Die Bedeutung von freien Radikalen für die Toxizität proinflammatorischer Zytokine und die Apoptoseinduktion in insulinproduzierenden Zellen

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Kurzzusammenfassung

Der Typ 1 Diabetes mellitus wird durch eine selektive Zerstörung der insulinsezernierenden β-Zellen des Pankreas verursacht. Die Zerstörung wird insbesondere durch zytotoxische Zytokine mediiert, die eine zur β-Zellapoptose führende Signalkaskade auslösen. Dabei kommt der intrazellulären Bildung von reaktiven Sauerstoffspezies (ROS) eine besondere Bedeutung zu. Im Rahmen dieser Dissertation konnte gezeigt werden, dass in β-Zellen ein Ungleichgewicht zwischen dem antiapoptotischen Protein Bcl-2 und dem proapoptotischen Protein Bax herrscht. Dieses Ungleichgewicht wird durch die zytokinvermittelte Reduktion der Bcl-2 Expression zusätzlich verstärkt, so dass die Permeabilisierung der äußeren Mitochondrienmembran begünstigt wird. Durch die Überexpression von Katalase in Mitochondrien (MitoKatalase) konnte das Bax/Bcl-2 Ungleichgewicht kompensiert und die zytokinvermittelte Abnahme von Bcl-2 verhindert werden. Dies lässt den Schluss zu, dass in den Mitochondrien gebildetes H_2O_2 u.a. die Expression von Bcl-2 supprimiert. Um die zytokininduzierte ROS Bildung zu quantifizieren, wurde der H_2O_2-sensitive Fluoreszenz-sensor HyPer spezifisch im zytosolischen und mitochondrialen Kompartiment überexprimiert. So konnte gezeigt werden, dass Zytokine lediglich in Mitochondrien eine signifikant gesteigerte H_2O_2 Bildung bewirken. H_2O_2 kann über die metallkatalysierte Fenton Reaktion zum aggressiven Hydroxylradikal umgewandelt werden, welches eine hohe Reaktivität gegenüber zellulären Strukturen aufweist. Nach Exposition von β-Zellen mit Zytokinen konnten massive oxidative Schäden der mitochondrialen DNA sowie eine erhöhte Peroxidation von Cardiolipin nachgewiesen werden. Ferner wurde die Freisetzung von Cytochrom c und SMAC/DIABLO aus den Mitochondrien in das Zytosol und die daraus resultierende Aktivierung von Caspase-9 und -3 beobachtet. Die Detoxifizierung von H_2O_2 durch MitoKatalase verhinderte die oxidative Schädigung von zellulären Strukturen und damit die Destabilisierung der mitochondrialen Integrität, sowie die Aktivierung von Caspase-9 und -3. Zudem führte die Überexpression der NF-κB Untereinheit c-Rel über die Induktion der antiapoptotischen Proteine Bcl-X_L und c-IAP2 zu einer erhöhten Resistenz von β-Zellen gegenüber Zytokinen. Darüber hinaus konnte eine Beteiligung der ER-spezifischen Caspase-12 im zytokinvermittelten β-Zelltod sowie eine Interaktion mit der Caspase-9 ausgeschlossen werden. Diese Ergebnisse belegen die zentrale Bedeutung des mitochondrialen Apoptosesignalweges und der mitochondrialen Bildung von ROS für den zytokinvermittelten β-Zelltod beim Typ 1 Diabetes mellitus.

Schlagwörter: Diabetes mellitus, ROS, intrinsische Apoptosesignalkaskade

Abstract

Type 1 diabetes mellitus is caused by a selective destruction of insulin-secreting pancreatic β-cells. The destruction is particularly mediated by cytotoxic cytokines due to the induction of signalling pathways finally leading to β-cell apoptosis. At this process reactive oxygen species (ROS) generated during cytokine-mediated β-cell destruction are involved as additional mediators of β-cell death. The results presented in this thesis revealed that β-cells exhibit an imbalance between the antiapoptotic protein Bcl-2 and the proapoptotic protein Bax. This imbalance is further accelerated by the cytokine-induced reduction of the Bcl-2 expression facilitating the outer mitochondria membrane permeabilisation. However, the overexpression of catalase in mitochondria (MitoCatalase) could compensate for Bax/Bcl-2 imbalance and prevent the cytokine induced Bcl-2 reduction, indicating that H_2O_2 generated within the mitochondria suppresses the Bcl-2 expression. In order to quantify the cytokine-induced ROS formation, the H_2O_2-sensitive fluorescence sensor HyPer was specifically overexpressed in the cytosolic and mitochondial compartment. It could be shown that cytokines cause a significantly increased H_2O_2 formation only in mitochondria. H_2O_2 can be converted by the iron catalysed Fenton Reaction into aggressive hydroxyl radicals which possess high reactivity against cellular structures. Exposure of β-cells to cytokines resulted in massive oxidative damages of mitochondrial DNA and an elevated cardiolipin peroxidation. Moreover, a release of cytochrom c and SMAC/DIABLO from the mitochondria into the cytosol and the consequent activation of caspase-9 and -3 could be observed. Detoxification of H_2O_2 by MitoCatalase prevented the oxidative damage of cellular structures and thereby the destabilisation of the mitochondrial integrity as well as the activation of caspase-9 and -3. Furthermore, overexpression of the NF-κB subunit c-Rel led to a higher resistance of β-cells against cytokines by the induction of the anti-apoptotic proteins Bcl-X_L and c-IAP2. In addition the contribution of the ER-specific caspase-12 in the cytokine induced β-cell death and an interaction with caspase-9 could be excluded. These results revealed that the mitochondrial apoptotic pathway and the mitochondrial ROS formation are crucial for the cytokine induced β-cell death in type 1 diabetes mellitus.

Keywords: Diabetes mellitus, ROS, intrinsic apoptosis signalling pathways.

Inhaltsverzeichnis

1 Einleitung

1.1 Definition und Klassifikation des Diabetes mellitus

Der Diabetes mellitus ist charakterisiert durch eine Stoffwechseldysregulation, die durch den Leitbefund der chronischen Hyperglykämie definiert ist. Die Ursache ist entweder eine gestörte Insulinsekretion, eine verminderte Insulinwirkung oder eine Kombination beider Faktoren (Kerner *et al.* 2004). Die Prävalenz dieser endokrinen Stoffwechselerkrankung hat epidemische Ausmaße angenommen und steigt stetig an. Gegenwärtig sind weltweit mehr als 285 Millionen Menschen an Diabetes mellitus erkrankt, bis zum Jahr 2030 wird diese Zahl schätzungsweise auf 439 Millionen ansteigen (Shaw *et al.* 2010).

