxide (NaOH)	Fluka
Sodium orthovanadate (Na_3VO_4)	Sigma
N,N,N',N'-Tetra-methyl-ethylenediamine (TEMED)	Bio-Rad
Thymidine	Sigma
Tris-base	Acros organics
Triton X-100	Sigma
Trypsin/EDTA	Invitrogen Gibro
Tryptone/peptone	Roth

Tween 20	Merck
Tween 80	Sigma

3.1.2 Antibodies

Primary Antibody	**Dilution**	**Company**
Rabbit anti-β-actin, polyclonal	1:1000 (WB)	Abcam
Rabbit anti-p65 (C-20), polyclonal	1:1000 (WB) 1:150 (IF)	Santa Cruz Biotech
Mouse anti-phospho-IκBα ($Ser^{32/36}$), monoclonal	1:2000 (WB)	Cell Signaling Tech
Rabbit anti-phospho-p65 (Ser^{536}),polyclonal	1:1000 (WB)	Cell Signaling Tech
Guinea Pig Anti-insulin, polyclonal	1:500 (IF)	DAKO
Rat Anti-Mouse Ly-6G (Gr-1), monoclonal	1:150 (IF)	eBioscience
Rat Anti-Mouse CD11b (M1/70.15), monoclonal	1:150 (IF)	ImmunoTools
Secondary antibody		
Peroxidase-conjugated Goat anti-mouse-IgG	1:5000 (WB)	Dianova
Peroxidase-conjugated Goat anti-rabbit-IgG	1:5000 (WB)	Dianova
Rhod Red-X-APure Donkey Anti-Guinea Pig IgG	1:400 (IF)	Jackson ImmunoResearch
Rhod Red-X-APure Donkey Anti-Rabbit IgG	1:400 (IF)	Jackson ImmunoResearch
FITC-AffiniPure Donkey AntiGuinea Pig IgG	1:400 (IF)	Jackson ImmunoResearch
FITC-AffiniPure Donkey Anti-Rabbit IgG	1:400 (IF)	Jackson ImmunoResearch
Rhod Red-X-APure Donkey Anti-Rat IgG	1:400 (IF)	Jackson ImmunoResearch

3.1.3 Kits

Kits	**Manufacturer**
ABsoluteTM QPCR SYBR Green Mixes	Thermo ABgene
Power SYBR Green PCR Master Mix	Applied Biosystem
SuperScript® III Reverse Transcriptase	Invitrogen
RNeasy Mini	Qiagen
BCA Protein Assay Kit	Thermo Scientific Pierce
Mouse TNF-α ELISA kit	R&D Systems
Porcine insulin ELISA kit	DRG Instruments

In Situ Cell Death Detection Kit, Fluorescein	Roche

3.1.4 Recombinant proteins

Recombinant Proteins	Source
Recombinant Human TNF-α	R&D Systems
Recombinant Human Il-1β	R&D Systems
Recombinant Human IFN-γ	R&D Systems

3.1.5 Primer sequences for real-time PCR

Primer sequences for murine genes (5'→3')	
β-actin_for	GAGATTACTGCTCTGGCTCCTA
β-actin_rev	TCATCGTACTCCTGCTTGCT
F3_for	CAAACGCCCCAAAGTTTTTA
F3_rev	GTGCTTGAGCCTTTCCGATA
FasL_for	CCCCAGTACACCCTCTGAAA
FasL_rev	GCTGGTTGTTGCAAGACTGA
Ins2_for	CACCTGGTGGAGGCTCTCT
Ins2_rev	ACCCAGCTCCAGTTGTGC
ICAM1_for	GGAGACGCAGAGGACCTTAAC
ICAM1_rev	CGCTCAGAAGAACCACCTTC
IκBα_for	GGAGCACTTGGTGATTTG
IkBa_rev	CTTGGTAGGTTACCCTGTTG
IP10_for	AATCATCCCTGCGAGCCTAT
IP10_rev	TTTGGCTAAACGCTTTCATT
MCP1_for	TTAAAAACCTGGATCGGAACCAA
MCP1_rev	GCATTAGCTTCAGATTTACGGGT
Tnf_for	GAACTGGCAGAAGAGGCACT
Tnf_rev	AGGGTCTGGGCCATAGAACT
VCAM-1_for	AGTTGGGGATTCGGTTGTTCT
VCAM-1_rev	CCCCTCATTCCTTACCACCC

VEGFc_for	CCTGAATCCTGGGAAATGTG
VEGFc_rev	CACAGCGGCATACTTCTTCA

Primer sequences for porcine genes (5'→3')

Bax-α_for	TAACATGGAGCTGCAGAGGA
Bax-α_rev	AAAGTAGAAAAGCGCGACCA
BCL2_for	ATGTGTGTGGAGAGCGTCAA
BCL2_rev	CCTTCAGAGACAGCCAGGAG
BCL-xl_for	GAAACCCCTAGTGCCATCAA
BCL-xl_rev	GGGACGTCAGGTCACTGAAT
C-FLIP_for	GAGCAAGCCCCTAGGAATCT
C-FLIP_rev	GTCTTGGTGTTGGGGCATAC
HIF1α_for	GCTTGCTCATCAGTTGCC
HIF1α_rev	GCCTTCATTTCATCTTCAATATCC
RPL13A_for	CCTTCCTCCGCAAGCGCATGAA
RPL13A_rev	ACCGTCCGCCAGAAGATGCG
survivin_for	CCTGGCAGCTCTACCTCAAG
survivin_rev	TCTTCTATGGGGTCGTCGTC
VEGFA_for	CCTTGCCTTGCTGCTCTAC
VEGFA_rev	GGTTTCTGGTCTCCTTCTGC
VEGFR2_for	GAAATGACACTGGAGCCTACAAG
VEGFR2_rev	TCCATGCTGGTCACTAACAGAAG

3.1.6 Animals

Male inbred athymic NMRI nu/nu mice (12 weeks old) were purchased from Charles River (Sulzfeld, Germany). Animal research was approved by Regional Commission Giessen, Germany under the code number GI20/11-Nr.15/2006. Animal husbandry was performed according to the German Animal Welfare Law as published in the latest version under http://bundesrecht.juris.de/tierschg.

3.1.7 Cell Lines

The pancreatic beta cell line MIN6 (mouse insulinoma, 6. subclone) was kindly provided by Prof. Sigurd Lenzen, Institute of Clinical Biochemistry, Medizinische Hochschule Hannover, Germany.

3.2 Methods

3.2.1 Cell Culture

3.2.1.1 MIN6 cell culture

MIN6 insulinoma cells were cultured in 25-mmol/l glucose Dulbecco's modified Eagle's medium (DMEM), supplemented with 15% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mmol/L-glutamine, and 50 μmol/L β-mercaptoethanol at 37°C under conditions of 95% air and 5% CO_2.

The cells were passaged by trypsinization. Cells were washed with pre-warmed Dulbecco's PBS (without Ca^{2+} and Mg^{2+}, PAA), and then treated with trypsin/EDTA solution. After incubation at room temperature for 3-5 minutes, the detached cells were diluted with warm DMEM culture medium, centrifuged and split into new flasks.

For cell freezing, the concentrated cell suspension was diluted 1:1 with freshly prepared freezing medium (80% FCS and 20% DMSO) and incubated at -80ºC overnight followed by storage in liquid nitrogen. To thaw the cells, a vial was transferred to a water bath (37°C) for 1-2 minutes and then plated directly with the fresh medium.

3.2.1.2 Primary pancreatic islet isolation and culture

Porcine islets were isolated at the Islet Isolation Facility of Third Medical Department using previously described techniques of collagenase digestion and Ficoll purification (79; 80). Briefly, the retired breeder pigs, aged from 17 to 60 months, were slaughtered in a local abattoir. After mechanical induction of cerebral death, the time lag for complete bleeding was extended for at least 5 min to minimize pancreatic blood content. The splenic lobe of the glands was dissected ex situ, intraductally distended with 100 ml University of Wisconsin (UW) solution, and transported in ice-cold saline solution to the isolation laboratory. Within 1.5-2.0 hr of cold ischemia, pancreata were dissected free of surrounding fat and vascular tissue and further distended with freshly prepared University of Wisconsin (UW) solution containing 4.4 Unit/gram organ collagenase NB 8 (SERVA Electrophoresis, Germany) and 0.6 Unit/gram organ neutral protease NB (SERVA) at 8-10°C, at a ratio of 2 ml of UW solution per gram of pancreatic tissue. Distended pancreata were chopped into pieces and placed in a 450 ml stainless steel chamber including glass marbles and a 500 μm pore-sized steel mesh positioned inside. The entire system was filled with Hanks' balanced salt solution (HBSS; Biochrom, Germany) via a tubing system, passing a heat circuit to

increase temperature slowly to a digestion temperature of 30-33°C. While recirculating the solution (200 ml/min), the digestion chamber was set in vertical motion (300 oscillations/min) with an amplitude of 1.8 cm. Samples were taken to monitor the dissociation of the tissue. The digest was collected in pre-cooled (4°C) 250 ml conical centrifuge tubes containing 10% (vol/vol) newborn calf serum and was washed two times at 80×g. Subsequent to cold storage in UW solution for 30 min, the digest was washed again at 80×g, dissolved in 400 ml of isoosmolar Ficoll-sodium-diatrizoate (Biochrom) and loaded into a Cobe 2991 at room temperature. During spinning at 800×g, the Ficoll suspension was top layered with 150 ml of Medium-199 supplemented with 10% NCS (Biochrom). Subsequent to 5 min of centrifugation, the running Cobe 2991 was unloaded, and islet fractions were collected,, washed consecutively at 200×g and 80×g in Medium-199 plus 10% NCS, and finally suspended in culture medium.

After isolation, the quality of islet isolation was evaluated by Trypan Blue exclusion, dithizone staining and glucose-stimulated insulin secretion to check viability, purity and function. The purity and viability were both more than 90% for subsequent experiments. The culture medium was composed of CMRL 1066 medium (R1631, PAA), supplemented with 20% pig serum, 2,5mM L-Glutamine, 1mM Na-Pyruvat, 2mg/ml Glucoselsg, 10mM Nikotinamid , Penicilin-Streptomycin and Ciprobay 200. The islets were cultivated in transfer packs (PL2410, Baxter, Illinois), kept in a humidified air and room temperature incubator, and used within 10 days after isolation.

3.2.1.3 Hypoxia culture

Hypoxia experiments were carried out in a New Brunswick Innova CO-48 hypoxia chamber (New Brunswick Scientific, New Jersey) with active oxygen controls. As the oxygen tension of the portal circulation has been reported to be typically 10–15 mmHg (16), and the measured oxygen tension within the culture medium in a oxygen-regulated incubator set to 2% O_2 is approximately 14-16 mmHg (81), the O_2 level in the current study was set to 2% in most cases to mimic the moderate hypoxic environment in the portal vein system. In the islet apoptosis assays, however, the O_2 level was set to 1% to mimic the extreme shortage of oxygen supply in the intraportally transplanted islets which have been reported to possess a mean oxygen tension of 5 mmHg (25).

3.2.2 Protein extraction

3.2.2.1 Total cell extraction in NP-40 lysis buffer

After washing in ice-cold PBS, cells were collected by centrifugation. Cell pellet was resuspended in 80-120 µl of NP-40 lysis buffer (freshly added phosphatase and protease inhibitors) and incubated on ice for 20 min. Cell debris were removed by a 13,200 rpm centrifugation at 4°C for 15 min. The supernatant containing total cell extract was collected for further use.

NP-40 lysis buffer	20 mM	Tris/HCl pH 7.5
	150 mM	NaCl
	1% (v/v)	Nonidet P-40
Phosphatase and	10 mM	NaF
protease inhibitors	0.5 mM	Sodium vanadate
	10 µg/ml	Leupeptin
	10 µg/ml	Aprotinin
	1 mM	Phenylmethylsulfonylfluoride (PMSF)

3.2.2.2 Measurement of protein concentration

Protein concentrations were determined the BCA Protein Assay kit. This assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total protein. The method combines the well-known reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium (the biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{1+}) using a unique reagent containing bicinchoninic acid.1 The purple-colored reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. This water-soluble complex exhibits a strong absorbance at 562 nm that is nearly linear with increasing protein concentrations over a broad working range. Therefore, the OD562 value depends on the concentration of most soluble proteins. The OD562 value of the tested samples were measured and plotted against a reference curve obtained with known concentrations of BSA.

3.2.3 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE is used to separate proteins on the basis of their molecular size. The system contains two gels: a "stacking gel" with a low level of crosslinkage and of low pH, allowing proteins to enter the gel and compact without smearing; and a "seperation gel" of higher pH, where the proteins are separated according to molecular size. For an 8×10×0.1 cm gel, the following volumes were used:

Seperation gel (10%)		**Stacking gel**	
4 x lower buffer	1.25 ml	4 x upper buffer	0.5 ml
AA (30%)	1.66 ml	AA (30%)	0.25 ml
H2O	2.09 ml	H2O	1.25 ml
APS (10%)	25 µl	APS (10%)	20 µl
TEMED	4 µl	TEMED	2 µl

4 x Lower buffer	1.5 M Tris/HCl pH8.8
	0.4% SDS
4 x Upper buffer	0.5 M Tris/HCl pH6.8
	0.4% SDS

Protein samples derived from cell extracts were denatured by heating at 95°C for 5 min in 1 x SDS sample buffer, and then cooled immediately on ice. The samples were collected by brief centrifugation and then equal amounts of protein were loaded on a SDS gel. The electrophoresis was performed with a vertical gel chamber in 1 x SDS running buffer. After electrophoresis, the gel could be used for Western blotting analysis.

10 x SDS running buffer	250 mM Tris
	2 M Glycine
	1% SDS
	pH 8.3

3.2.4 Western blotting

The separated proteins were electrically transferred from the gel to a polyvinylidene difluoride (PVDF) membrane (Millipore) by semi-dry blotting (Bio-Rad), according to the manufacturer's

instructions. After blocking in 1 x TBS containing 0.1% Tween 20 and 5% non-fat milk powder or BSA for 1-2 hour at room temperature, the membrane was incubated overnight with the appropriate primary antibodies at 4°C.

Followed by 4-5 washes in TBST (1 x TBS with 0.1% Tween 20), the membrane was incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. After another 4-5 washes in TBST, the bound proteins were then detected using the Thermo Scientific Pierce enhanced chemiluminescence system (ECL).

Transfer buffer	50 mM Tris
	40 mM Glycine
	0.04% (w/v) SDS
	20% Methanol
10 x TBS	250 mM Tris/HCl pH 7.6
	1.37 M NaCl
	50 mM KCl
	7 mM $CaC1_2 \cdot 2H_2O$
	1 mM $MgCl_2 \cdot 6H_2O$

3.2.5 Immunofluorescence analysis

The subcellular localization of NF- κB p65 subunit was studied by immunofluorescence analysis. MIN6 cells were grown on cover slips in 12-well dishes. The medium was aspirated off and the cells were washed twice in cold PBS. Then the cells were fixed by incubating them for 1 minute in a 1:1 solution methanol: acetone. Afterwards, the fixing solution was aspirated off. Prior to staining, the cells were rehydrated in PBS for 20 minutes and blocked for 1 hour by shaking in 1× PBS containing 10% (v/v) goat serum. The cells were incubated with the primary antibody diluted in 1× PBS containing 1% (v/v) goat serum for the next 2 hours. Subsequently, the cells were washed twice for 10 minutes in PBS. Then the secondary, fluorescence dye-coupled antibody diluted in PBS containing 1% (v/v) goat serum was incubated with the fixed cells for 2 hours. Thereafter, two washing steps of 10 minutes in PBS followed. The nuclear DNA could be stained by incubating the cells for 10 minutes with DAPI. Again the cells were washed twice in PBS. Then one drop of pre-warmed Kaiser's glycerol gelatine was put on a microscope slide. The cover slip was mounted on the gelatine and gently pressed. The slide was left at 4°C overnight. The next

day, the cover slip and slide were sealed with nail polish. The stained proteins were analyzed using an inverted Nikon Eclipse 2000E microscope.

3.2.6 Enzyme-linked immunosorbent assay (ELISA)

The quantification of murine TNF-α was performed by Mouse TNF-α Immunoassay kit (MTA00, R&D System, UK). The supernatant was analyzed directly. The cell lysis was diluted 40 x prior to assay.

Porcine insulin was quantified by the porcine-specific insulin ELISA kit (EIA-4747, DRG Instrument, Germany). The supernatant was diluted with IRI buffer 20 x prior to assay, and the islet lysis with 50 x.

ELISA assays were carried out following the instructions of the manufacturers. The results were standardized with the protein content measured by BCA assay in the respective sample.

3.2.7 RNA isolation and gene expression analysis

3.2.7.1 Isolation of total RNA

To prevent contamination with RNases, gloves were worn and RNase-free tubes, filter pipette tips, glassware and solutions were used. Total RNA was extracted using the RNeasy Mini kit, according to the manufacturer's instructions (Qiagen).