Unter ätiologischen und pathogenetischen Gesichtspunkten werden der insulinpflichtige Typ 1 Diabetes mellitus (T1DM), früher auch als jugendlicher bzw. juveniler Diabetes mellitus oder IDDM bezeichnet, vom nicht-insulinpflichtigen Typ 2 Diabetes mellitus (T2DM), auch als Altersdiabetes oder NIDDM bezeichnet, unterschieden. Am Typ 1 Diabetes mellitus (einschließlich der spät manifestierenden Form: LADA, *Late Autoimmune Diabetes in the Adult*) leiden ca. 10-20 % aller Erkrankten. Die Inzidenz dieses Typs ist am höchsten bei Kindern und Jugendlichen. Etwa 90 % aller Diabetesfälle im jungen Lebensalter zählen zum Typ 1 Diabetes und ca. 40 % dieser Patienten entwickeln die Krankheit bereits vor dem 20. Lebensjahr. Deshalb ist der Typ 1 Diabetes eine der häufigsten chronischen Erkrankungen im Kindes- und Jugendalter (Daneman 2006; Jahromi & Eisenbarth 2007). Dagegen sind dem Typ 2 Diabetes mellitus, der weiterhin in 2a (ohne Adipositas) und 2b (mit Adipositas) unterteilt wird, 80-90 % der Erkrankten zuzuordnen (Waldhäusl & Lenzen 2007). Die steigende Inzidenz dieser Form tritt vermehrt im höheren Lebensalter auf.

An der Entstehung beider Diabetesformen sind sowohl genetische als auch unterschiedliche exogene Faktoren beteiligt, so dass beide Formen des Diabetes mellitus als multifaktorielle und polygenetische Krankheiten beschrieben werden. Als genetisch prädisponierende Faktoren für die Entstehung des insulinpflichtigen Typ 1 Diabetes gelten insbesondere Histokompatibilitätsantigene des HLA (Humanes Leukozyten Antigen)-Systems, die auf dem kurzen Arm des Chromosoms 6 lokalisiert sind. Darüber hinaus sind genetisch determinierte Loci außerhalb des HLA-Systems, wie z.B. der Insulin-, CTLA4- (*cytotoxic T-lymphocyte antigen* 4) und der PTPN22-Locus (*phosphatase non-receptor type* 22) für die Diabetessuszeptibilität von Bedeutung. Infolge einer T-Zell-vermittelten Autoimmunreaktion mit lymphozytärer Insulitis kommt es hierbei zur selektiven Zerstörung der insulin-produzierenden β-Zellen des Pankreas und damit zum absoluten Insulinmangel, was eine

lebenslange Substitution des Hormons erforderlich macht (Kim & Polychronakos 2005; Daneman 2006; Jahromi & Eisenbarth 2007; Concannon *et al.* 2009). Diese immungenetische Prädisposition allein genügt jedoch nicht, um den Typ 1 Diabetes zu manifestieren. So werden exogene Triggerfaktoren postuliert, die in Interaktion mit den immungenetischen Faktoren zur selektiven Zerstörung der insulinproduzierenden β-Zellen des Pankreas führen (Waldhäusl & Lenzen 2007).

Als mögliche exogene Faktoren, die diese Autoimmunreaktion auslösen können, werden virale Infektionen und Umwelteinflüsse, wie allergene Nahrungsbestandteile (z.B. gluten- und kuhmilchhaltige Lebensmittel) vermutet (Akerblom & Knip 1998; Knip *et al.* 2005; Daneman 2006; van der Werf *et al.* 2007; Goldberg & Krause 2009; Richer & Horwitz 2009).

Der Typ 2 Diabetes dagegen ist genetisch stärker determiniert als der Typ 1 Diabetes. Es besteht jedoch kein direkter Zusammenhang zwischen der Entstehung des Typ 2 Diabetes und dem HLA-System. Beim Typ 2 Diabetes mellitus handelt es sich zu Beginn um eine ausgeprägte Störung der Insulinwirkung und einen Insulinsekretionsdefekt der ansonsten intakten β-Zellen (Rahier *et al.* 2008). Im weiteren Verlauf der Diabeteserkrankung kommt es zur Abnahme der β-Zellmasse (Del Prato & Marchetti 2004), was über das Stadium der gestörten Glucosetoleranz letztlich zu einer chronischen Hyperglykämie führt.

Aufgrund der chronisch erhöhten Blutglucosekonzentration kommt es bei beiden Diabetesformen, in Abhängigkeit von der Qualität der Glucosestoffwechseleinstellung, zu diabetischen Folgeschäden. Zu diesen zählen kardiovaskuläre Komplikationen, Nephropatie, Retinopathie und Polyneuropathie. Eine Ursache für diese Schädigungen sind u.a. nicht-enzymatische Glykosylierungen, die zu einer Strukturveränderung oder einem Funktionsverlust der betroffenen Proteine führen (Waldhäusl & Lenzen 2007).

1.2 Pathogenese des Typ 1 Diabetes mellitus

Der immunpathologische Prozess des Typ 1 Diabetes ist schleichend und beginnt schon Jahre vor der Diabetesmanifestation. Während dieser progressiven β-Zellzerstörungsphase, der sog. prädiabetischen Phase, werden bereits immunologische und metabolische Veränderungen wie z.B. inselspezifische Autoantikörper und aktivierte Lymphozyten im peripheren Blut nachgewiesen (Pihoker *et al.* 2005; Jahromi & Eisenbarth 2007).

Autoantikörper gelten als besondere immunologische Marker zur Prädiktion einer Autoimmunpathogenese. Von Bedeutung sind hierbei Antikörper gegen zytoplasmatische Inselzellbestandteile (ICA), Insulin (IAA), die 65-kD-Isoform des Enzyms Glutamatdecarboxylase (GAD65), Tyrosinphosphatase-homologen Proteine (IA-2) und gegen

den kürzlich entdeckten Zinktransporter 8 (ZnT8). So weisen um die 90 % aller neu diagnostizierten Typ 1 Diabetiker Autoantikörper gegen ein oder mehrere dieser o.g. Antigene auf. Der prädiktive Wert der Autoantikörper nimmt sogar bis auf 98 % zu, wenn Antikörper gegen den ZnT8 in Kombination mit weiteren Autoantikörpern nachgewiesen werden (Pihoker *et al.* 2005; Wenzlau *et al.* 2007; Achenbach *et al.* 2009). Während dieser pädiabetischen Phase sind die Patienten klinisch asymptomatisch.

Mit zunehmendem Verlust der β-Zellmasse treten metabolische Störungen, wie z.B. eine gestörte Glucosetoleranz in den Vordergrund. Nach einer β-Zellzerstörung von mehr als 80 % wird eine erhöhte Nüchternblutglucosekonzentration beobachtetet (Knip 2002). Erst zu diesem Zeitpunkt wird bei Patienten klinisch der Typ 1 Diabetes diagnostiziert, da nun die ausgeprägte Hyperglykämie zu den typischen diabetischen Symptomen wie Polyurie, Polydipsie, Ketoazidose und allgemeiner körperlicher Schwäche führt. Die bereits zum Zeitpunkt der klinischen Manifestation des Typ 1 Diabetes vorangeschrittene β-Zellzerstörung führt zwingend zu einer lebenslangen Substitution des lebenswichtigen Insulins.

Die genaue Ätiologie des Typ 1 Diabetes mellitus ist nach wie vor ungeklärt. Das liegt zum größten Teil an der Komplexität der pathogenetischen Prozesse, welche zur Entwicklung und Manifestation dieser Krankheit führen.

Als mögliche Ursache für die Zerstörung der insulinproduzierenden β-Zellen werden die unter Punkt 1.1 erwähnten exogenen Triggerfaktoren vermutet. Potentielle Trigger des gegen die β-Zellen gerichteten Autoimmunprozesses können Viren, insbesondere Entero-, Rubella-, Rubula-, Zytomegalie- und vor allem die Coxsackie-B4-Viren sein (van der Werf *et al.* 2007; Goldberg & Krause 2009; Richer & Horwitz 2009). Die starke Sequenzhomologie zwischen dem hoch exprimierten Enzym Glutamatdecarboxylase (GAD65) und dem PC2-Protein des Coxsackie-B4-Virus wird als mögliche Ursache für die Virusgenese vermutet. So wäre es daher möglich, dass die gegen Coxsackie-B4-Virus gerichteten Immunzellen auch die eigenen β-Zellen attackieren (van der Werf *et al.* 2007; Richer & Horwitz 2009), was zu deren Zerstörung führt.

Diese Triggerereignisse, wie Viren und allergene Nahrungsbestandteile führen zu einer Aktivierung von autoaggressiven Immunzellen, die die β-Zellen gezielt attakieren können. Dabei wird angenommen, dass diese exogenen Faktoren die Selektion von autoaggressiven Zellen durch molekulare Mimikry positiv beeinflussen (Pickup & Williams 2003; Knip *et al.* 2005).

1.2.1 Kopenhagener Modell der Pathogenese des Typ 1 Diabetes

Als Konsensusmodell der autoimmunen β-Zellzerstörung hat sich das von Nerup *et al*. entwickelte Kopenhagener-Modell etabliert, dass das Zusammenspiel von zellulären und humoralen Immunmediatoren bei der β-Zellzerstörung beschreibt (Nerup *et al.* 1994). Während der 90er Jahre wurden jedoch weitere wichtige Befunde zur Ätiopathogenese des Typ 1 Diabetes erhoben, die eine Revision des 1994 formulierten Kopenhagener-Modells notwendig machte (Freiesleben De Blasio *et al.* 1999). Dieses revidierte Modell macht deutlich, dass mehrere Faktoren eine β-Zellzerstörung initiieren können und der darauf folgende pathologische Prozess von vielen sich gegenseitig beeinflussenden Vorgängen abhängt (Abb.1.1). So beginnt die Autoimmunzerstörung der β-Zellen mit einem initialen Angriff der Immunzellen, die zur Freisetzung von β-Zellproteinen führt. Diese Proteinfreisetzung kann direkt durch Viren und Chemikalien, sowie indirekt durch Nahrungsbestandteile oder Zytokine initialisiert werden. In diesem Zusammenhang spielt die genetische Prädisposition der Patienten eine wichtige Rolle (Hürter & Lange 1997). So hat der Nachweis der engen Beziehung zwischen MHC Antigenen und dem T1DM (Cudworth & Woodrow 1974; McDevitt & Bodmer 1974; Nerup *et al.* 1974) sowohl das Konzept des multifaktoriellen Erbmodus als auch die These der genetischen Heterogenität bestätigt (Cudworth 1978; Rotter & Rimoin 1978).

Die freigesetzten β-Zellproteine werden von antigenpräsentierenden Immunzellen wie Makrophagen, Monozyten oder dendritischen Zellen aufgenommen und als Antigen präsentiert (antigenpräsentierende Zellen, APC). Diese bewirken die Aktivierung spezifischer T-Helferzellen durch Präsentation von Antigenfragmenten auf der Oberfläche der MHC-II Moleküle. Aktivierte T-Helferzellen sezernieren proinflammatorische Zytokine (Interleukin-1β (IL-1β), Tumornekrosefaktor–α (TNF-α) und Interferon-γ (IFN- γ)), von denen insbesondere IFN-γ in einer Feedback-Stimulierung die Expression von MHC-II Molekülen induziert. Des Weiteren wird die Zytokinproduktion der anderen antigenpräsentierenden Zellen weiter angeregt, die zur Generierung von freien Radikalen, wie Stickstoffmonoxid ($NO^{\bullet}$), und Superoxidanion in den β-Zellen des Pankreas führt (Lenzen 2008). Dieser *Circulus vitiosus* aus Antigenfreisetzung und Antigenpräsentation verstärkt sich selbst und löst über die sezernierten löslichen Todesmediatoren Signalkaskaden aus, die letztlich die β-Zellapoptose bewirken. Ergebnis dieses Prozesses sind Langerhanssche Inseln, die keine β-Zellen mehr enthalten und als so genannte „*End-Stage Islets*“ bezeichnet werden (Freiesleben De Blasio *et al.* 1999).

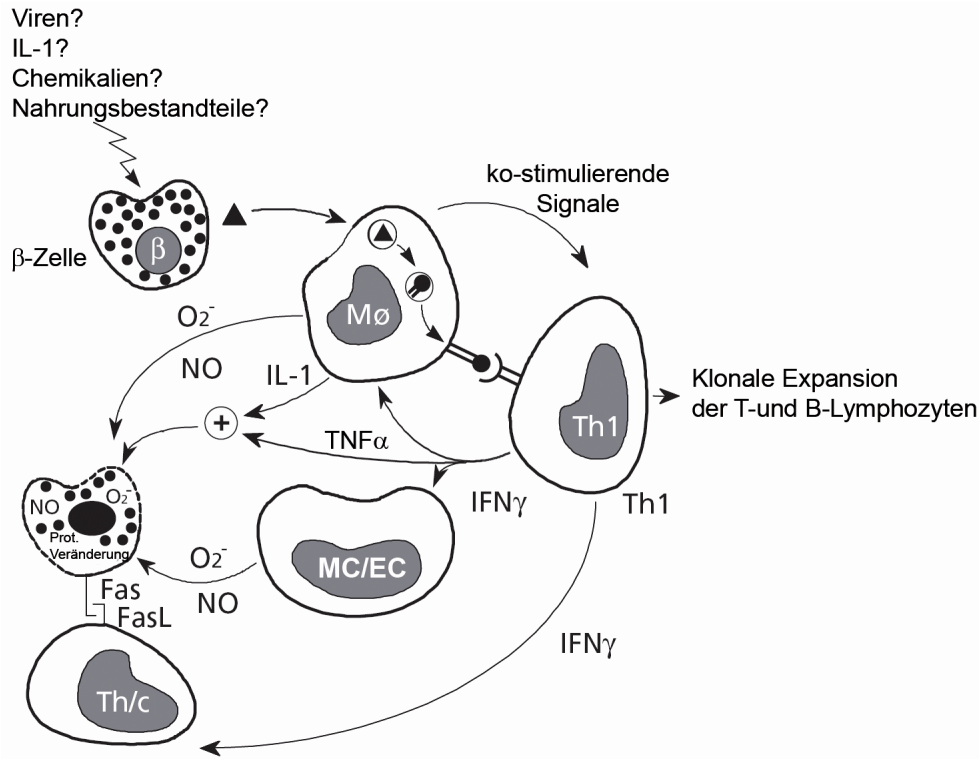

Abb.1.1 Das Kopenhagener-Modell der Pathogenese des Typ 1 Diabetes mellitus nach Freiesleben De Blasio *et al.* (1999)