3.2.7.2 Measurement of RNA concentration

The RNA concentration and quality was measured using the NanoDrop 1000 Spectrophotometer (NanoDrop, Wilmington). The purity of RNA was checked by ratio of sample absorbance at 260 and 280 nm (260/280), which should be approximately 2.0 for pure RNA. Sample concentration was given in ng/μl based on its absorbance at 260 nm.

3.2.7.3 Synthesis of cDNA

Reverse transcription was employed to convert mRNAs into cDNAs by using Oligo (dT) primers and reverse transcriptase (Invitrogen). Briefly, 300 ng (porcine islets) or 1 μg (MIN6 cells) of total RNA was mixed with 10 mM dNTPs (final concentration), 0.5 μg $Oligo(dT)_{20}$ and H_2O to 12 μl. This mixture was heated at 65°C for 5 min, and quickly chilled on ice. Then, 4 μl of 5 x first-strand buffer and 2 μl of 0.1 M DTT were added to the sample and incubated at 42°C for 2 min.

Finally, 1 µl of SuperScript II RT (200 U) was added to the mixture (total volume 20 µl), followed by incubating at 42°C for 50 min and heating at 70°C for 15 min.

3.2.7.4 Real-time PCR analysis

Real-time PCR was used to amplify a segment of a known sequence from cDNA with gene-specific primers.

A typical 10 µl reaction mixture consists of:

5 µl	SYBR Green Master MIX (2 x)
0.5 µl	cDNA template
X µl (70 nM)	Forward primer
X µl (70 nM)	Reverse primer

RNase-free H_2O to a final volume of 10 µl

Quantitative real-time PCR was performed on StepOne Plus real-time PCR cycler (Applied Biosystems) using the following programs:

	Temp.	**Time**	**Number of cycles**
Enzyme activation	95°C	15 min	1 cycle
Denaturation	95°C	15 sec	40 cycles
Annealing	60°C	30 sec	
Extension	72°C	30 sec	

After amplification, a melting curve was performed to confirm the specificity of the reaction.

	Temp.	**Time**	**Number of cycles**
Denaturation	95°C	30 sec	1 cycle
Starting temp.	60°C	30 sec	1 cycle
Melting step	60°C	10 sec	80 cycles

The threshold value ct for each individual PCR product was calculated by the instrument's software and ct values obtained for the target gene were normalized by subtracting the ct values obtained with the reference gene. The resulting Δct values were then used to calculate relative changes of mRNA expression as the ratio (R) of mRNA expression of stimulated/unstimulated cells according to the equation:

$R=2^{(\Delta ct(stimulated)-\Delta ct(unstimulated))}$.

3.2.8 Cell viability analysis

3.2.8.1 MTT viability assay

Viable MIN6 cell number was determined using a Vybrant MTT kit (Molecular Probes, Invitrogen) following the manufacturer's instructions. The basic principle of the assay involves the conversion of the MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) to formazan crystals by viable cells in culture. The formazan crystals are solubilized by the addition of SDS-HCl and the resultant color is measured in a spectrophotometer at 570nm. The absorbance correlates directly and linearly with cell number.

3.2.8.2Annexin V/PI apoptosis assay

Islet cell death was assessed by using FITC conjugated Annexin V and propidium iodide (PI) staining to visualize early and late apoptotic (and/or necrotic) cells simultaneously (82).
Loss of plasma membrane is one of the earliest features of cell apoptosis. In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment. Annexin V is a 35-36 kDa Ca^{2+} dependent phospholipid-binding protein that has a high affinity for PS, and binds to cells with exposed PS. Annexin V may be conjugated to fluorochromes including FITC. This format retains its high affinity for PS and thus serves as a sensitive probe for flow cytometric analysis of cells that are undergoing apoptosis. Since externalization of PS occurs in the earlier stages of apoptosis as well as the latest stages of cell death resulting from either apoptotic or necrotic processes, staining with FITC Annexin V is typically used in conjunction with a vital dye such as propidium iodide (PI) to identify early apoptotic cells (PI negative, FITC Annexin V positive). Viable cells with intact membranes exclude PI, whereas the membranes of dead and damaged cells are permeable to PI. For example, cells that are considered viable are FITC Annexin V and PI negative; cells that are in early apoptosis are FITC Annexin V positive and PI negative; and cells that are in late apoptosis or already dead are both FITC Annexin V and PI positive.

Samples of approximately 2000 IEQ per group were cultured with or without hypoxia (1% O_2) in 24-well plates, with culture medium supplemented with or without cytokine cocktail (10 ng/ml TNF-α, 5 ng/ml IL-1β and 100 ng/ml INF-γ), for 24 hours before the islets were dispersed into

single cell suspension with Cell Dissociation Buffer (Gibco Invitrogen). After washings with PBS, islet cells were resuspended in 100 μL of Annexin V binding buffer (BD Pharmingen). Fluorescent Annexin V (2.5 μL; BD Pharmingen™) and PI (5 μg/ml; Sigma) were added to the cell suspension and incubated for 10 minutes on ice, and then diluted with 300 μL binding buffer. A total of 15,000 cells were analyzed by using FACSCalibur (Becton Dickinson). Controls included cells incubated with Annexin-V only, PI only, or no marker. Early apoptotic cells were identified as a population of Annexin V single positive cells. Necrotic or late apoptotic cells were identified as Annexin V-PI double positive cells. The results were expressed as a percentage of apoptotic cells (Annexin V single positive) or necrotic cells (Annexin V-PI double positive).

3.2.9 Glucose-stimulated insulin secretion (GSIS)

Isolated porcine islets were treated with 3μM BMS-345541 before subjecting to different culture conditions for 24 hours as described in the apoptosis flow cytometry assay. The islets were then gently transferred to TCM-199 (Biochrom, Germany) medium supplemented with 9.3 mM (basal) or 23.2 mM (stimulation) glucose and incubated for 90 minutes, at which time the medium was sampled for insulin measurement. The islets were then homogenized in IRI buffer by sonication and subsequently measured for protein content. Insulin assay was performed using porcine-specific insulin ELISA kit (DRG Instruments, Germany). The insulin secretion results were standardized by protein content to ensure equal amount of islets in each test condition. The stimulation index was defined as the ratio of stimulated secretion to the basal secretion.

3.2.10 Islet transplantation in mice

3.2.10.1 Surgical Procedure

Male inbred athymic mice (NMRI nu/nu, 12 weeks old, Charles River) were used as recipients of porcine islets. Before transplantation, diabetes was induced in the recipients by a single injection of 200 mg/kg streptozotocin intraperitoneally, and non-fasting blood glucose levels were measured from the tail vein using a glucometer (Elite, Bayer). Mice with a blood glucose value higher than 300 mg/dL for at least two consecutive days were used as recipients. Briefly, the islets were removed from the culture bags in a room temperature incubator, concentrated and aspirated into a 1 ml syringe equipped with a 27-gauge needle (BD). After sedimentation, the culture medium was removed from the concentrated islets and replaced with Ficoll solution (total volume 200 μl). Recipient mice were anaesthetized with avertine and maintained with isoflurane. Around 4000 IEQ porcine islets were infused into the liver by a puncture into the portal vein. After the

bleeding was arrested, the wound was closed in two layers with absorbable sutures.

3.2.10.2 Experimental groups

Diabetic nu/nu mice were randomly assigned into two groups, namely vehicle group or treatment group. The mice in treatment group received a bolus injection of 1mg BMS-345541 on day 0 at 30 minutes before transplantation, with day 0 being the day of transplantation. BMS-345541 was formulated as 10 mg/ml solution in water containing 3% (v/v) Tween 80 and administered intraperitoneally. Prior to transplantation, the porcine islets for this group were incubated at 37°C for 30 minutes in culture medium supplemented with 3 μM BMS-345541. The mice in the vehicle group received same amount of 3% (v/v) Tween 80 solution and the porcine islets were incubated with normal culture medium prior to transplantation.

3.2.10.3 Graft survival

Blood-glucose levels were measured daily before 9:00 a.m. for 60 days. Diabetes recurrence was defined as the day of blood glucose level exceeding 300 mg/ml. Mice were euthanized after confirmation of diabetes recurrence, and graft survival period was calculated as the number of days before diabetes recurrence.

3.2.10.4 Immunohistochemistry

For immunofluorescence detection of NF-κB activation, graft livers were recovered and snap frozen in O.C.T medium (Sakura, Netherland). Five μm sections were cut and mounted onto SuperFrost Ultra Plus (R.Langenbrinck, Germany) slides, air dried and fixed in Zamboni fixative for 10 min. After blocking in PBS containing 1% BSA and 2% donkey serum, the cryosections were probed with primary antibodies and incubated at 4°C overnight. After 3 times washing in PBS, appropriated secondary antibodies were applied on the next day for 1 hour at room temperature. The nuclei were visualized by Hoechst 33342 (Sigma). The cover slips were mounted with Prolong Gold (Invitrogen), visualized and photographed using a Leica DMLB microscope (Leica, Germany) equipped with Leica DFC420C CCD and processed in Leica Application Suite.

Immunofluorescence detection of neutrophil migration/infiltration into the islet grafts was done with the same protocol but using the antibody against CD11b (M1/70.15, Immunotools, Germany).

Immunofluorescence detection of caspase-3 positive cells in the grafted liver tissue was done with

the same protocol but using the antibody against active caspase-3 (C8487, Sigma).

Cell death visualized by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) technique was carried out following the protocol from the manufacturer (In Situ Cell Death Detection Kit, Fluorescein, Roche) using cryosection prepare as described. After the standard TUNEL labeling procedure, the slices were washed 3 times in PBS, followed by overnight probing with anti-insulin antibody and standard secondary antibody labeling and imaging procedure on the second day for visualizing islet grafts.

3.2.11 Statistics

Results are given as mean ± SEM. Statistical significance was determined using student's t-test or one-way ANOVA with *posthoc Bonferroni's* test, as appropriate. Islet graft survival curves were analyzed with Mantel-Cox log-rank test (Prism Software, GraphPad, CA). A value of $p < 0.05$ was considered as statistically significant.

4. Results

4.1 Hypoxia compromises NF-κB activity in islet cells

4.1.1 Hypoxia abrogates NF- κB signaling in a time-dependent manner

To investigate the impact of hypoxia on inflammatory cytokine-induced NF- κB signaling, we monitored the critical phosphorylation events in the NF-κB cascade by Western blotting. Primary isolated porcine islets were subjected to hypoxic culture (2% O_2) for different periods, followed by 10 minutes TNF-α stimulation and analysis of NF-κB activation with phospho-specific antibodies. TNF-α was used as a classical inducer of NF-κB activation. As shown in *Figure 1A*, pancreatic islets are quickly responsive to inflammatory cytokine as10 minutes TNF-α stimulation resulted in the strong phosphorylation of IκB-α ($Ser^{32/36}$) and NF-κB p65 (Ser^{536}), which are the key markers of NF-κB activation. Primary islets were very sensitive to hypoxia stress as TNF-induced NF-κB activation was compromised by hypoxia within 2 hours. TNF-α induced NF-κB activation in islet cells was largely diminished upon prolonged incubation for 8 hours under hypoxic conditions.

Mouse β-cell line MIN6 cells were employed to assist the investigation due to their constant availability. MIN6 cells were subjected to the same hypoxia culture and TNF-α stimulation as primary islets had, and similar results were yielded. Although short-term hypoxia for 2 hours induced NF- κB activation without TNF stimulation in MIN6 cells, this was not observable after 8 hours. On the contrary, prolong hypoxia for 8 hours almost completely abrogated MIN6 cells' response to TNF-α stimulation (*Figure 1B*).

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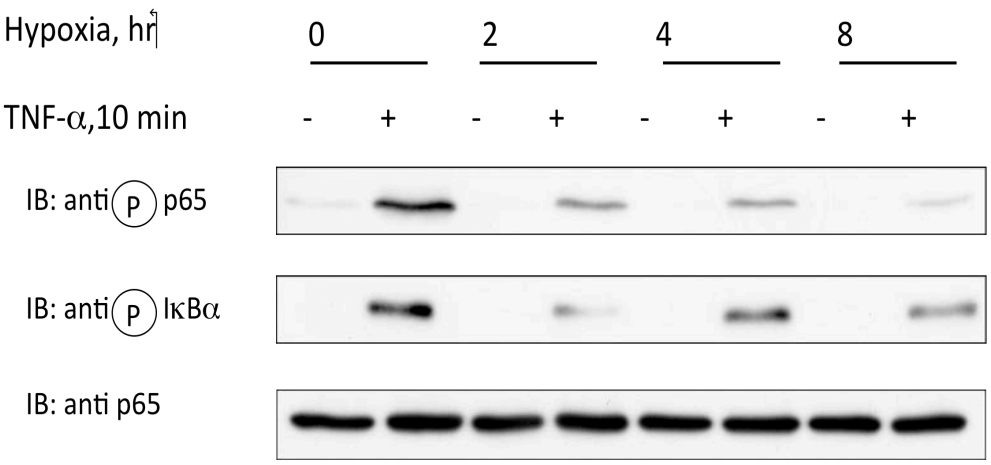

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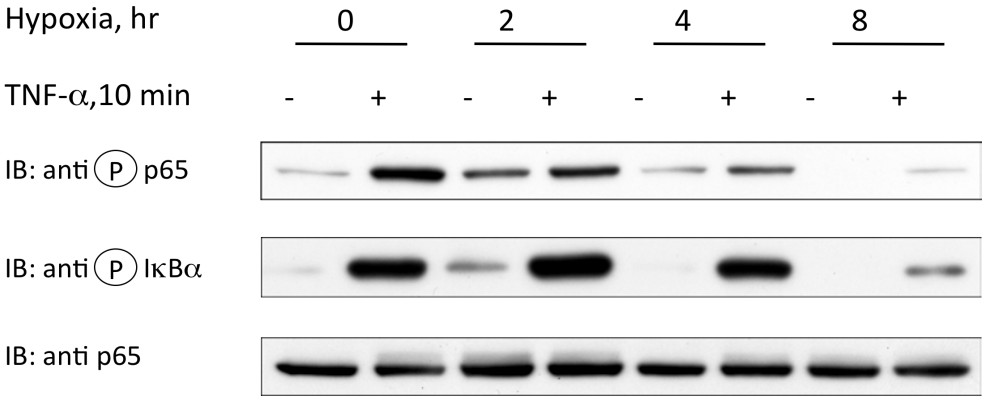


Figure 4-1 Prolonged hypoxia inhibits TNF-α-induced NF-κB-activating signaling pathways. Primary isolated porcine islets (A), or MIN6 cells (B), were subjected to different time of hypoxia as indicated, followed by 10 minutes TNF-α stimulation to check NF-κB activation. Equal amounts of protein contained in cell extracts were separated by SDS-PAGE and analyzed by Western blotting for the occurrence and phosphorylation of NF-κB signaling proteins. Phospho-specific antibodies were used to measure the phosphorylation of IκBα ($Ser^{32/36}$) and the NF-κB subunit p65 (Ser^{536}).

The effect of hypoxia on TNF-α-induced NF- κB activation in MIN6 cells was confirmed by immunofluorescence detection of subcellular localization of NF- κB subunit p65. As shown in *Figure 2*, p65 protein was retained in the cytoplasm in unstimulated cells under normoxia (first upper panel). The cytosolic p65 protein translocated to the nucleus upon TNF-α stimulation of normoxic cells. Preincubation under hypoxic conditions for 2 hours did not have a significant impact on TNF-induced p65 translocation, whereas 4 hours hypoxia moderately inhibited the nuclear translocation of p65. Finally, prolonged hypoxia for 8 hours totally abrogated TNF-α-induced p65 nucleus translocation in MIN6 cells.