Eine genetische Prädisposition kombiniert mit exogenen Triggern wie Infektionen mit β-zellzytotoxischen Viren oder Nahrungsbestandteilen initiiert die β-Zellzerstörung. Dabei werden β-zellspezifische Proteine freigesetzt, die durch antigenpräsentierende Zellen (APCs) aufgenommen, prozessiert und präsentiert werden. In einer zweiten Phase (Verstärkungsphase) werden durch die aktivierten APCs Zytokine (IL-1β), freie Radikale ($O_2^{\bullet-}$, NO) und ko-stimulierende Faktoren sezerniert. Gleichzeitig werden die von APCs präsentierten Antigene durch spezifische T-Helferzellen (Th1) erkannt. Die Aktivierung der T-Helferzellen stimuliert die Sekretion von Zytokinen, von denen insbesondere das IFN-γ die Expression von MHC II-Molekülen induziert. Zusätzlich wird die Sekretion von IL-1β und TNF-α durch die APCs in einer IFN-γ-bedingten Feedback-Stimulierung verstärkt. Als Folge der hohen lokalen Konzentrationen der Zytokine IL-1β, TNF-α und IFN-γ wird die β-Zellzerstörung durch die Freisetzung von Sauerstoffradikalen und NO aus Makrophagen, Endothelzellen (MC/EC) und T-Lymphozyten (Th/c) potenziert. Darüber hinaus kommt es zur Modifikation des Expressionsprofils vieler Proteine und zur Induktion der Oberflächenrezeptoren wie z.B. Fas. Das Ergebnis dieser Vorgänge ist die Selbstverstärkung des eingeleiteten Autoimmunprozesses bis zur völligen Zerstörung der insulinproduzierenden β-Zellen.

1.3 Apoptose als Form des β-Zelltodes

Die Zerstörung der insulinproduzierenden β-Zellen im Autoimmundiabetes ist ein komplexer Prozess, der durch zelluläre Elemente des Immunsystems sowie durch proinflammatorische Zytokine vermittelt wird. Diese löslichen Entzündungsmediatoren können Signalkaskaden, bestehend aus Caspasen und anderen proapoptotischen Proteinen auslösen, die schließlich den apoptotischen β-Zelltod initiieren (Mandrup-Poulsen 2001; Pirot *et al.* 2008).

Die molekularen Mechanismen, die zur progressiven Zerstörung der insulinproduzierenden β-Zellen führen, sind im Detail noch nicht erforscht. In früheren Studien konnte jedoch

gezeigt werden, dass die Apoptose für die Progression der Erkrankung ausschlaggebend ist (Eizirik & Mandrup-Poulsen 2001; Kim & Lee 2009).

Der Begriff Apoptose stammt aus dem Griechischen und steht für das Fallen der Blätter im Herbst. Er wurde Anfang der 70er Jahre von Kerr und seinen Mitarbeitern geprägt, um die morphologischen und biochemischen Veränderungen apoptotischer Zellen zu beschreiben (Kerr *et al.* 1972). Die Apoptose ist ein lebenswichtiger evolutionär stark konservierter Prozess und spielt eine essentielle Rolle bei der Embryogenese und der Aufrechterhaltung der Gewebshomöostase mehrzelliger Organismen (Danial & Korsmeyer 2004). Morphologisch beginnt die Apoptose mit der Kondensation des nukleären Chromatins, der Schrumpfung des Zytoplasmas und Ausstülpung der Plasmamembran. Die intranukleosomale DNA wird durch die Aktivierung unspezifischer DNasen in einzelne 180-200 bp Histon-assoziierte Fragmente zerlegt und kann als sogenanntes DNA-*laddering* detektiert werden. Am Ende des apoptotischen Zelltodes zerfällt die Zelle in kleine Apoptosekörper, so genannte *apoptic bodies*, die von immunkompetenten Phagozyten (z.B. Makrophagen) aufgenommen und eliminiert werden (Saraste & Pulkki 2000; Grodzicky & Elkon 2002). Im Gegensatz zur Nekrose, der ein akuter Verlust der Zellhomöostase zugrunde liegt, und die mit einer Erhöhung des osmotischen Drucks, früher Ruptur der Plasmamembran und Austritt des intrazellulären Inhalts zur Inflammation führt, kommt es bei der Apoptose zu keiner entzündlichen Reaktion des umliegenden Gewebes.

Eine fehlerhafte Regulation dieses Prozesses führt infolge einer Imbalance zwischen Zelltod und Proliferation zu schwerwiegenden Erkrankungen. Bei einer verminderten Apoptoserate kommt es zur Entstehung von Tumoren, während eine übermäßige Apoptoserate zu neurodegenerativen Erkrankungen wie Alzheimer und Parkinson oder zu Typ 1 Diabetes mellitus führen kann (Thompson 1995; Kurrer *et al.* 1997; Augstein *et al.* 1998; Hengartner 2000; Thomas *et al.* 2009).

Die Apoptose der β-Zellen kann verschiedene Auslöser haben: (1) Der direkte Kontakt mit zytotoxischen Molekülen wie Perforin und Granzym, die von $CD8^+$-T-Lymphozyten sezerniert werden, (2) die Initiierung der Signaltransduktionskaskaden durch sezernierte pro-inflammatorische Zytokine oder (3) der Zytokin-induzierte iNOS- und NO-Signalweg (Eizirik & Mandrup-Poulsen 2001; Mandrup-Poulsen 2001; Lenzen 2008). All diese extra- und intrazellulären apoptotischen Stimuli führen zwangsläufig zur Aktivierung intrazellulärer Proteasen (Caspasen).

1.3.1 Caspasen

Die Gruppe der Caspasen umfasst derzeit 14 einander verwandte Proteasen, die in ihrem aktiven Zentrum einen Cysteinrest tragen und die Eigenschaft besitzen, Proteine substratspezifisch hinter einem Aspartatrest zu spalten. Sie zeichnen sich durch das konservierte Pentapeptidmotiv „QACXG“ in ihrem aktiven Zentrum aus, wobei X für die Aminosäuren R, Q oder D steht (Fan *et al.* 2005). Die Caspasen werden als inaktive Zymogene oder Procaspasen exprimiert, die im Verlauf der Signaltransduktion durch sequenzielle Proteolyse oder Autoaktivierung aktiviert werden. Die Zymogene bestehen aus einer N-terminalen Prodomäne, sowie aus einer großen (20 kDa/ p20) und einer kleinen (10 kDa/ p10) katalytischen Untereinheit, die durch eine kurze *Linker*-Sequenz voneinander getrennt sind. Bei der Aktivierung des Zymogens werden die Untereinheiten proteolytisch an der Konsensussequenz voneinander getrennt und von der Prodomäne abgespalten. Anschließend lagern sich je zwei große und zwei kleine Untereinheiten zu einem aktiven Heterotetramer mit zwei katalytischen Zentren zusammen (Abb.1.2) (Bleackley & Heibein 2001; Lavrik *et al.* 2005b).