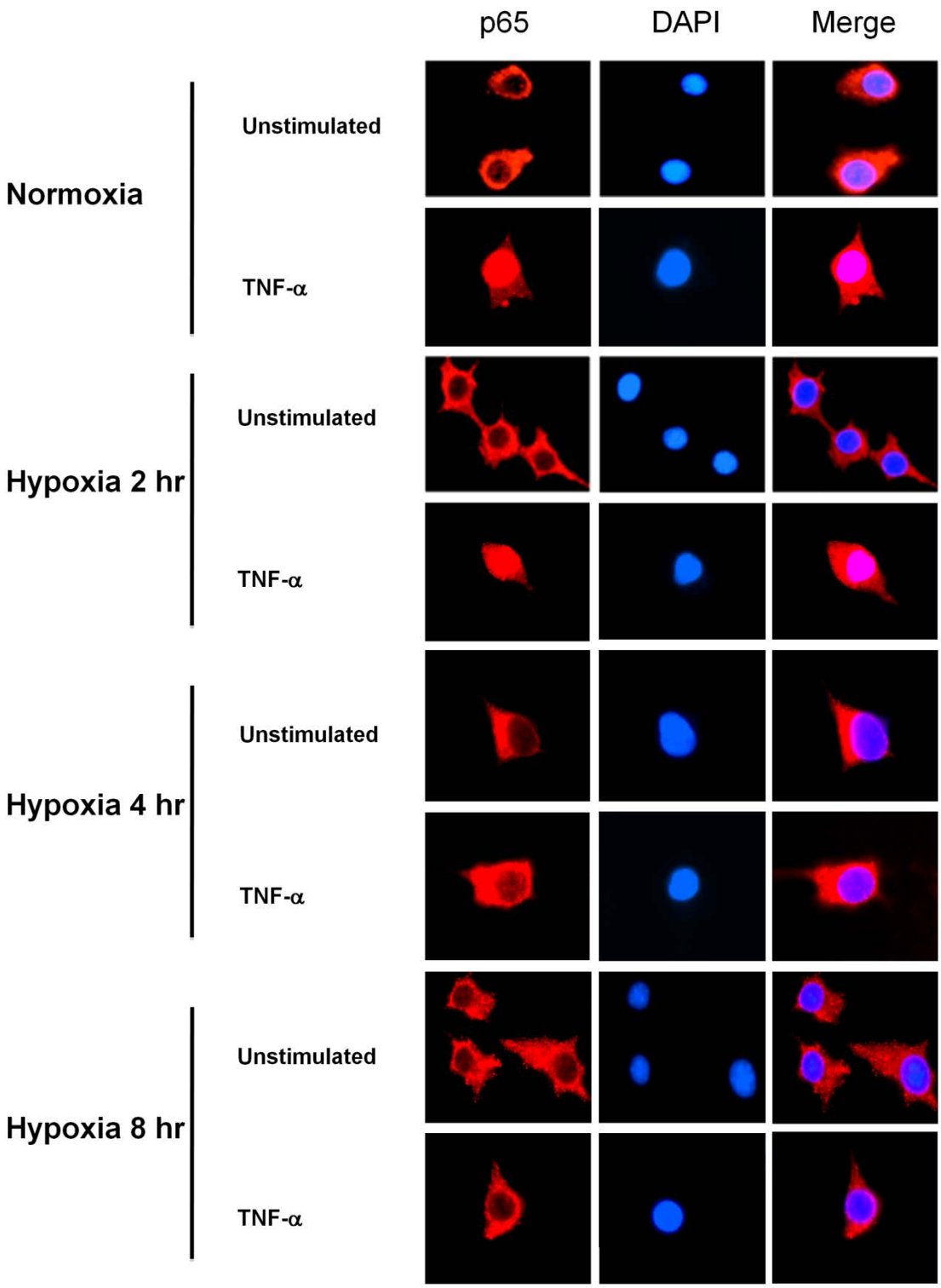


Figure 4-2 Prolonged hypoxia inhibits TNF-α-induced nuclear translocation of NF-κB p65. MIN6 cells were subjected to different period of hypoxic culture as shown and then left untreated or stimulated with TNF-α (10 ng/ml) for 30 minutes. Localization of NF-κB p65 was analyzed by indirect immunofluorescence as shown. Nuclear DNA was stained with DAPI (blue fluorescence, middle) and merged images are displayed at the right. All TNF stimulations were done under normoxic conditions.

To investigate the reversibility of NF-κB signaling after prolonged hypoxia, MIN6 cells were kept for 8 hours under hypoxic conditions, followed by normoxia cultivation for different periods before TNF-α stimulation. NF-κB signaling was still blocked 2 days after reoxygenation and was partially restored after 4 days under normoxic conditions (*Figure 3*). These data show that hypoxia-mediated loss of NF-κB function is slowly reversible.

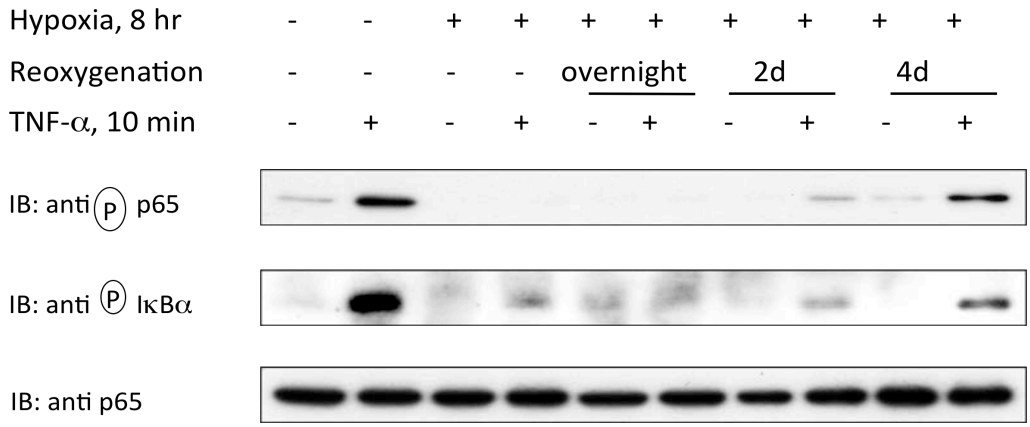


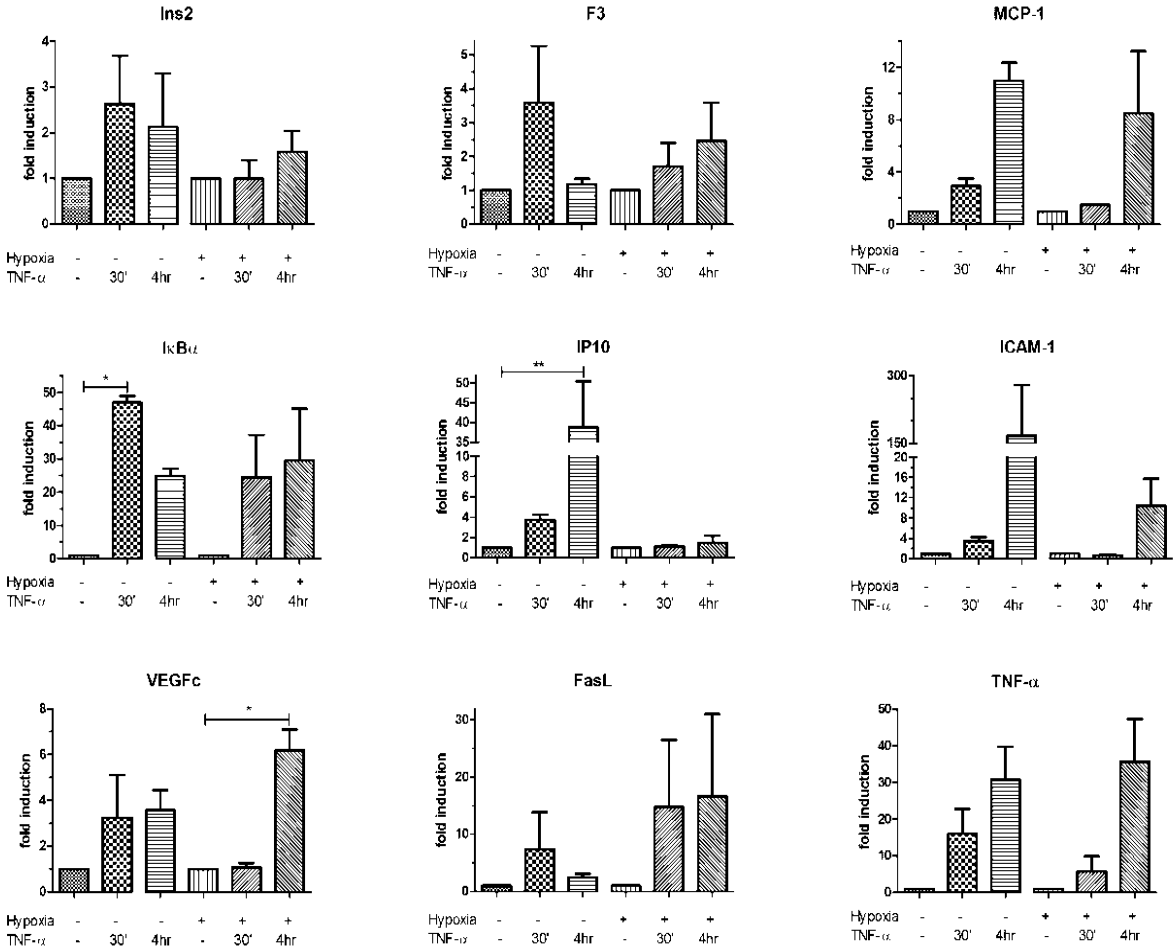
Figure 4-3 Reversibility of TNF-induced NF-κB activation after prolonged hypoxia. MIN6 cells were exposed to hypoxic conditions for 8 hours, followed by cultivation at normoxia for the indicated periods and induction of NF-κB by stimulation with TNF-α for 10 minutes. NF- κB activation was investigated with phospho-specific antibodies as in Figure 4-1.

4.1.2 Hypoxia diversely influences NF-κB-targeted gene expression in β-cells

Since NF- κB is a key transcription factor controlling the expression of its numerous target genes, we investigated the impact of hypoxia on NF- κB's target gene expression in MIN6 cells. For this aim, MIN6 cells were exposed to hypoxia for 12 hours followed by TNF stimulation for 30 minutes or 4 hours to induce NF- κB targeted gene expression, which was quantified by real-time PCR (*Figure 4A*). The analyzed genes could be functionally divided into different groups. In one group of genes including IκBα, (chemokine (C-X-C motif) ligand 10) IP10 and ICAM-1, the TNF-α-triggered induction of mRNA expression was inhibited under hypoxic conditions. On the other hand, another group of genes (insulin (Ins2), tissue factor (F3) and monocyte chemoattractant protein-1 (MCP-1)) were not suppressed under hypoxic conditions and some genes (VEGFc, Fas ligand (FasL) and TNF-α) were amplified after prolonged hypoxic incubation. Since TNF-α caused a strong induction of its own gene, synthesis of the endogenous TNF-α protein and its secretion was quantified in ELISA experiments. To differentiate between the TNF-α added for stimulation and TNF-α synthesized and secreted by the MIN6 cells, we used humanized TNF-α for stimulation and highly selective mouse TNF-α ELISA kit for detection. As

shown in *Figure 4B*, synthesis of intracellular TNF-α was strongly increased after combined reoxygenation and TNF-α stimulation for 24 hours. Secretion of this cytokine was mainly induced by hypoxia and could be augmented by the combination of reoxygenation and TNF-α stimulation.

A



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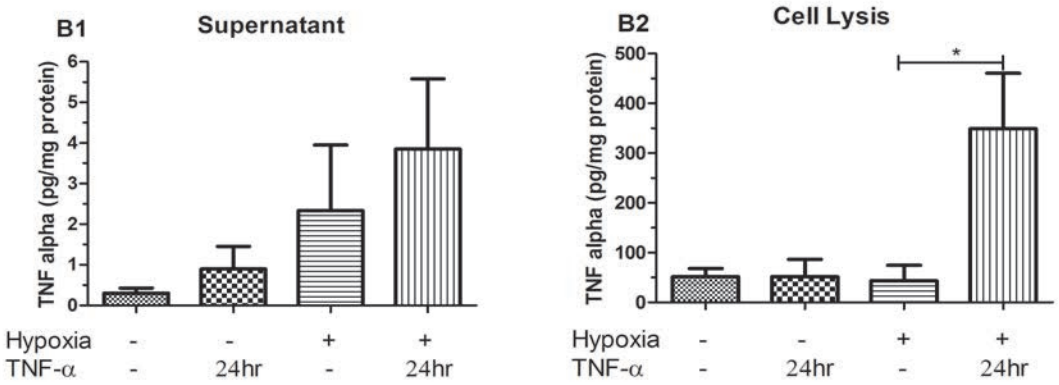


Figure 4-4 Analysis of gene expression and TNF-α synthesis after prolonged hypoxia. (A) MIN6 cells were incubated for 12 hours under hypoxic conditions and stimulated for 0.5 or 4 hours with humanized

TNF-α. After RNA isolation and cDNA synthesis, the relative expression of the indicated genes was quantified by real time PCR. The effects of different stimulations on individual gene expression were calculated as fold induction with the gene expression level in the control samples as fold one. All experiments were performed in triplicate. (B) TNF-α protein synthesis and secretion was quantified by ELISA. TNF-α ELISA was carried out immediately after 12 hours hypoxic incubation or after additional 24 hours of combined stress of reoxygenation and TNF-α stimulation. Secreted murine TNF-α was measured in the cell supernatant, while intracellular TNF-α was determined in cell extracts. Experiments were performed in duplicates, n=3. $^{*}p<0.05$ *vs* normal control; $^{**}p<0.01$ *vs* normal control.

4.1.3 Hypoxia transforms TNF-α into a death signal in β-cells

To investigate the impact of hypoxia and/or reoxygenation on β-cell survival, MIN6 cells were subjected to hypoxia for 12 hours followed by reoxygenation with or without combined TNF-α stimulation. As shown in *Figure 5A*, hypoxia or TNF-α stimulation alone for 12 hours did not cause noticeable cell death in MIN6 cells. However, reoxygenation for 2 days resulted in significant death which could be further augmented when TNF-α was combined with. The supernatant transferred from these cells impaired cell survival in normal-cultured cells (*Figure 5B*), indicating soluble factors signals were released into the supernatant which are detrimental to adjacent cells. These data indicate that, while TNF-α is not a direct killing factor for MIN6 cells under normoxia, it is transformed into a death signal after prolonged hypoxia exposure.

A B

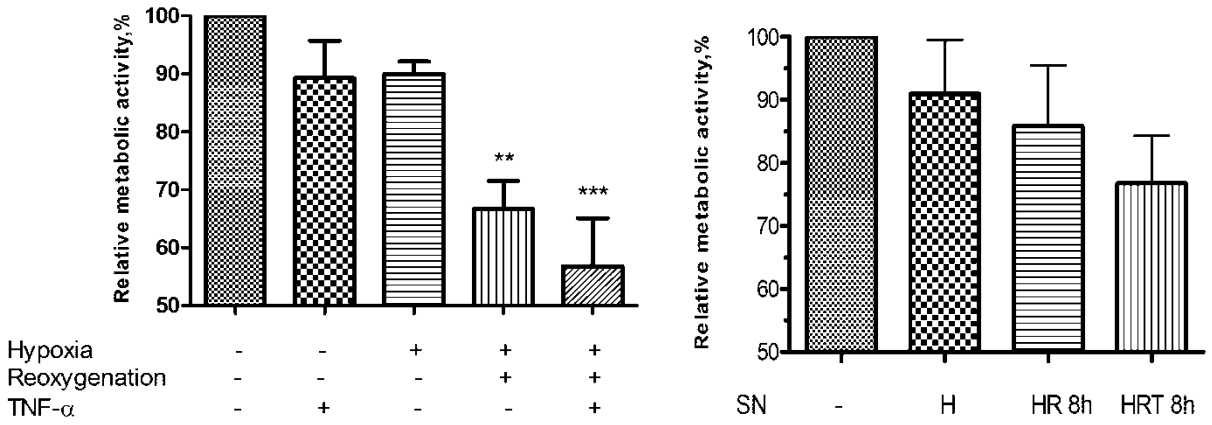


Figure 4-5 TNF-α is transformed to a death signal after hypoxia exposure. (A) MIN6 cells were subjected to 12 hours hypoxia incubation followed by reoxygenation with or without combined TNF-α stimulation for 2 days. Cell viability, as reflected by relative metabolic activity by viable cells, was measured by MTT assay. n=3. $^{**}p<0.01$ *vs* normal control; $^{***}p<0.001$ *vs* normal control. (B) The culture medium for normal-cultured cells was replaced by the supernatant (SN) transferred from the stressed cells. The cells were cultured in the supernatant for another 2 days before subjecting to MTT assay. H: hypoxia

12 hours; HR 8h: hypoxia followed by reoxygenation for 8 hours; HRT 8h: hypoxia followed by combined reoxygenation and TNF-α stimulation for 8 hours.

4.2 Cytokine-induced NF- κB activity is pro-apoptotic in pancreatic islets under hypoxia

The significant effect of hypoxia on NF-κB activity and its target gene expressions in MIN6 cells indicates that NF- κB signaling might play an important role in determining cell survival in pancreatic islets. This prompted us to search for a NF- κB inhibitor which should have *in vivo* activity at low concentrations. The highly selective IKKβ inhibitor BMS-345541, which has been shown to block NF-κB activation *in vivo* at nanomolar range and have an excellent pharmokinetics profile (83), enables us to investigate the roles of NF-κB on pancreatic islets without gene manipulation.