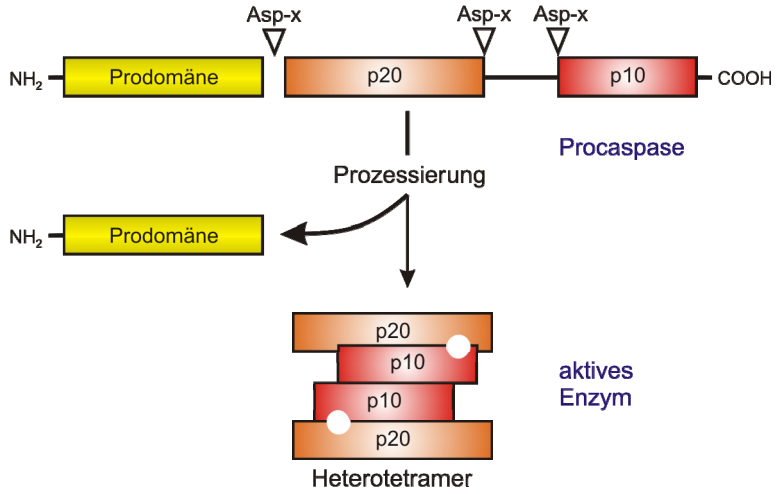

Abb.1.2 Schematische Darstellung der Aktivierung von Caspase-3 nach Lavrik *et al.* (2005)
Die Procaspase besteht aus einer N-terminalen Prodomäne sowie aus einer großen und kleinen Untereinheit. Die Spaltung zwischen der großen und der kleinen Untereinheit führt zur autoproteolytischen Freisetzung der Prodomäne und zur Bildung des aktiven Enzyms, eines Heterotetramers aus je zwei kleinen und zwei großen Untereinheiten mit zwei katalytischen Zentren.

Hinsichtlich ihrer Strukturhomologie und Substratspezifität können die Caspasen in zwei Hauptgruppen unterteilt werden: (1) Die inflammatorischen Caspasen (Caspase 1, 4, 5, 11, 12, 13, 14), die für die Prozessierung und Reifung pro-inflammatorischer Zytokine (IL-1β und IL-18) während einer Immunantwort essentiell sind, und (2) die apoptotischen Caspasen, die weiterhin in Initiatorcaspasen (Caspase 2, 8, 9, 10) und Effektorcaspasen (Caspase 3, 6, 7)

unterteilt werden. Die Initiatorcaspasen enthalten im Gegensatz zu Effektorcaspasen eine lange Prodomäne mit charakteristischen Motiven wie CARD (*caspase recruiting domain*) oder DED (*death effector domain*). Die CAR-Domäne ermöglicht eine elektrostatische und die DE-Domäne eine hydrophobe Interaktion mit komplementären Domänen der Adapterproteine, wodurch die Aktivierung der Caspasen vermittelt wird. Dagegen besitzen die Effektorcaspasen kurze Prodomänen und werden durch Initiatorcaspasen proteolytisch prozessiert und aktiviert (Abb.1.3) (Fuentes-Prior & Salvesen 2004; Lavrik *et al.* 2005b; Logue & Martin 2008).

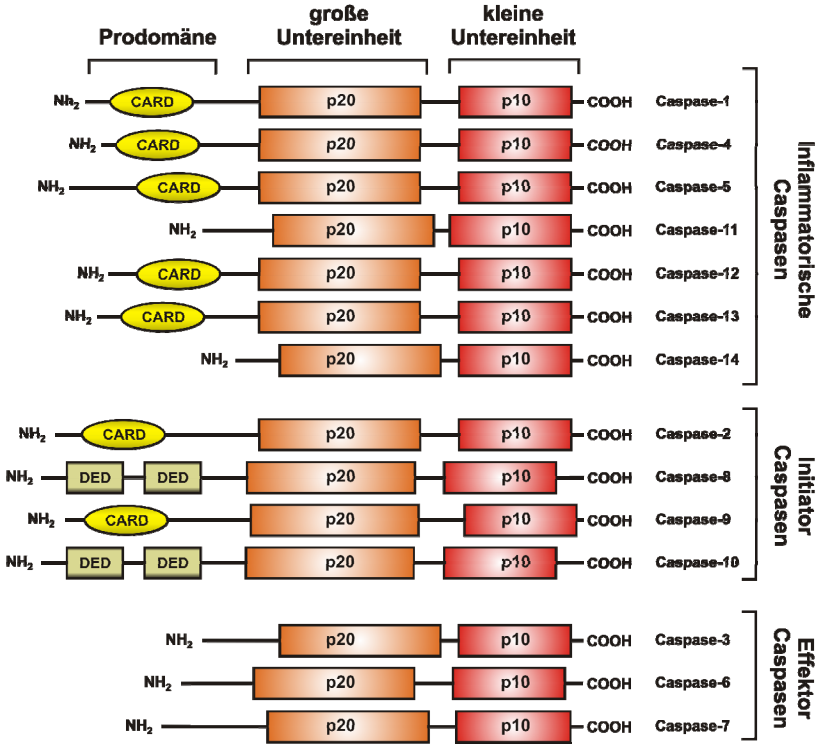

Abb.1.3 Schematische Darstellung der bekannten Caspasen-Familienmitglieder nach Lavrik *et al.* (2005)

Die Familie der Caspasen wird in drei Gruppen eingeteilt: Gruppe I umfasst die inflammatorischen Caspasen, Gruppe II die Initiatorcaspasen und Gruppe III die Effektorcaspasen. (CARD: *caspase recruitment domain*; DED: *death effector domain*)

1.3.1.1 Aktivierungssignalwege der Caspasekaskade

Die Aktivierung der Caspasenkaskade und damit die Apoptoseinduktion kann auf zwei grundlegend unterschiedlichen Wegen erfolgen, (1) dem Rezeptor-abhängigen (extrinsischen) und (2) dem Rezeptor-unabhängigen (intrinsischen) Weg (Abb. 1.4).

(1) Extrinsischer Signalweg:

Der extrinsische Signalweg erfolgt über die Aktivierung membranständiger Todesrezeptoren, zu denen die TNF-(Tumor-Nekrose-Faktor)-Rezeptorfamilie (TNFR1, Fas/CD95/APO) sowie die TRAIL (TNF-*related apoptosis-inducing ligand*)-Rezeptoren (DR4 und DR5) gehören

(Ashkenazi 2002). Diese Rezeptoren sind durch extrazelluläre cysteinreiche Domänen, welche die Bindung der Liganden und deren Trimerisierung vermitteln, sowie durch eine intrazelluläre Todesdomäne (DD, *death domain*), die die Rekrutierung der Adapterproteine ermöglicht, charakterisiert (Daniel 2000; Lavrik *et al.* 2005a).