4.2.1 BMS-345541 inhibits TNF-α-induced NF- κB activation in β-cells

The effect of BMS-345541 on TNF-α-induced NF- κB activation was evaluated in MIN6 cells by immunofluorescence. For this purpose, MIN6 cells were grown on 8 well chamber slides and stimulated with TNF-α for 10 minutes before subjecting to immunofluorescence detection of NF- κB subunit p65 subcellular localization. As shown in *Figure 6*, TNF-α stimulation resulted in p65 translocation from the cytoplasm to the nucleus in MIN6 cells. While BMS-345541 at 1 μM concentration did not have an obvious effect on p65 translocation, 3 μM BMS-345541 almost completely abrogated p65 translocation. Based on this observation, BMS-345541 was used at 1 μM or 3 μM range to evaluate the dose-dependency of NF- κB inhibition in the following studies.

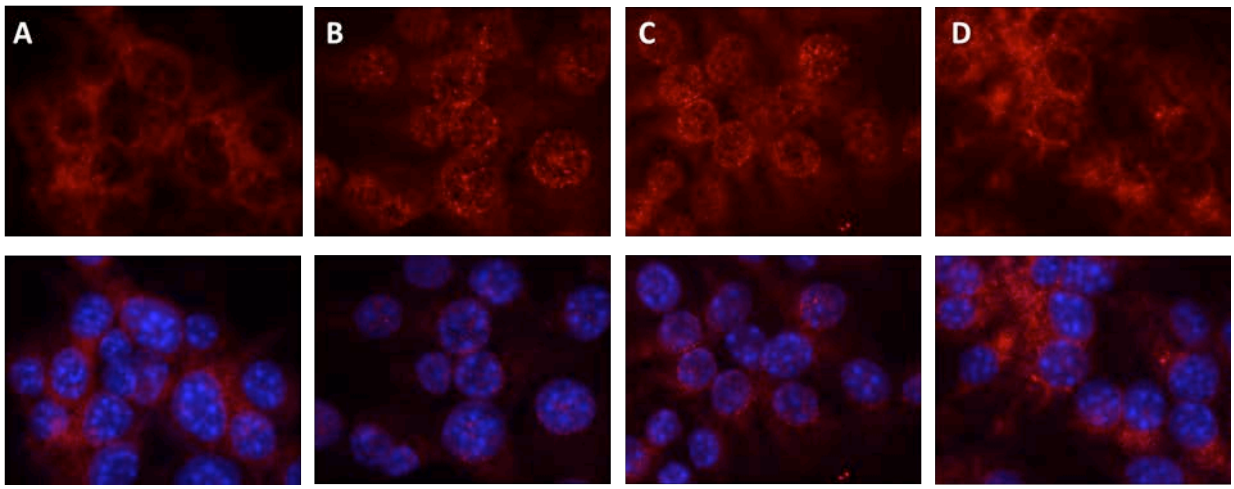


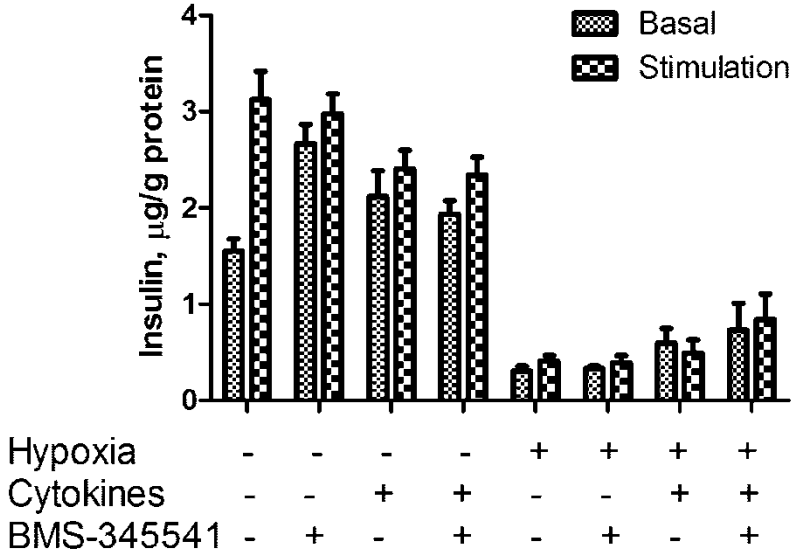
Figure 4-6 BMS-345541 inhibits NF-κB subunit p65 nuclear translocation dose-dependently. MIN6 cells were cultured in 8-well chamber slide for 2 day followed by 10 minutes TNF-α (10 ng/ml) stimulation

and immunofluorescence detection. p65 protein was stained red by sc109 antibody; nuclei were stained blue by Hoechst. BMS-345541 was added to the culture medium 30 minutes before TNF-α stimulation. (A) Unstimulated cells. (B) TNF-α stimulated cells. (C) TNF-α stimulated cells with 1 μM BMS-345541 treatment. (D) TNF-α stimulated cells with 3 μM BMS-345541 treatment. Upper panel: p65 (red) staining images; Lower panel: overlay images of p65 (red) and nuclei (blue).

4.2.2 BMS-345541 has no significant impact on glucose-stimulated insulin secretion

To evaluate its potential application in islet transplantation, the impact of BMS-345541 on islet function was examined by glucose-stimulated insulin secretion (GSIS) in primary isolated porcine islets. Primary isolated islets were pretreated with 3 μM BMS-345541 for 30 minutes before subjecting to culture with or without hypoxia and cytokine cocktail for 24 hours followed by GSIS, insulin ELISA and protein quantification. As shown in *Figure 7*, while BMS-345541 treatment increased basal insulin release under normoxia, it has no significant effect on insulin secretion in other test conditions. The calculated stimulation index revealed that, while the treatment decreased the stimulation index under normoxia due to the reason that it increased the basal insulin release, BMS-345541 treatment improved insulin secretion function when the islets were stressed by combined hypoxia and cytokine cocktail. As the latter situation models on intraportal transplantation environment, these data indicate that NF-κB inhibition may enhance the glucose-controlling function of the transplanted islets, or at least is not harmful to their functionality.

A



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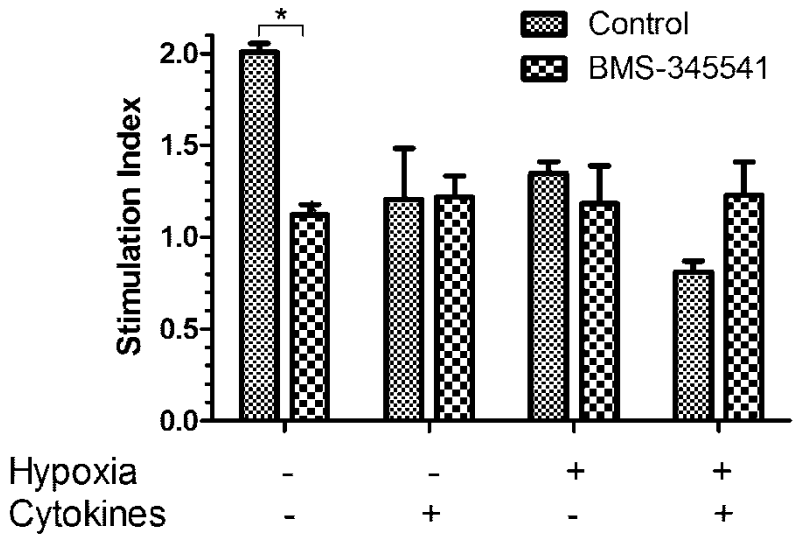


Figure 4-7 BMS-345541 has no impact on glucose-stimulated insulin secretion in primary islets. Isolated islets were pretreated with 3 μM BMS-345541 starting 30 minutes before subjecting to culture with or without hypoxia and cytokine cocktail for 24 hours. The islets were then gently transferred to TCM-199 medium supplemented with 9.3 mM (basal) or 23.2 mM (stimulation) glucose and incubated with gentle shaking for 90 minutes, at which time the medium was sampled for insulin measurement. The islets were then homogenized and subsequently measured for insulin and total protein content. (A) The insulin secretion results were standardized by protein content to ensure equal amount of islets in each test condition. (B) The stimulation index was defined as the ratio of stimulated secretion to the basal secretion. N=3. $^{*}p<0.05$ *vs* control.

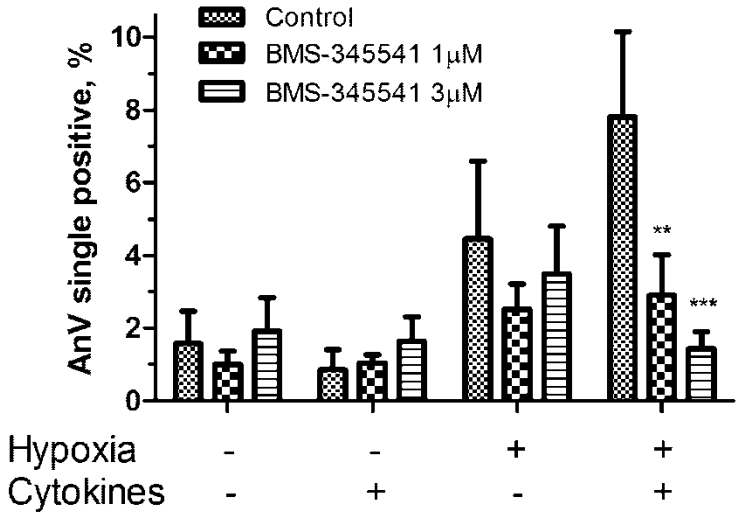
4.2.3 Cytokine-induced NF-κB activity has distinct roles under normoxia and hypoxia

The potentially beneficial effect of BMS-345541 on insulin secretion makes it a suitable candidate for studying the role of NF-κB in pancreatic islets. Next, we evaluate the impact of BMS-345541 treatment on islet survival under environmental stress. As controversial NF-κB roles were reported using diverse conditions, we investigated its role in a more comprehensive way.

Isolated porcine islets were cultured under different conditions for 1 day before flow cytometric cell death assay. As shown in *Figure 8*, in normoxia (absence of hypoxia), the addition of cytokine cocktail slightly reduced both apoptotic and necrotic cell death in the control samples, indicating a pro-survival effect of cytokine-induced signaling pathways. However, BMS-345541 treatment did not have an evident effect either with or without addition of cytokines under normoxia. These data indicate that cytokine-induced NF-κB activity has no dominant impact on islet cell viability under normoxic conditions.

In contrast, the cytokine cocktail increased cell death under hypoxic conditions. Analysis of cell death by Annexin V-FITC and propidium iodide (PI) showed that the combination triggered both apoptosis and necrosis. Cytokine cocktail-induced cell death under hypoxic conditions was dose-dependently inhibited by BMS-345541 (*Table 1*). Interestingly, BMS-345541 treatment only interfered with cytokines-triggered cell death as it had no significant impact on cell survival under hypoxia when cytokines were absent, confirming the specificity of BMS-345541's inhibition on cytokine-induced NF-κB signaling. Taking together, these data indicate that cytokine-induced NF-κB activity under hypoxia has a dominantly pro-apoptotic role in pancreatic islets.

A



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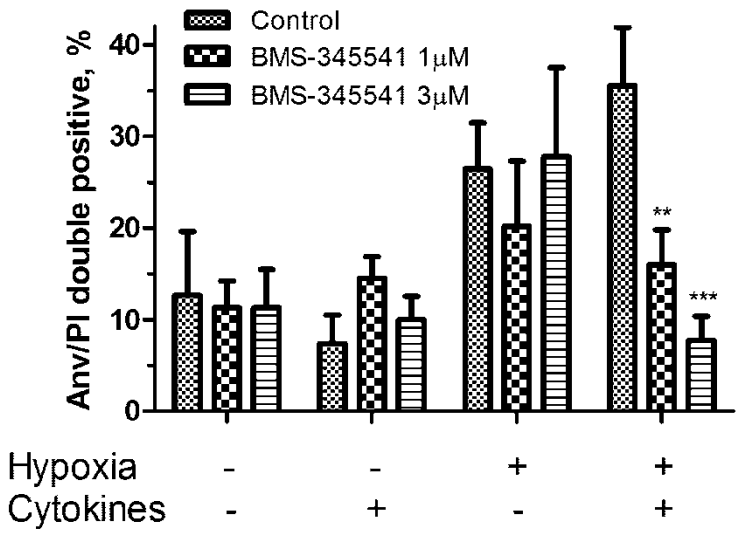


Figure 4-8 BMS-345541 treatment improved islets survival only under combined hypoxia and cytokine cocktail stress. Isolated porcine islets were treated with indicated dose BMS-345541 starting 30 minutes before subjecting the culture with or without hypoxia and cytokine cocktail, as indicated, for 24

hours. The islets were dissociated and subjected to flow cytometric cell death assay. Apoptotic cells were identified by Annexin V single positive (A), whereas necrotic cells were identified as Annexin V-PI double positive (B). BMS-345541 treatment dose-dependently reduced apoptosis and necrosis only under combined stress by hypoxia and cytokine cocktail. $^{*}p<0.05$ and $^{**}p<0.01$ *vs* control (no BMS-345541 treatment); n=4.

Table 4-1 BMS-345541 treatment dose-dependently improves pancreatic islet cell survival under combined hypoxia and cytokine cocktail stress.

	Apoptosis	Necrosis
Control	7.81±2.35	35.55±6.36
BMS-345541 1μM	2.91±1.11	15.98±3.82*
BMS-345541 3μM	1.42±0.48*	7.75±2.64**

$^{*}p<0.05$ *vs* Control; $^{**}p<0.01$ *vs* Control; n=4.

4.2.4 Cytokine-induced NF-κB activity represses anti-apoptotic gene expression under hypoxic conditions

To investigate the mechanisms behind the different roles of NF-κB under normoxia and hypoxia, porcine islets were subjected to the above mentioned stress conditions with or without 3 μM BMS-345541 treatment for 24 hours before total RNA extraction and two-step real-time PCR. We analyzed BCL-2, BCL-xL and cellular FLICE-inhibitory protein (c-FLIP), which are anti-apoptotic genes regulated by NF-κB (84). In addition, we examined gene expression of survivin, vascular endothelial growth factor A (VEGFa), vascular endothelial growth factor receptor 2 (VEGFR2) and HIF-1alpha (HIF-1α). Survivin is an anti-apoptotic gene and a member of the inhibitor of apoptosis (IAP) family which is not regulated by NF-κB (69). VEGFa is an anti-inflammatory cytokine that has been shown to be an extremely important mediator of islet revascularization process (36; 85) and is targeted by both NF-κB and HIF-1α (66; 86). VEGFR2 functions as the primary mediator of VEGF activation in endothelial cells and its transcriptional regulation may involve multiple elements including NF-κB (87). HIF-1α is another transcription factor which controls genes involved in energy metabolism and angiogenesis under hypoxia (86).
As shown in *Figure 9*, under normoxia, cytokine cocktail increased gene expression of BCL-xL, c-FLIP, BCL-2 and VEGFa. These genes are NF-κB targeted and BMS-345541 treatment abolished the increased expression induced by cytokines. Cytokines also increased the expression of survivin, VEGFR2 and HIF-1α. These genes are not directly regulated by NF-κB and BMS-345541 treatment did not have an evident impact on the expression levels. Interestingly, cytokines

treatment promoted both anti-apoptotic BCL-2 and pro-apoptotic Bax-α gene expression, indicating a competition between anti-apoptotic and pro-apoptotic machineries. However, the ratio of BCL-2/Bax was increased by cytokine cocktail which is in consistence with the pro-survival effect of cytokines under normoxia in the previous apoptosis assay. Collectively, these data indicate that the predominant effect of cytokines-induced NF-κB activity under normoxia is pro-survival.

In contrast, under hypoxia, cytokine cocktail treatment repressed the expression of BCL-xL, c-FLIP, survivin, VEGFa and VEGFR2 genes, all of which could be positively restored by BMS-345541. In addition, BMS-345541 significantly increased the expression of HIF-1α. Moreover, while cytokine addition did not suppress BCL-2 expression under hypoxia, NF-κB blockage by BMS-345541 increased the expression ratio of BCL-2/Bax, which is again in favor of cell survival. These data indicate that cytokine-induced NF-κB activity under hypoxia represses anti-apoptotic gene expressions and promotes cell death in pancreatic islets, which is consistent with our previous results on apoptosis and necrosis.