Aktiviert werden die Todesrezeptoren durch die Bindung an entsprechende Liganden. Dabei kommt es zur Trimerisierung des Rezeptors, die intrazellulär mittels der DDs die Assoziation des Adapterproteins FADD (Fas *associated death domain*) ermöglicht. FADD verfügt neben der DD, mit der das Adapterprotein an die DD des Rezeptors bindet, noch zusätzlich über eine DED, die mit der komplementären DED der Procaspase-8 interagieren kann, und den sogenannten DISC (*death inducing signaling complex*) bildet.

Die Aktivierung der rekrutierten Initiatorcaspasen erfolgt anschließend durch das Prinzip der induzierten Nähe (*induced proximity*). Hierbei kommt es zu einer lokalen Akkumulation der Procaspasen-8/10 am DISC, die sich gegenseitig durch ihre geringe intrinsische Proteaseaktivität autoproteolytisch aktivieren (Muzio *et al.* 1998). Sie können nun durch Prozessierung nachgeschalteter Caspasen die Caspasekaskade initiieren, was letztlich zum apoptotischen Zelltod führt. Die Caspase-8 (aktivierte Procaspase) kann zudem die Spaltung des proapoptotischen Bcl-2-Familienmitglieds Bid zu tBid (*truncated* Bid) vermitteln, das zum Mitochondrium transloziert und den intrinsischen Signalweg auslöst (Taylor *et al.* 2008).

(2) Intrinsischer Signalweg:

Der intrinsische Signalweg wird hingegen durch oxidativen Stress, DNA-Schädigung und Entzug von Wachstumsfaktoren ausgelöst. An diesen intrazellulären Signalwegen sind die Mitochondrien ganz wesentlich beteiligt. Apoptotische Signale führen zum Verlust der Integrität und Stabilität der Mitochondrienmembran und infolgedessen zur Freisetzung von Cytochrom c aus dem Intermembranraum ins Zytosol. Das Cytochrom c bindet nun an ein als Apaf-1 (*apoptotic protease-activating factor-1*) bezeichnetes Protein, das anschließend ATP-abhängig oligomerisiert und zur Bildung des Apoptosoms führt. Das Apaf-1 Protein besitzt wie die Procaspase-9 eine CAR-Domäne, anhand derer die Procaspase-9 über homotypische Interaktion rekrutiert und aktiviert wird (Riedl & Salvesen 2007).

Neben dem Cytochrom c können auch weitere proapoptotische Faktoren aus den Mitochondrien freigesetzt werden, insbesondere SMAC/DIABLO (*second mitochondria-derived activator of caspases/direct* IAP *binding protein with low* PI), Serinprotease HrtA2/Omi (*high-temperatur requirement protein* A2), AIF (*apoptosis-inducing factor*) sowie EndoG (Endonuklease G). SMAC/DIABLO und HrtA2/Omi binden dabei an sog. IAPs (*inhibitor of apoptosis protein*) und neutralisieren deren antiapoptotische Wirkung. Diese

Proteine wirken also synergistisch mit dem freigesetzten Cytochrom c während der Aktivierung der Caspasekaskade (Yan & Shi 2005; Armstrong 2006; Eilers 2009). EndoG und AIF können dagegen caspaseunabhängig die apoptosefördernden Prozesse induzieren.

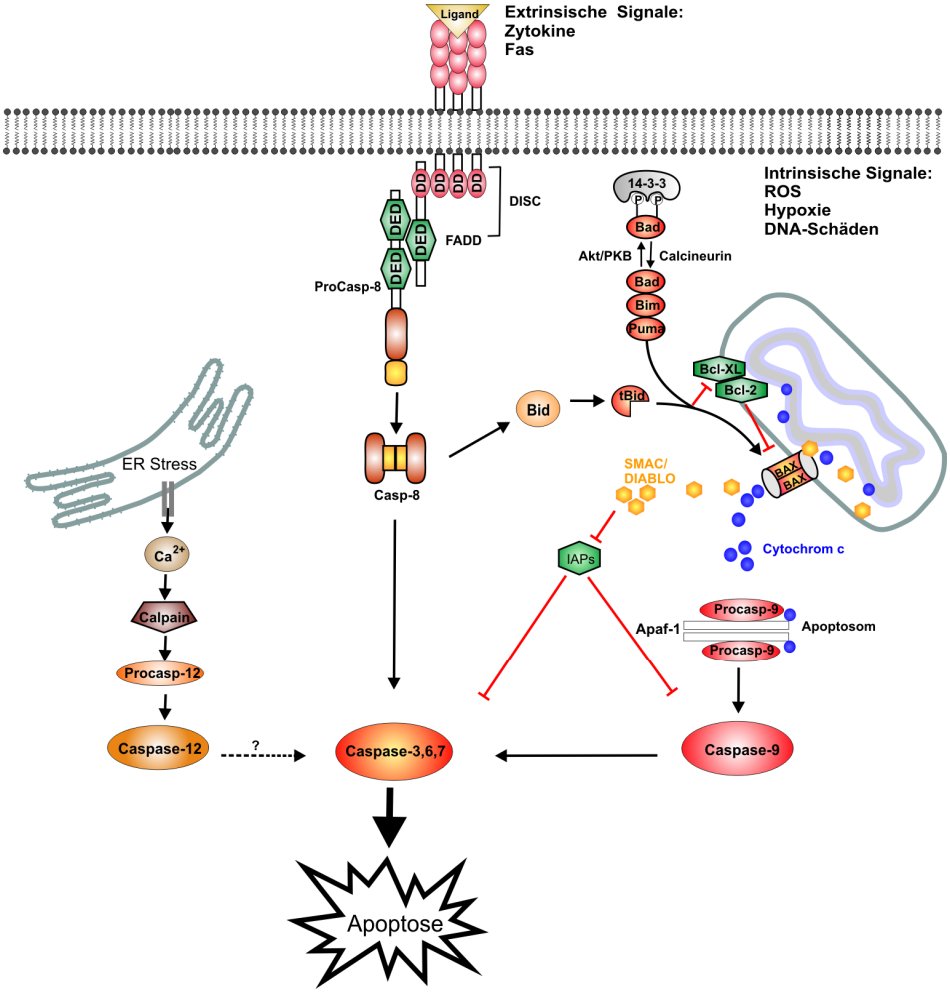

Abb. 1.4 Schematische Darstellung des extrinsischen und intrinsischen Apoptosesignalwegs
Der extrinsische Signalweg wird von den Todesrezeptorfamilienmitgliedern vermittelt. Die Bindung des entsprechenden Liganden am Rezeptor induziert eine Trimerisierung, die über die DD das Adaptormolekül FADD rekrutiert. Der dabei entstehende DISC interagiert über seine DED mit der Procaspase-8, die nach dem Prinzip der induzierten Proximität aktiviert wird. Die Caspase-8 kann direkt die Effektorcaspasen prozessieren und/oder über die Spaltung von Bid zu tBid eine Verbindung zum intrinsischen Signalweg herstellen. Der intrinsische Signalweg wird hingegen durch eine Vielzahl von Stressoren, wie z.B. oxidativen Stress oder DNA-Schäden, ausgelöst. Dabei kommt es zur Aktivierung proapoptotischer Bcl-2 Proteine, die zur Permeabilisierung der äußeren Mitochondrienmembran, Freisetzung von Cytochrom c und anderen proapoptotischen Faktoren führen. Cytochrom c bindet an Apaf-1, das daraufhin die Procaspase-9 rekrutiert und hierdurch das Apoptosom bildet. Die Procaspase-9 spaltet sich autokatalytisch zur aktiven Caspase-9, die wiederum Effektorcaspasen katalytisch prozessiert. Analog hierzu wird derzeitig eine ER-spezifische Apoptoseinduktion postuliert. Dieser Signalweg wird durch die ER-spezifische Caspase-12 vermittelt, die infolge des ER-Stresses aktiviert wird.

In jüngsten Studien wird eine Beteiligung des endoplasmatischen Retikulums (ER) an der intrinsischen Apoptosevermittlung beschrieben. Die Hauptfunktion des ERs besteht neben der Biosynthese und posttranslationalen Modifikationen von Proteinen, naszierende Polypeptide

in ihrer nativen dreidimensionalen Form zu falten. Wird jedoch die Faltungskapazität des ERs überlastet, so kann es zu einer Akkumulation ungefalteter sowie missgefalteter Proteine im ER-Lumen und infolgedessen zum ER-Stress kommen (Zhang & Kaufman 2008). Anschließend wird die UPR (*unfolded protein response*) durch die drei ER-lokalisierten Stress-Sensoren IRE1 (*inositol requiring ER-to-nucleus signal kinase*), ATF6 (*activating transcription factor*) und PERK (*protein kinase RNA (PKR)-like ER kinase*) induziert. Alle drei Sensoren sind unter physiologischen Bedingungen an das Chaperon Bip (*immunglobulin heavy-chain-binding protein*) gebunden und somit inaktiv. Während des ER-Stresses interagiert Bip hingegen mit ungefalteten oder missgefalteten Proteinen und setzt somit die ER-Stress-Sensoren frei. Diese setzen Signalkaskaden in Gang, die zum einen die Proteintranslation reduzieren und zum anderen die Expression von ER-Chaperonen erhöhen, um die korrekte Proteinfaltung zu begünstigen und dadurch dem ER-Stress entgegenzuwirken. Misslingt es jedoch bei anhaltendem ER-Stress die ER-Homöostase wiederherzustellen, wird die Expression von proapoptotischen Genen wie CHOP (*C/EBP homologous protein*) induziert und dadurch die Apoptose eingeleitet (Ron & Walter 2007; Zhang & Kaufman 2008; Eizirik & Cnop 2010).

Weiterhin wird eine direkte Aktivierung der ER-spezifischen Caspase-12 durch die Ca^{2+}-abhängige Cysteinprotease Calpain beschrieben. Dabei wird postuliert, dass die Apoptose unabhängig vom Rezeptor- und Mitochondrien-vermittelten Signalweg ausgelöst wird (Nakagawa *et al.* 2000; Szegezdi *et al.* 2003). Bisher konnte die Rolle der Caspase-12 als Initiatorcaspase beim ER-Stress-induzierten Apoptoseweg nicht bestätigt werden und ist daher z.Zt. noch stark umstritten (Hotchkiss & Nicholson 2006).

Im letzten Schritt der apoptotischen Signalkaskade gehen der extrinsische und der intrinsische Signalweg mit der Aktivierung der Effektorcaspasen einher. Diese attackieren und spalten eine Vielzahl regulatorischer und struktureller Proteine und leiten hierdurch endgültig die Exekution der Apoptose ein. Substrate der Effektorcaspasen sind z.B. Zellstrukturproteine wie Lamin A und Fodrin sowie DNA-Reparaturenzyme wie PARP1 (Poly [ADP-ribose] polymerase 1). Ein wesentliches Substrat der Effektorcaspasen ist ICAD (*inhibitor of caspase-activated* DNase), ein Inhibitor der Caspase-aktivierten DNAse (CAD), die für das charakteristische DNA-*laddering* verantwortlich ist (Wyllie *et al.* 1980; Fan *et al.* 2005).

1.3.2 Bcl-2 Proteinfamilie

Die Initiierung des intrinsischen Apoptosesignalwegs und die Integrität der an diesem Signalweg beteiligten Zellkompartimenten, insbesondere der Mitochondrien, werden durch

die Bcl-2-Familienmitglieder streng reguliert. Diese Proteinfamilie besteht aus pro- und antiapoptotisch wirkenden Mitgliedern, die mindestens über eine der vier evolutionär konservierten α-helikalen Homologiedomänen (Bcl-2 *homology*: BH) verfügen (Adams & Cory 1998; Tsujimoto & Shimizu 2000a). Entsprechend ihrer Struktur und Funktion werden sie in drei Gruppen unterteilt: (1) Antiapoptotische Proteine (Bcl-2, Bcl-X_L, Bcl-w, A1/Bfl-1, Mcl-1, Boo/Diva), die über alle 4 α-helikalen BH-Domänen und eine Transmembrandomäne (TM) verfügen, (2) proapoptotische Bax-homologe Multidomänenproteine (Bax, Bak, Bok), die die BH1-BH3-Domänen sowie eine TM tragen, und (3) proapoptotische BH3-only Proteine (Bad, Bik/Nbk, Bid, Hrk/DP5, Blk, Bim/Bod, Bmf, Noxa und Puma). Diese enthalten nur die BH3-Domäne, die für die proapoptotische Wirkung dieser Proteine ausschlaggebend ist und daher namensgebend war (Cory & Adams 2002; Danial 2007).

Eine Übersicht über die Klassifikation und Struktur der Bcl-2-Familienmitglieder ist in Abb. 1.5 dargestellt.