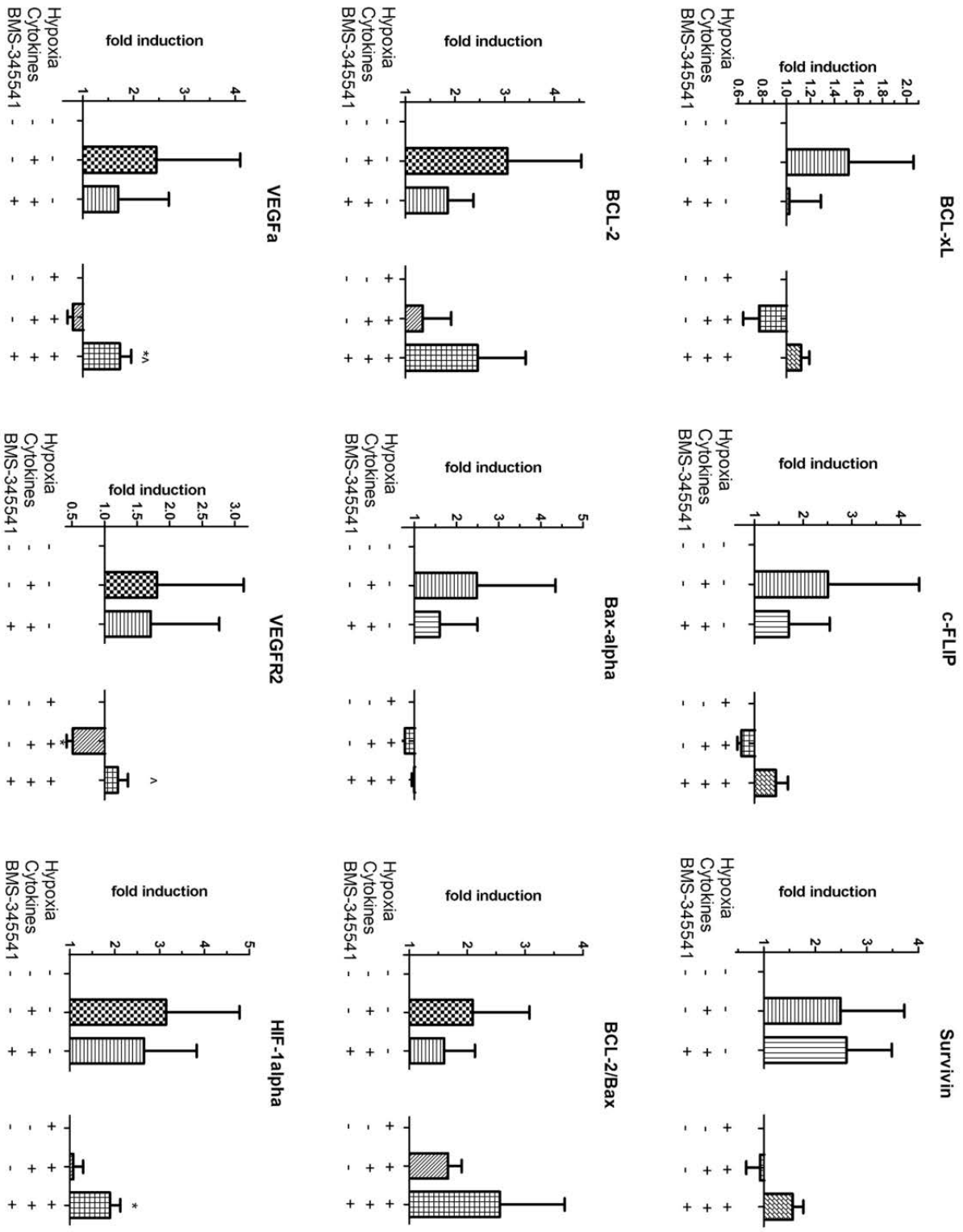


Figure 4-9 BMS-345541 treatment increases anti-apoptotic genes expression under hypoxic conditions. Isolated porcine islets were treated with the same conditions as indicated in Figure 4-4. After RNA isolation and cDNA synthesis, the relative expression of the indicated genes was quantified by real time PCR. The effects of different treatment on individual gene expression were calculated as fold

induction with the gene expression level in the control samples, normoxia or hypoxia respectively, as fold one. Results are mean ± SEM. Values are normalized relatively to PRL13A. $^{*}p<0.05$ *vs* hypoxia control; $^{\wedge}p<0.05$ *vs* hypoxia plus cytokines control; n=3.

4.3 Systemic NF-κB inhibition improves islet transplantation outcome in mice

Since NF-κB inhibition by BMS-345541 dose-dependently improved pancreatic islet survival under combined hypoxia and cytokines stress, which models on intraportal transplanted islets, we next tested whether this effect could be translated into intraportal transplantation using an animal model.

4.3.1 NF-κB is activated in islet grafts shortly after intraportal transplantation

First, we investigated whether NF-κB is activated in the transplanted islets. For this purpose, 4,000 IEQ porcine islets were transplanted intraportally to male NMRI nu/nu mice. Grafted livers were retrieved subsequent to transplantation; cryosections were prepared and subjected to immunohistochemical staining to investigate NF-κB p65 subunit distribution in the grafted liver. NF-κB activation, as evidenced by p65 nuclear translocation was detected in islet grafts within 30 minutes after intraportal transplantation (*Figure 10*). In contrast, endogenous islets in the native pancreas demonstrate no NF-κB activation. The neighboring hepatocytes exhibited no NF-κB activation at this time point.

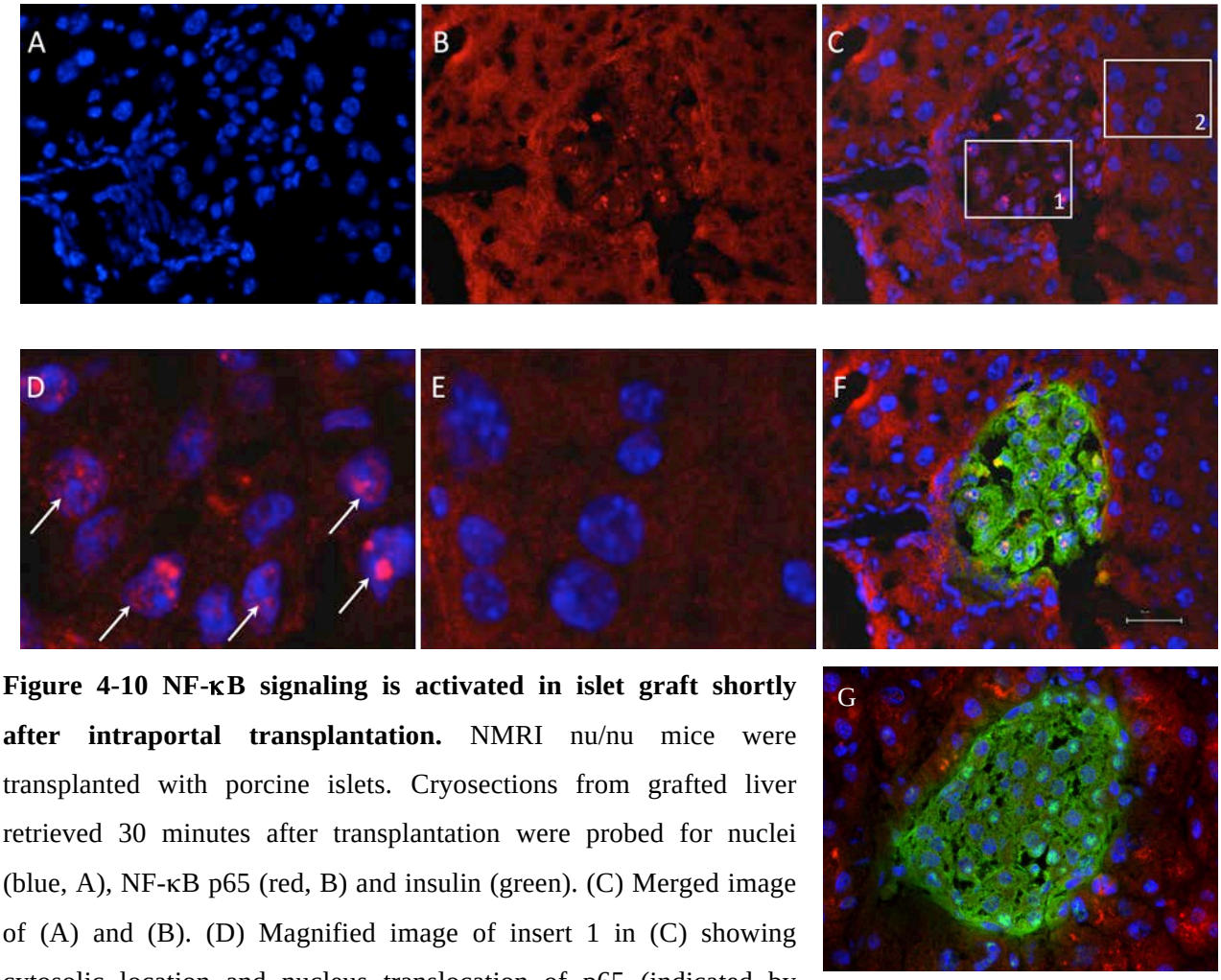
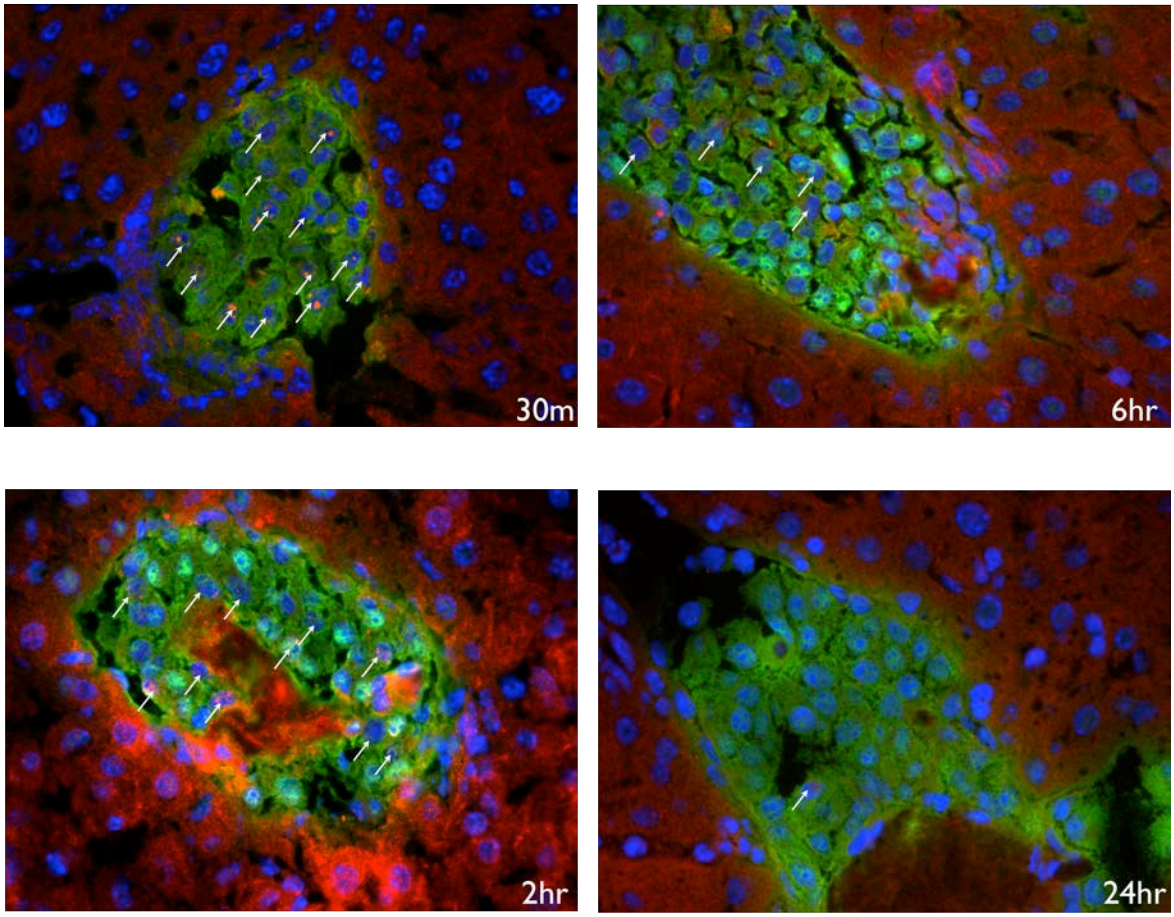


Figure 4-10 NF-κB signaling is activated in islet graft shortly after intraportal transplantation. NMRI nu/nu mice were transplanted with porcine islets. Cryosections from grafted liver retrieved 30 minutes after transplantation were probed for nuclei (blue, A), NF-κB p65 (red, B) and insulin (green). (C) Merged image of (A) and (B). (D) Magnified image of insert 1 in (C) showing cytosolic location and nucleus translocation of p65 (indicated by arrows) in β-cells. (E) Magnified image of insert 2 in (C) showing only cytosolic location of p65 in hepatocytes. (F) Overall merged image of (A), (B) and insulin (green). (G) Native mouse pancreatic islets. Magnification x400; bar=25μM.

4.3.2 NF-κB activation in islet grafts diminishes by time

When the kinetics of p65 translocation in the transplanted islets was checked by immunohistochemistry, we found NF- κB activation in islet grafts was diminished by time. As shown in *Figure 11*, p65 translocation into nucleus was clear and strong in the transplanted islets at 30 minutes after transplantation. The magnitude of p65 nucleus translocation decreases within 24 hours. When the number of p65 translocation positive cells was counted and its ratio to total cells number in each islet graft was calculated, a significant reduction in p65 translocation positive cell percentage was revealed 24 hours after transplantation (percentage of p65 positive nucleus: 7.76±2.69 *vs* 39.03±7.01, $p<0.05$).

A



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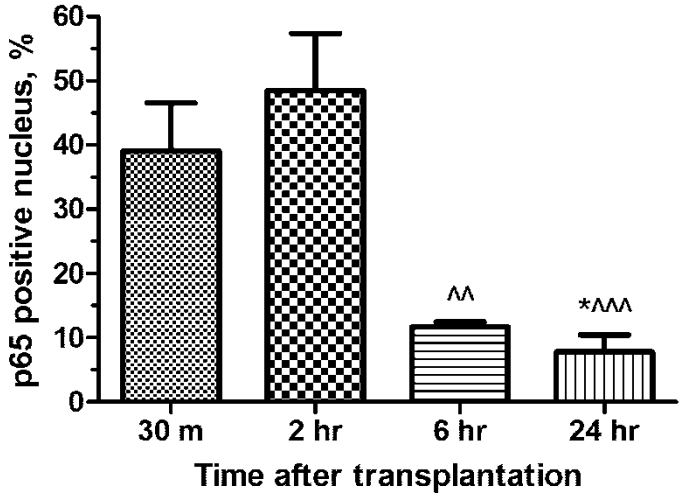


Figure 4-11 NF-κB activation in islet grafts diminished by time (A) Representative pictures of immunohistochemical detection of NF-κB subunit p65 nucleus translocation in the early grafted liver sections. The grafted livers were retrieved after 30 minutes, 2 hours, 6 hours or 24 hours after intraportal islet transplantation. Immunohistochemistry was done as described. **(B)** The number of p65 translocation positive nuclei was counted and its ratio to total nucleus number in each islet graft was calculated. n≥4. *p<0.05 *vs* 30 minutes; ^^p<0.01 *vs* 2 hours; ^^^p<0.001 *vs* 2hours.

4.3.3 Systemic BMS-345541 administration inhibits NF-κB activation in islet grafts

The recipient mice were treated with either vehicle or a single injection of BMS-345541 30 minutes before transplantation to achieve systemic NF-κB inhibition in the peri-transplantation stage.

The effective of BMS-345541 treatment on NF-κB activation within the transplanted islets were studies by immunohistochemistry. As the magnitude of NF-κB activation in islet grafts was maximal at 30 minutes and 2 hours post-transplantation, we retrieved grafted livers from both groups at these time points. As shown in *Figure 12*, BMS-345541 administration inhibited NF-κB activation in most of the islet cells at 30 minutes and essentially all the islet cells at 2 hours post transplantation. These results show that a single dosage of 1mg per mouse (equal to around 30 mg/kg) BMS-345541 is effective and sufficient for NF- κB inhibition in transplanted islets.

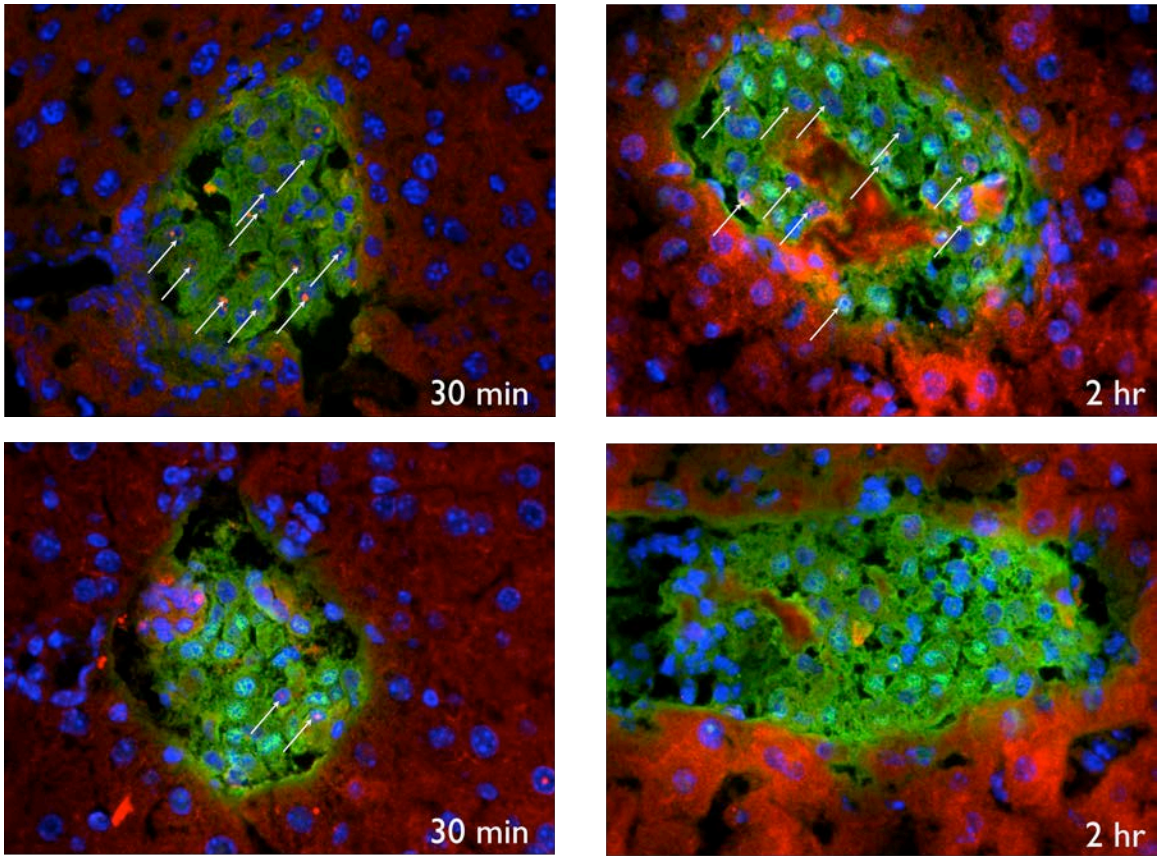


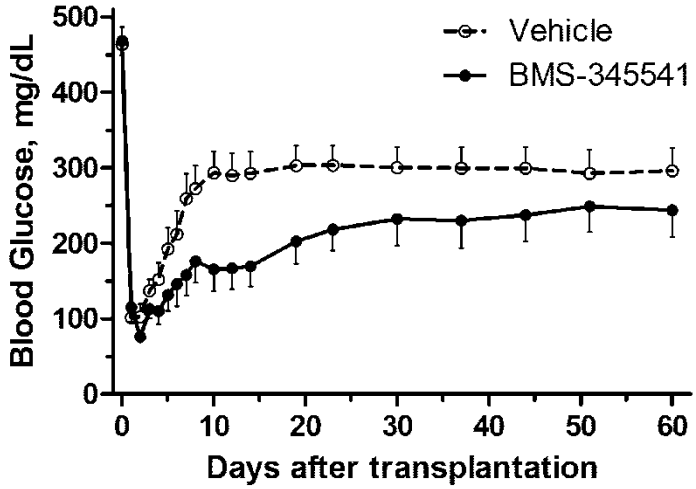
Figure 4-12 BMS-345541 at 1 mg per mouse dosage is effective and sufficient for NF-κB inhibition in transplanted mice. Representative images showing immunohistochemical detection of NF-κB subunit p65 translocation (indicated by arrows) in the grafted liver sections from 30 minutes or 2 hours after transplantation (upper panel: vehicle group; lower panel: treatment group).

4.3.4 Systemic NF-κB blockage improves islet engraftment

No side effects of BMS-345541 treatment at the current dosage were observed in the study. The safety of this treatment was confirmed when the body weight in the recipient mice was recorded and no difference was found between the two groups. As the diabetic recipients lost body weight before transplantation, we observed in both groups a gradually increased body weight in the transplanted mice which reflected improved general health conditions by islet transplantation.

As shown in *Figure 13*, all recipient mice were highly diabetic and became euglycemic after transplantation, indicating the functionality of the graft. However, 73 % (11/15) in the vehicle group lost islet grafts within 10 days (median survival: 7 days). In contrast, 46% (6/13) of the mice in the BMS-345541 treatment group lost graft function in 30 days, and the rest kept graft function after 60 days of observation period (median survival: 45 days). Log-rank analysis of the survival curve confirmed a significant benefit from BMS-345541 treatment (Chi square 5.625, p=0.0177). The areas under the 60-day glucose curves were significantly decreased in the treated group (8326 ± 689 *vs* 5891 ± 676 mg/dl·day, p=0.0180) confirming improvement of islet engraftment after transplantation.

A



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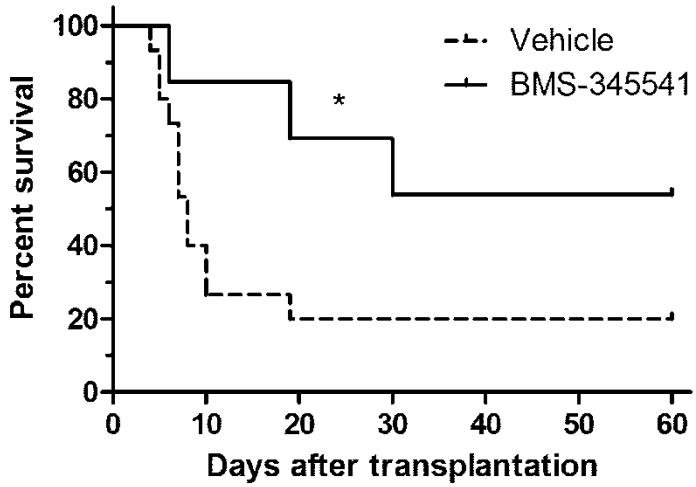

Figure 4-13 Systemic BMS-345541 administration improves islet engraftment after intraportal transplantation. Mice were treated as described. (A) Blood glucose level in the recipient mice was recorded daily and mean±SEM values were shown. Mice were euthanized after confirmation of diabetes recurrence and the last values were carried-on till the end for demonstration. n=15 in vehicle group; n=13 in BMS-345541 treatment group. (B) Islet graft survival curve in 60 days after transplantation. *p<0.05 *vs* vehicle.

5. Discussion

Although intensive investigation focused on the role of NF-κB on β-cell destruction during progress of T1D, little attention was drawn on its role in pancreatic islet transplantation. This study shows that hypoxia compromised NF-κB activity in a time-dependent manner in pancreatic islets. Furthermore, we found that cytokine-induced NF-κB activity plays a predominantly pro-apoptotic role under hypoxic conditions. Finally, we demonstrated a protective effect of NF-κB blockage in pancreatic islets under combined stress of hypoxia and cytokine cocktail *in vitro* and after intraportal islet transplantation *in vivo*.

5.1 Hypoxia as a modulator of NF-κB activity in pancreatic islets

Despite plenty of observations that indicate hypoxia as a key factor in determining the fate of the transplanted islets, there is no observation addressing how hypoxia interferes with NF- κB signaling. Thus, the effect of hypoxia on NF-κB signaling was studied in the current study which showed a dynamic reaction of NF-κB to hypoxia. While short-term hypoxia for 2 hours activated NF- κB signaling in immobilized β-cells (MIN6 cells), prolonged hypoxia for 8 hours substantially abrogated TNF-α induced NF- κB signaling in both MIN6 cells and primary islets. These *in vitro* observations were confirmed *in vivo* when the transplanted islets were examined by immunohistochemistry for p65 translocation after intraportal transplantation. NF- κB activation was detectable in transplanted islets within 30 minutes after transplantation; the magnitude peaked at 2 hours post-transplantation but diminished substantially after 6 hours. The time-course of hypoxia's inhibitory effect on NF- κB signaling is largely consistent in both models.

The molecular mechanisms underlying how hypoxia abrogates NF- κB signaling in pancreatic islets remain unclear. Although no direct answer could be found from the literature, reactive oxygen species (ROS, including superoxide anions, hydrogen peroxide and hydroxyl radicals) could serves as a potential mediator.

The major source of intracellular ROS is the mitochondrial respiratory chain. Aerobic life yields most of its energy from oxidative phosphorylation, a process in which oxygen serves as acceptor of electrons. During respiration, most of the oxygen consumed is reduced to water. However, an estimated 1–2% of oxygen consumed during respiration is not completely reduced to water but is instead partially reduced to $O_2^{\cdot -}$, a moderately reactive species that can generate hydrogen peroxide (H_2O_2), which in turn can produce highly reactive hydroxyl radicals (-OH), especially in the presence of transition metals such as iron (88). Extra-mitochondrial sources of ROS also exist,

including peroxisomes, the endoplasmic reticulum (ER), the plasma membrane and the cytosol, where these species are formed through the action of cytochrome P450, xanthine and nicotinamide adenine dinucleotide phosphate (NADPH) oxidases and other enzymes, including those participating in arachidonic acid metabolism such as lipoxygenases (LOXs) and cyclooxygenases (COXs).

Owing to their high reactivity, ROS represent a serious hazard for the cell, as they can oxidize macromolecules, thus damaging proteins, lipids and DNA. To cope with these harmful effects of ROS, aerobic cells evolved powerful mechanisms aimed at preventing accumulation of these species, including antioxidant enzymes such as superoxide dismutases (SODs), catalases, glutathione peroxidases (GPx) and peroxiredoxin, and non-enzymatic systems such as vitamins C and E, reduced glutathione (GSH) and thioredoxin (Trx). The development of these safeguard systems is critical for enabling cells to control and later exploit the reactivity of ROS for signal transduction (89).

ROS is an active modulator of NF-κB signaling. Interestingly, whereas ROS has been proposed as an activator of NF-κB almost two decades ago when H_2O_2 was found to be able to induce NF-κB activation and antioxidant blocked the activation (90), our understanding of the mechanisms for ROS-mediated activation of NF-κB signaling is still poor. Although the nature of redox regulation of NF-κB signaling remains to be fully elucidated, it is generally agreed that ROS production in the cytoplasm can mediate NF-κB activation whereas the DNA binding activity of oxidized NF-κB is suppressed and requires a thioredoxin-dependent reduced status to restore its action in the nucleus (89; 91; 92). Importantly, it has been pointed out that high level of ROS would probably lead to an inhibition of NF-κB rather than its activation (93; 94). The mechanisms responsible for this inhibition have been elegantly reviewed (89; 94). Briefly, *1)* High levels of oxidized glutathione were found to hinder the activity of ubiquitin conjugating enzymes, and accordingly, block proteolysis of IκBα and release of active NF-κB. *2)* comparative analysis between the structure of the kinase domains of IKKs and related kinases indicated that, oxidation of Cys-179 in either IKKα or IKKβ would lead to the inhibition of kinase activity. *3)* ROS directly interfere with the DNA-binding activity of NF-κB dimers by oxidation of a conserved, redox-sensitive cysteine residue, Cys-62, located in a region of the Rel-homology domain (RHD) of p50 that directly contacts DNA. However, the threshold amount by which ROS leads to the activation or inhibition of NF-κB signaling remains unknown.

On the other hand, several studies have shown that hypoxia increases cellular ROS generation in cells or tissue (95-98), including isolated pancreatic islets and transplanted ones (33). While mitochondrial complex III has been demonstrated to be involved in ROS generation during

hypoxia (95; 98; 99), the exact mechanism by which hypoxia increase mitochondrial-generated ROS is not known. Recently, it has been suggested that the increased oxidant generated by mitochondria under hypoxia is not $O_2 \bullet -$ but rather ONOO- formed from a reaction between mitochondrially generated $O_2 \bullet -$ and $NO \bullet$ (88). As shown in *Figure 5-1A*, ONOO- concentrations dependent on oxygen levels, increasing under hypoxia. The hypoxia-increased production of ROS can modulate NF-κB activation as described above. Interestingly, NF-κB seems to be a key negative regulator of ROS formation. For instance, NF-κB has been demonstrated to suppress the TNF-induced ROS accumulation and cell death by inducing the expression of antioxidant ferritin heavy chain (FHC) (100; 101). As hypoxia gradually compromised NF-κB activity up to 8 hours in the current study, it might be plausible to assume that there might be an auto-amplifying loop between the compromised NF-κB and ROS production under hypoxia (*Figure 5-1B*). In this loop, NF-κB under hypoxia is not sufficient to induce antioxidant expression to eliminate accumulating ROS. High availability of ROS further compromises NF-κB activity and finally NF-κB activity is eliminated.

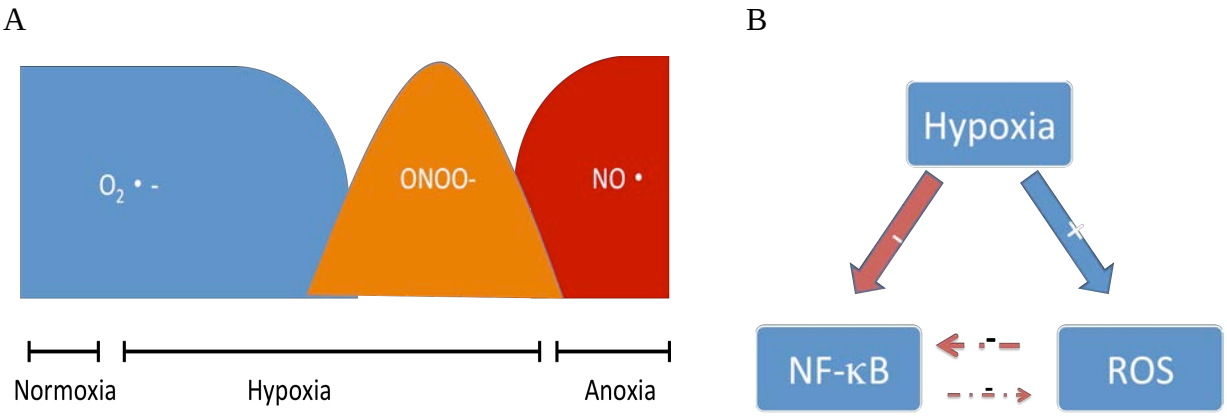

Figure 5-1 ROS and NF-κB activity under hypoxia. *(A)* Effect of oxygen availability on $O_2 \bullet -$, $NO \bullet$ and ONOO- production. While $O_2 \bullet -$ is the dominant free radical species under normoxia, both ONOO- and $O_2 \bullet -$ are available under hypoxic conditions. Mitochondrially generated $NO \bullet$ is expected to be the major mitochondrial free radical species produced under anoxic conditions. Adapted from *Poyton et al. 2009* (88). *(B)* Under prolonged hypoxia, ROS production is increase whereas NF-κB activity is inhibited. The compromised NF-κB activity is not sufficient to induce enough antioxidant expression to eliminate the ROS production, thus ROS accumulate by time which further compromise NF-κB activity and finally eliminate NF-κB activity.

Since pancreatic islets are poorly equipped with antioxidant enzymes including SODs, CAT and GPx, they could be highly sensitive to hypoxia-ROS-compromised NF-κB loop. The transplanted islets are subjected to an even more proinflammatory environment as the IBMIR-triggered infiltrated immune cells produce both ROS and inflammatory cytokines. These factors, together

with the hypoxic environment in the portal circulation, could have accelerated the hypoxia-ROS-compromised NF-κB loop as discussed above. Indeed, a fast dissolution of NF-κB activation in the transplanted islets is observed in the current study. While the number of cells showing NF-κB nuclear translocation increased at 2 hours post-transplantation, the intensity of the staining had decreased which dropped even further at 6 and 24 hours post-transplantation.

However, since the threshold of ROS leading to the activation or inhibition of NF-κB signaling remains unknown and the redox status of the islet cells in the current study was not checked, more detailed investigations are needed to elucidate the mechanisms.

5.2 Hypoxia-compromised NF-κB in pancreatic islets is pro-apoptotic

To clarify the controversially reported roles of NF-κB activity in pancreatic islets, we compared in parallel its function under normoxia or hypoxia, with and without combined cytokines stress. By utilizing a small molecular inhibitor, we demonstrated that NF-κB can exert distinct roles in pancreatic islets depending on the activation context. NF-κB blockage by BMS-345541 did not affect cell viability under normoxia in the absence of cytokines, indicating that BMS-345541 is non-toxic to pancreatic islets. However, BMS-345541 slightly increased cell death when cytokines presented, indicating a pro-survival role of NF-κB activity in normoxia. This is supported by the observation that cytokine cocktail, which leads to NF-κB activation, actually reduced islet cell death by both apoptosis and necrosis under normoxia. On the other hand, BMS-345541 did not have a significant impact on cell viability under hypoxic stress, indicating that hypoxia alone does not activate NF-κB signaling or NF-κB does not have a dominant role in determining cell death under such condition. In contrast, cell death was significantly augmented by cytokines cocktail under hypoxia, which could be reversed by BMS-345541 significantly and dose-dependently. These data indicate that cytokines-augmented islet cell death under hypoxia is NF-κB dependent and that NF-κB activity under this situation is predominantly pro-apoptotic.

Gene expression results give insight into the underlying events. Under normoxia, cytokine cocktail promoted expression of anti-apoptotic genes, including BCL-xL, c-FLIP, BCL-2 and VEGFa, all of which could be inhibited by IKKβ inhibitor. These data support an anti-apoptotic role of NF-κB activity in islets under normoxic conditions. In contrast, under hypoxia, cytokines induced a reduction in BCL-xL, c-FLIP, survivin, VEGFa and VEGFR2 gene expression, all of which could be restored by IKKβ inhibitor. These data indicate a pro-apoptotic role of cytokine-induced NF-κB activity under hypoxic conditions. The gene analysis data are in consistence with the results from flow cytometric cell apoptosis assay, and support the concept that NF-κB activity in

pancreatic islets is anti-apoptotic in normoxia but pro-apoptotic under hypoxia.

The opposite characteristics exerted by NF-κB under normoxic and hypoxic conditions in the current study indicate oxygen level as a key factor in determining the fate of pancreatic islets. As described above, we found that NF-κB activation in pancreatic islets was compromised both *in vitro* by hypoxia and *in vivo* by the hypoxic transplant environment. The compromised NF-κB activity by hypoxia provides an important hint for its different characteristics in hypoxia and normoxia.

It is known that the activity of NF-κB can be modulated by post-translational modifications and the functions of NF-κB at specific promoters and enhancers depend on its association with different co-activators and co-repressors (102). Several post-translational modifications of RelA have been described, the most important ones being the phosphorylation and acetylation (103). Basal repression of NF-κB in resting cells is controlled primarily by p50 homodimers associated with histone deacetylases-1 (HDAC-1), or the corepressor complex of silencing mediator for retinoic acid and thyroid hormone receptor (SMRT)/HDAC3, that bind to DNA and repress NF-κB-dependent gene expression. Following stimulation, RelA is phosphorylated at key residues both in its RHD and transactivation domain (TAD), which enhances transcriptional response. Phosphorylation events also modulate DNA binding and oligomerization of NF-κB, and weaken the affinity of its subunits for IκBα, all resulting in a more persistent NF-κB response (63). Meanwhile, a remodeling cascade is usually initiated by acetylation of histones H3 and H4 within regulatory regions and depends on p65-dependent recruitment of CBP or its homologue, p300, a coactivator protein that contains intrinsic histone acetyltransferase activity and mediates the acetylation of p65 (104; 105). Further modifications may also involve chromatin remodeling and conformational changes that lead to interaction of the enhancer with a proximal regulatory region (106).

In the current study, the activation of NF-κB signaling pathway was examined by checking the phosphorylation of p65 at serine 536, which is inhibited by hypoxia time-dependently in islet cells. Phosphorylation at Ser^{536} is known to affect the activity of NF-κB/RelA (57). Ser^{536} phosphorylation was shown to be critical for RelA's recruitment of CBP/p300 and its subsequent acetylation and transcriptional activity (107-109). Phosphorylation of Ser^{536} on RelA has been shown to favor interleukin-8 transcription mediated by TATA-binding protein-associated factor II31 in HELA cell, whereas in the absence of phosphorylation the hydrogen bond promotes preferential binding of the region around Ser-536 to the corepressor amino-terminal enhancer of split (AES) and represses p65 activity (108). Reconstitution of RelA-deficient murine embryonic fibroblasts with a non-phosphorylatable S536A mutant decreased TNF-α-induced p300

recruitment, acetylation of lysine 310, and subsequently decreased the transcription activity of NF-κB (107). Thus, the hypoxia-decreased Ser^{536} phosphorylation on p65 in islet cells observed in the current study could have led to decreased recruitment with its co-activator CBP/p300 and affect the transcription activity of NF-κB.

On the other hand, MAP kinase pathways have been shown to participate in the regulation of the transactivation process of NF-κB. Both p38 and extracellular signal-regulated kinase (ERK) pathways have been shown to be required for NF-κB-driven gene transcription in response to TNF in murine fibrosarcoma cells (110). Numerous studies reveal that MAPKs, especially p38 MAPK, can affect NF-κB activity during the multi-steps in the NF-κB activation process. p38 activity was shown to be required to enhance the accessibility of the cryptic NF-κB binding sites contained in H3 phosphorylated promoters, thus indicates that p38-dependent H3 phosphorylation may mark promoters for increased NF-κB recruitment (111). Mitogen- and stress-activated protein kinase-1 (MSK1), a downstream target of p38 and ERK MAPKs, was demonstrated to be activated by TNF, and subsequently associated with p65 in a stimulus-dependent manner and specifically phosphorylated p65 at Ser^{276} which is an essential element for engagement of nuclear cofactors and their associated HAT activity, thus leading to its positioning at NF-κB-containing promoter sections and selective stimulation of particular NF-κB-driven genes (112; 113). In addition, p38 MAPK was shown to be able to directly phosphorylate p300, which is a co-activator of NF-κB (114). In a recent study, cytokine stimulation in astrocytes leads to p38-dependent phosphorylation of p65 coactivator p300, which results in the association of p300 with p65 and subsequent acetylation of p65 at K310 which is important for transcriptional activity of NF-κB (115). Blockage of p38 activity has been shown to attenuate or delay NF-κB activation in the above mentioned studies. All these evidences highlight p38 as a relevant mediator of NF-κB transactivation. Therefore, p38 and ERK MAPK signaling pathways were investigated in the current study in parallel to the NF-κB pathway (*Figure 5-2*). Interestingly, a similar kinetics of hypoxia's effect on MAPK pathways was found. While short-term hypoxia for 2 hours triggers p38 pathway activation in both MIN6 and primary islets, this effect was largely inhibited after 4 hours hypoxia. TNF-triggered p38 pathway activation was confirmed in islet cells, but was abrogated by 8 hours hypoxia in MIN6 cells. While TNF-triggered p38 pathway activation sustained in primary islets after 8 hours, the intensity had decreased, and could be expected to diminish afterwards (need further confirmation). A similar kinetics was observed with ERK1/2 pathway. Based on these observations and the above mentioned studies, the hypoxia-impaired activity of p38 and ERK1/2 may lead to decreased association of RelA with its co-activators and

inhibits NF-κB transactivation in pancreatic islets under hypoxic environment.

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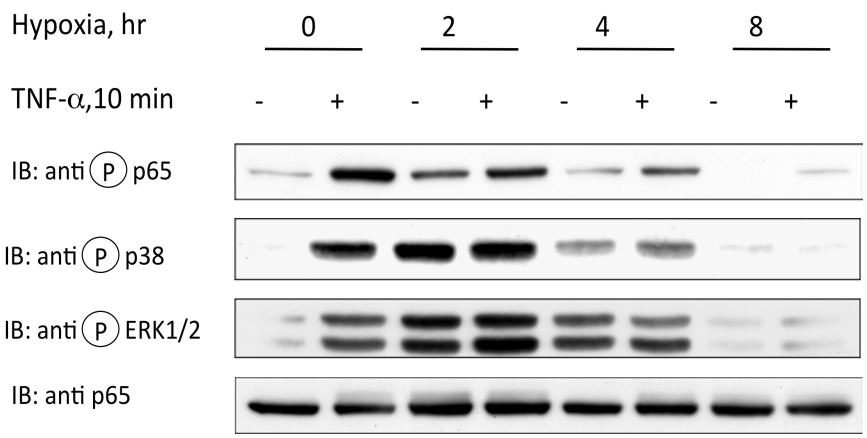

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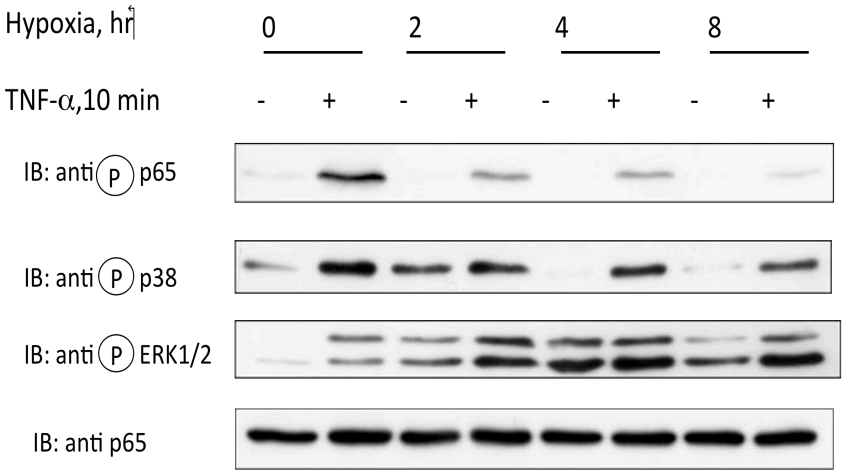

Figure 5-2 Prolonged hypoxia impairs p38 and ERK MAPK signaling pathways. MIN6 cells (A), or primary islets (B), were subjected to different time of hypoxia as indicated, followed by 10 minutes TNF-α stimulation followed by WB for checking NF-κB and MAPK signal pathways activation. Equal amounts of protein contained in cell extracts were separated by SDS-PAGE and analyzed by Western blotting for the occurrence and phosphorylation of NF-κB and MAPK signaling proteins. Phospho-specific antibodies were used to measure the phosphorylation of the NF-κB subunit p65 (Ser^{536}), p38 MAPK (Thr^{180}/Tyr^{182}) and p44/42 MAPK (Erk1/2) (Thr^{202}/Tyr^{204}).

Another factor that could potentially affect the transactivation of NF-κB in the current study is the hypoxia itself. Histone deacetylases (HDACs) are enzymes that catalyze the removal of acetyl groups from lysine residues in both histone and non-histone proteins. The activity and expression of HDAC were shown to be regulated by oxygen tension in human hepatoblastoma cells and increases under hypoxia (116). In a recent study, it was demonstrated that while it does not affect HDAC protein expression, hypoxia enhances phosphorylation of HDAC1 and HDAC2 by

enhancing CK2 kinase activity (117). The mechanism of HDAC activation in response to hypoxia was proposed in that study. The authors proposed that CK2 is activated by hypoxia and the catalytic subunits of CK2 subsequently shuttle to the nucleus and phosphorylate HDAC, leading to HDAC activation which contributes to tumor progression by pVHL downregulation and HIF-1α stabilization. Interestingly, increase p65-associated HDAC2 activity, a phenomenon that has been shown to mediate deacetylation of p65 and switch NF-κB from an activator to a repressor of transcription (119; 120), was observed in Hela cells under hypoxia (118). Indeed, the association of p65 and HDAC had been shown to be important for the active repression of anti-apoptotic gene expression by p65 following treatment with cytotoxic stimuli (69).

Taking together, the decreased Ser^{536} phosphorylation on p65 and impaired p38 and ERK MAPK pathway activation could lead to inhibited NF-κB transactivation by decreasing the association of p65 with its co-activator CBP/p300, whereas hypoxia could potentially increase the association of p65 with its co-repressor HDACs and increase the latter activity. Collectively, decreased association with the co-activators and increased association with the co-repressors might be responsible for the transformation of NF-κB from an activator of anti-apoptotic gene expression and a death inhibitor under normoxia into a repressor of these genes and a death promoter under hypoxia as demonstrated in the current study.

Based on these observations and inferences, we developed a schematic model for NF-κB's role on β-cell survival. As shown in *Figure 5-3*, under normoxic conditions where NF-κB activity is intact, cytokines induced normal NF-κB activation resulting in its nuclear translocation, association with the co-activator CBP/p300 and subsequent transcription of its anti-apoptotic target genes and improved cell survival; under hypoxic conditions, although cytokines can still induce NF-κB activation, NF-κB activation is compromised by hypoxia in this case and its activation by cytokines leads to its interaction with the co-repressor HDACs and result in the repression of anti-apoptotic genes and promotes cell death.

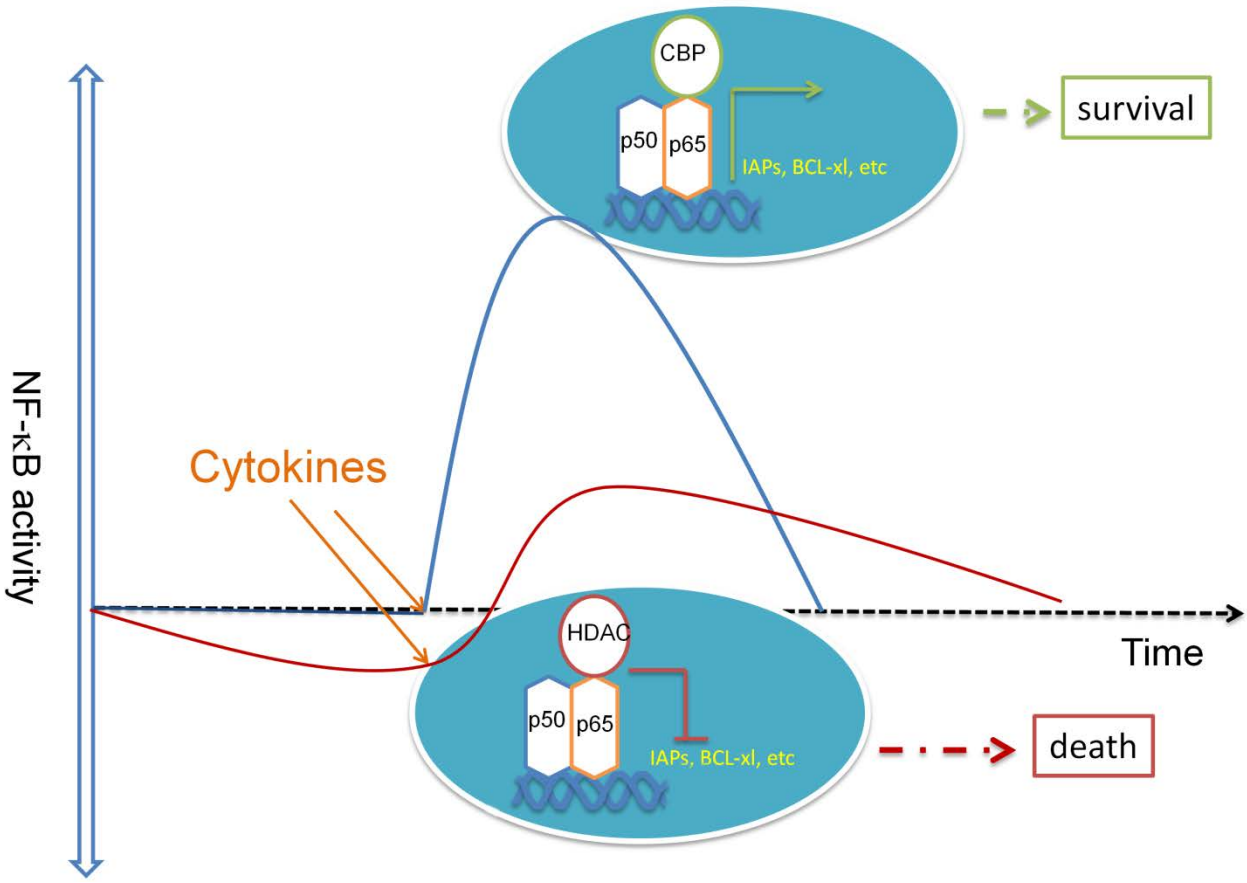

Figure 5-3 Possible mechanism for NF-κB's distinct roles on islet cell death under normoxic and hypoxic conditions in pancreatic islets. *(Blue curve)* Under normoxia when NF-κB activity in pancreatic islets is intact, stimulation with cytokines (TNF-α, IL-1β and IFNγ) leads to normal NF-κB activation and association with co-activator (CBP/p300), and preceding transcription of its target genes, including anti-apoptotic genes (BCL-xL, c-FLIP, survivin etc.), and promotes cell survival. *(Cardinal curve)* Under hypoxia, NF-κB activity is compromised by hypoxia. While cytokine stimulation can still lead to NF-κB activation, the compromised NF-κB activation results in the association of p65 with its co-repressor (HDACs), and subsequently swifts NF-κB into an active repression of anti-apoptotic genes and promotes cell death.

While this model needs further confirmations, the current findings extend our understanding of the complicated functions of transcription factor NF-κB. Particularly, our data support the concept that NF-κB activation will lead to cell death when IKKβ or NF-κB activity have been compromised (84). While this hypothesis has been proposed for years, its pathophysiological relevance remains unclear. Here, we demonstrated that hypoxia compromises NF-κB activity and transforms it into a pro-apoptotic transcription factor. Importantly, both the compromised NF-κB

activity and its pro-apoptotic function were confirmed in our *in vivo* study where IKKβ inhibitor significantly prolonged the survival of the transplanted islets. To our knowledge, this is the first evidence providing the *in vivo* relevance of the compromised NF-κB activity and its subsequent pro-apoptotic function under a pathophysiological environment.

5.3 The primary target of systemic NF-κB inhibition is the transplanted islet

In the current study, we achieved systemic NF-κB inhibition in the transplanted mice by intraperitoneal BMS-345541 injection. This could have resulted in NF-κB inhibition in different organs and cell types; therefore, we examined the impact of NF-κB inhibition on other cell types which have been suggested to be involved in the engraftment of the transplanted islets in intraportal transplantation.

5.3.1 BMS-345541 treatment has no significant effect on liver apoptosis/necrosis

Post-transplantation liver injury, as detected by elevated liver enzyme aspartate aminotransferase (AST), was reported in clinical islet transplantation (121). In a recent study, islet embolism in the portal vein has been suggested as a major cause of functional loss following intraportal islet transplantation (52). In the latter study, the authors demonstrated that multi-foci of liver necrosis and proinflammatory cytokines (IL-1β, IL-6 and TNF-α) production from the ischemic grafted liver tissue were quickly induced by islet transplantation. Ischemic-preconditioning (IP), a procedure of pre-exposure of the liver to transient ischemia and reperfusion, protected the liver from embolism induced ischemic injury and prevented early islet graft failure. Recently, liver tissue NF-κB activation was demonstrated and associated with liver necrosis and proinflammatory cytokine production following intraportal islet transplantation (122). These reports highlight the importance of liver tissue injuries in the process of islet engraftment.

Therefore, liver tissue NF-κB activation and cell apoptosis/necrosis were examined in this study. As shown in *Figure 4-11,* in contrast to the report (122) where *in vivo* bioluminescence imaging (BLI) technique was applied and NF-κB activation in the liver tissue was detectable in 3 hours and increased at 24 hours post-transplantation, immunohistochemistry (IHC) on grafted liver sections reveals that liver NF-κB activation was only detectable at 2 hours after transplantation. Different techniques (BLI *vs* IHC) used to detect NF-κB activation may account for the different kinetics of NF-κB activation in the liver tissue. The mice used in the BLI assay were transgenic mice expressing the proximal 5' human immunodeficiency virus long terminal repeat (referred to as HIVLTR/Luciferase, HLL mice). While BLI technique offers the possibility of real time and

quantitative assessment of tissue NF-κB activity, it has several limitations (123). Firstly, the bioluminescence signal depends on the luciferase-catalyzed bioluminescent reaction which requires ATP, oxygen, and the substrate luciferin. However, oxygen and ATP availability is limited in the hypoxic areas where islets embolized; and the availability of luciferin depends on tissue penetration which is alerted after islet embolism. Secondly, bioluminescence of the non-transplanted tissues in these mice revealed high basal signals. Lastly and most importantly, maximum bioluminescence, even in the same mouse, occurred at different times over the 30 minutes imaging session and at different times on different days, thus the results have to be normalized by each session and experimental group which makes the results not as strong as expected. On the contrary, while IHC can offer only qualitative assessments, it is more specific and sensitive. Nevertheless, both methods revealed the activation of NF-κB signaling in the liver tissue, which might contribute to the cellular inflammatory reactions and islet graft loss.

Liver necrosis was also examined the current study. IHC on the early grafted liver tissue showed that, TUNEL positive hepatocytes were detectable within 2 hours after transplantation (*Figure 5-4A*) and prevailed by time. In contrast, caspase-3 positive liver cells were only detected at 24 hours after transplantation, interestingly, mostly stained on the border of necrotic live tissue and normal liver tissue. As TUNEL staining was shown to detect cell death in both apoptosis and necrosis forms (124; 125) and caspase-3 is more specific for apoptosis pathway, our data indicate that islet emboli induce liver cell death by both primary apoptosis pathway and (secondary) necrosis.

Interestingly, IHC on the transplanted islets shows no TUNEL positive islets until 24 hours post-transplantation when very few TUNEL positive islet grafts were found. All the early grafted islets (within first 24 hours after transplantation) checked by IHC were negative for caspase-3, indicating that the transplanted islets died majorly by necrosis after transplantation which is in consistence with our *ex vivo* islet apoptosis analysis.

By comparing the IHC images from vehicle mice and BMS-345541 treated mice, we found that BMS-345541 treatment was initially protective for the liver tissue stressed by hypoxia following islet embolism. Very few TUNEL positive cells were found in the treated mouse liver sections at 2 hours post-transplantation, at which time point many TUNEL positive were found in the vehicle mouse liver sections in the areas where the transplanted islets took residence. However, no noticeable differences were found between the groups at the later time points (6 & 24 hours). We conclude that systemic NF-κB inhibition at the current dosage had no significant effect on liver necrosis/injury induced by liver embolism. However, as the BMS-345541 treatment was tailored

to the pancreatic islets in the current study, we cannot exclude that a higher dosage of BMS-345541 administered for a longer time has some effect on the destruction of liver cells.

While the anti-apoptotic function of NF-κB has been long known for protecting hepatocytes from TNF-induced apoptosis (126), it was demonstrated that NF-κB inhibition by IKKβ knockout is not sufficient for induction of liver failure or hepatocyte apoptosis, even in the presence of high level of circulating TNF (127). Later studies revealed that IKKβ deletion does not significantly impair NF-κB activation in hepatocytes in response to TNF (which is confirmed in the IHC on p65 translocation in hepatocytes at 2hr, *Figure 4-12*), and sensitivity of the hepatocytes to TNF is mainly determined by the extent of inhibition of the NF-κB response (128). Interestingly, a chemical inhibitor of IKKβ, AS602868, was demonstrated to protect mice from liver ischemia/reperfusion injury without sensitizing them toward TNF-induced apoptosis (129). The protective effect of IKKβ inhibition on liver tissue was confirmed in this study at early time point (2 hours post-transplantation). Our data, together with the above mentioned studies indicate that IKKβ inhibition could be potentially protective for the liver tissue against liver injury induced by islet embolism; or at least not detrimental for the grafted liver.

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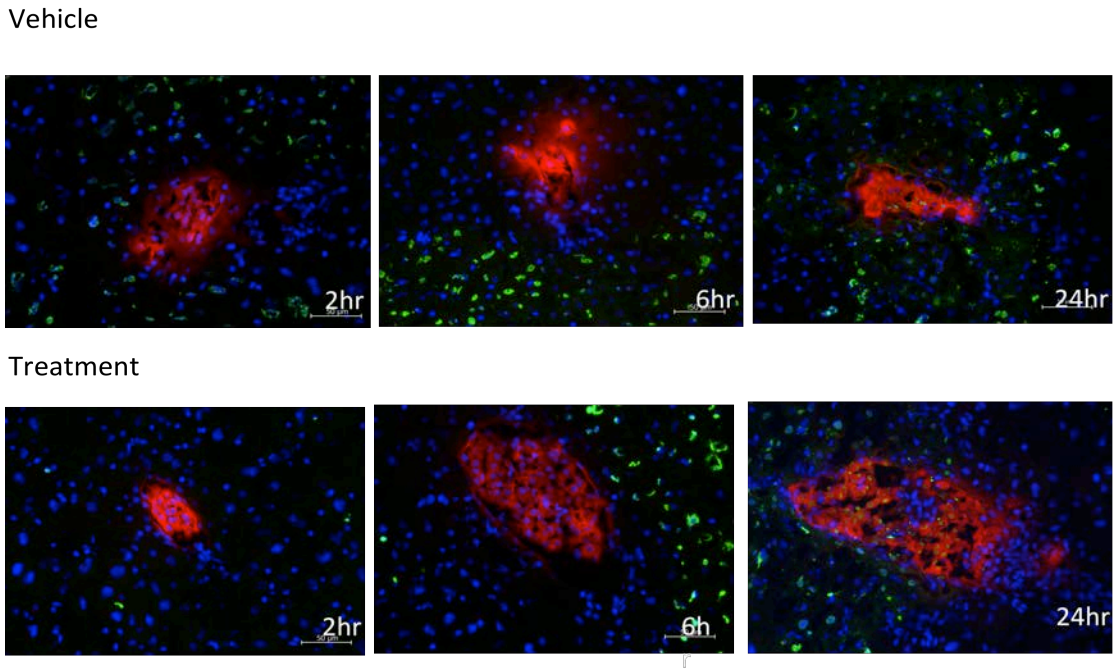

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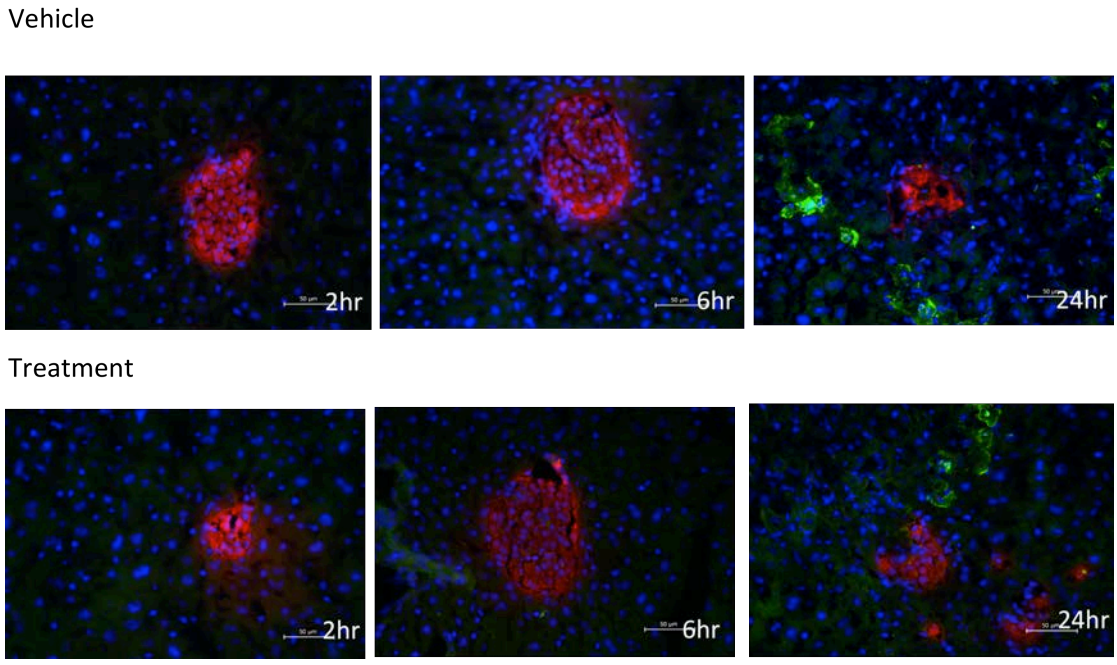

Figure 5-4 Representative images of TUNEL (A) and caspase-3 (B) IHC staining on the early grafted liver sections. Early grafted livers were recovered as described. Cryosections from these grafted livers were probed for nuclei (blue), TUNEL or caspase-3 (green) and insulin (red). Upper panels: cryosections from the vehicle mice livers; lower panels: cryosections from the treated mice livers at the corresponding time points. Magnification x200, bar=50 μm.

5.3.2 BMS-345541 has no impact on neutrophil migration/infiltration

Neutrophilic granulocytes have been implicated to be an important player in early islet destruction. They are shown to be the predominant cell type infiltrating pancreatic islets after incubation with blood in a loop tube system (46). In this system, neutrophilic granulocytes appeared in the islets after 15 minutes of incubation, increased at 1 hour and peaked at 2 hours. The infiltrated neutrophils can negatively affect islet graft function by cytotoxic attack and phagocytosis. Importantly, these cells can generate superoxidase to form reactive oxygen species (ROS) and release cytokines upon activation (46), which further deteriorate the inflammation and destroy the transplanted islets.

Recently, the contribution of neutrophils in the early graft loss was confirmed in a recent islet transplantation mouse study (130). Gr-1^{+}CD11b^{+} neutrophils, through their production of IFN-γ, were suggested to be the major effector cells responsible for early islet destruction after intraportal transplantation. Importantly, the injection of monoclonal antibody against Gr-1 or CD11b was able to prevent early islet destruction, highlighting the importance of neutrophils in early islet destruction.

Therefore, we examined neutrophil migration/infiltration in the islet grafts (*Figure 5-5*). IHC on the grafted liver sections with antibody against CD11b (or Gr-1) revealed neutrophil migration at 2 hours after transplantation when most of the neutrophils appeared surrounding the islet graft. Neutrophils migration/infiltration peaked at 6 hours post-transplantation when infiltration could be clearly detected inside the islet grafts. Neutrophil infiltration faded out 24 hour after transplantation.

No obvious difference was found between the vehicle mice and BMS-345541 treated mice.

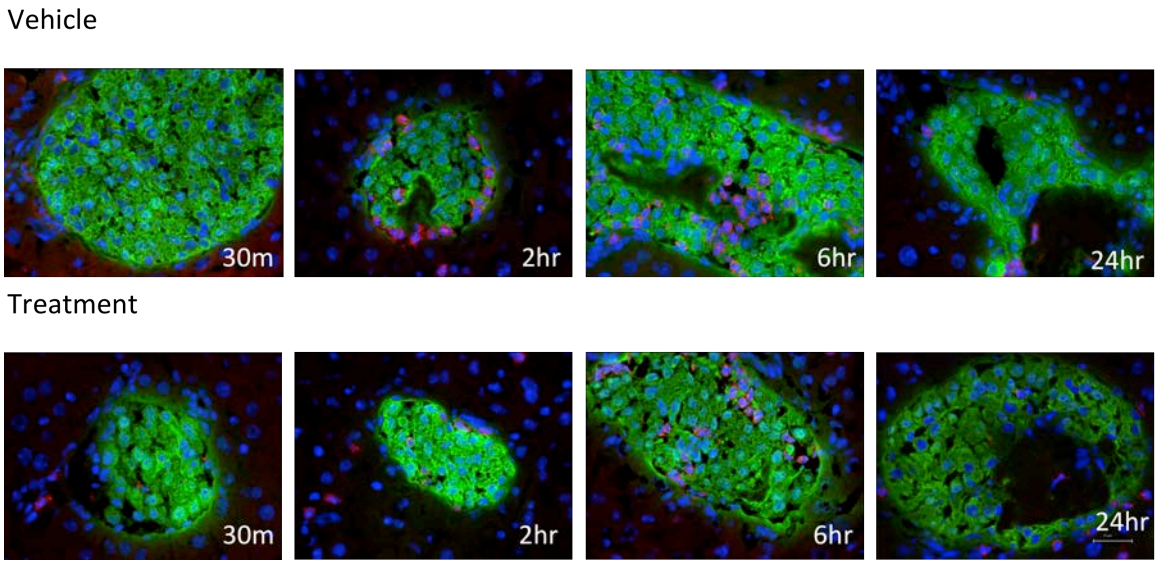

Figure 5-5 BMS-345541 administration has no impact on neutrophil migration and infiltration into islet grafts within 24 hours. Early grafted livers were recovered as described. Cryosections from these grafted livers were probed for nuclei (blue), CD11b (red) and insulin (green). Upper panel: cryosections from the vehicle mice livers; lower panel: cryosections from the treated mice livers at the corresponding time points. Magnification x400, bar=25 μm.

Together with our liver necrosis analysis, these results are in agreement with the concept that the function NF-κB is regulated by a cell and tissue specific manner (131). Meanwhile, these data indicate that the primary target of systemic NF-κB inhibition is the transplanted islets, and that the improvement in islet transplantation by NF-κB inhibition resulted mainly by improving the resistance of the transplanted islets to the hypoxic transplantation stress and thus prolonging the survival of transplanted islets.

5.4 Implications for clinical islet transplantation

Currently, clinical islet transplantation is hampered by low transplantation efficiency caused by massive islet graft destruction after transplantation. In the current study, the cytokine-induced NF-κB activity is found to be the dominant pro-apoptotic signal in the pancreatic islets under hypoxic transplantation environment. Its blockage improved pancreatic islet survival under combined hypoxia and cytokines stress *in vitro* and improved islet transplantation outcome *in vivo*.

While the effector molecules by which hypoxia modulates the transcription activity of NF-κB in pancreatic islets remains to be elucidate in the future, our findings have several implications for the clinical islet transplantation.

Firstly, systemic NF-κB blockage can improve islet graft survival in the immediate stage after transplantation. At this stage when transplanted islets suffer from the combined stress of hypoxia and proinflammatory cytokines, cytokine-induced NF-κB activation will result in cell death because NF-κB activity is compromised by hypoxia and is pro-apoptotic.

Secondly, NF-κB blockage should not be continued when islet engraftment is completed and new microvasculature has established. As NF-κB activity is slowly reversible after hypoxia stress, it might have gained normal activity when the engraftment is finished. Under this circumstance, NF-κB activity is anti-apoptotic and its blockage can lead to cell death when cytokines are present.

Finally, while the potential side-effects need to be carefully examined, pharmaceutical IKKβ inhibition could be a practical strategy to improve primary islet engraftment in intraportal islet transplantation.

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7. Declaration

I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and referenced all text passages that are derived literally from or are based on the content of published or unpublished work of others, and all information that relates to verbal communications. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen in carrying out the investigations described in the dissertation.

_____ _____

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8. Appendix

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Curriculum Vitae

Der Lebenslauf wurde aus der elektronischen Version der Arbeit entfernt.

The curriculum vitae was removed from the electronic version of the paper.

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