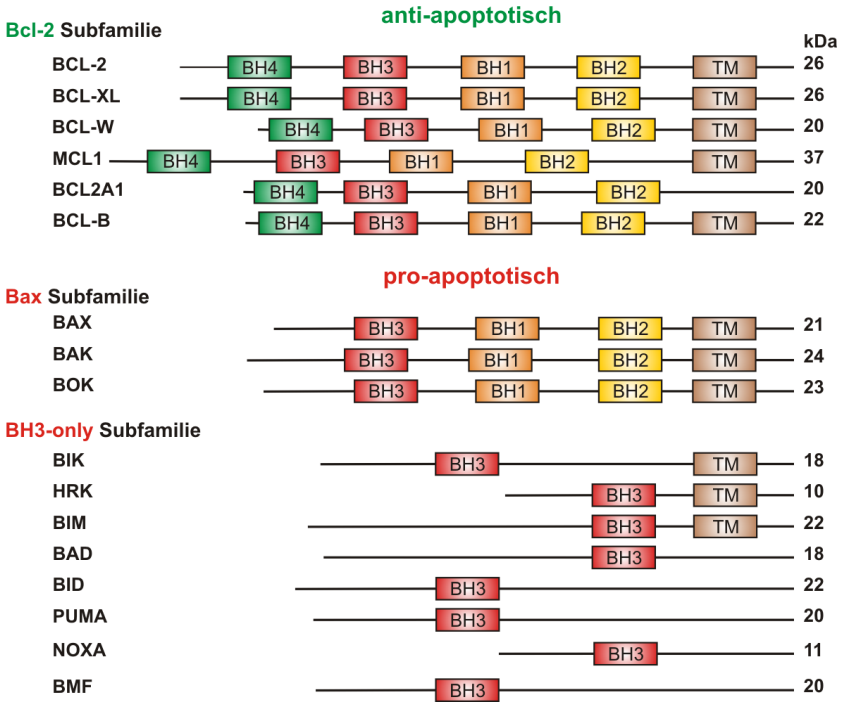

Abb. 1.5 Schematische Übersicht struktureller und funktioneller Klassifizierung der Bcl-2 Proteinfamilie nach Taylor *et al* (2008)

Die Bcl-2-Familienmitglieder werden nach Bcl-2-Homologiedomänen (BH) und anti- bzw. pro-apoptotischen Eigenschaften differenziert. BH1 bis BH4 sind hoch konservierte Sequenzbereiche. Die meisten antiapoptotischen Proteine weisen im Vergleich zu proapoptotischen Proteinen alle vier BH-Domänen auf. Die proapoptotischen Bax-Familienmitglieder tragen die BH-Domänen 1-3, während die BH3-only-Proteine nur über die BH3-Domäne verfügen. Die Transmembrandomäne, die nicht bei allen Proteinen vorhanden ist, wird für die Insertion in die äußere Mitochondrienmembran, Kernmembran und dem endoplasmatischen Retikulum benötigt.

Als erstes Mitglied dieser Proteinfamilie wurde das namensgebende Protoonkogen Bcl-2 (B *cell lymphoma gene* 2) beschrieben, das infolge einer chromosomalen t(14;18)-Translokation in follikulären non-Hodgkin-Lymphomen überexprimiert wird (Tsujimoto *et al.* 1985; Cleary *et al.* 1986). Im Gegensatz zu anderen Protoonkogenen besteht die Funktion des Bcl-2 darin, die Zellen vor Apoptose zu schützen und nicht die Proliferation zu stimulieren (Hockenbery *et al.* 1990). Bcl-2 ist über die C-terminale hydrophobe TM-Domäne konstitutiv an der äußeren Mitochondrienmembran, dem endoplasmatischen Retikulum (ER) und der Kernmembran assoziiert. Die homologen und ebenfalls antiapoptotisch wirkenden Proteine Bcl-X_L, Bcl-w und Mcl-1 liegen sowohl zytosolisch als auch teilweise membrangebunden vor und translozieren während der Apoptoseinitiierung zur äußeren Mitochondrienmembran und dem ER (Youle & Strasser 2008). Von proapoptotischen Bax-homologen Multidomänen-proteinen ist nur das Bak ein membranständiges Protein, das konstitutiv in der äußeren Membran von Mitochondrien und dem ER lokalisiert ist. Nicht aktives Bax hingegen liegt als Monomer zytosolisch oder in leichter Assoziation mit intrazellulären Membranen vor. Im monomeren Zustand ist seine Transmembrandomäne in einer durch die BH1-BH3 Domänen geformten hydrophoben Tasche maskiert. Durch apoptotische Stimuli durchläuft Bax eine Konformationsänderung, wodurch die C-terminale hydrophobe Transmembrandomäne aus der hydrophoben Tasche gelöst wird und als Homodimer in die äußere Mitochondrienmembran insertiert wird (Cory & Adams 2002; Scorrano & Korsmeyer 2003).

Die proapoptotische Bax/Bax-Homodimerisierung kann dabei durch Bcl-2/Bax-Heterodimerisierung und weiteren antiapoptotischen Proteinen der Bcl-2-Familie verhindert werden (Gross *et al.* 1999; Zhang *et al.* 2004). Die Mitglieder der BH3-only Proteinfamilie liegen hingegen zytosolisch vor und werden durch unterschiedliche apoptotische Signale aktiviert. Die Aktivierung dieser Proteinfamilie erfolgt auf transkriptioneller Ebene, sowie durch posttranslationale Modifikationen. So wird Bid durch die Caspase-8 zum trunkierten Bid (tBid) proteolytisch gespalten und aktiviert. Das so entstehende tBid transloziert zu den Mitochondrien und induziert dort über Interaktion mit Bax bzw. Bak die Freisetzung von Cytochrom c (Li *et al.* 1998; Gross *et al.* 1999; Oh *et al.* 2005). Bad dissoziiert durch Dephosphorylierung des Ser^{112} und Ser^{136} von seinem Bindungspartner 14-3-3 und wird so aktiviert. Dieses dephosphorylierte Bad wandert anschließend zu den Mitochondrien, wo es mit antiapoptotisch wirkenden Bcl-2 Proteinfamilenmitgliedern interagiert und seine proapoptotische Wirkung entfaltet (Zha *et al.* 1996; Chiang *et al.* 2001). Bim und Bmf sind hingegen mit Elementen des Zytoskeletts Mikrotubuli (Bim) bzw. Aktin (Bmf) verbunden. Infolge einer Apoptoseinduktion kommt es zur Dissoziation und Translokation beider

Proteine zu den Mitochondrien, wobei sie ebenfalls mit antiapoptotischen Bcl-2-Proteinen interagieren, deren antiapoptotisches Potential antagonisieren und somit die intrinsische Apoptosekaskade anregen (Puthalakath *et al.* 1999; Puthalakath *et al.* 2001).

Als möglicher Mechanismus der Freisetzung beider Proteine aus dem Dyneinkomplex wird die JNK-vermittelte Phosphorylierung von Thr^{112} angenommen (Lei & Davis 2003). Die Regulation bzw. Aktivierung der anderen BH3-only Familienmitglieder wie z.B. Noxa, Puma und Hrk/DP5 erfolgt vornehmlich auf transkriptioneller Ebene (Willis & Adams 2005).

1.3.2.1 Regulation des intrinsischen Apoptosesignalwegs durch die Bcl-2 Familienmitglieder

Die antiapoptotische Wirkung der Bcl-2 Familienmitglieder beruht auf der Hemmung der mitochondrialen Freisetzung apoptotischer Signalmoleküle, sowie auf der Stabilisierung der mitochondrialen Integrität (Cory & Adams 2002; Youle & Strasser 2008). Die genauen molekularen Grundlagen, wie diese komplexe Proteinfamilie den intrinsischen Apoptosesignalweg reguliert, konnten bisher noch nicht vollständig aufgeklärt werden. Als gesichert gilt jedoch, dass im Rahmen der Apoptoseinduktion die pro- und antiapoptotischen Mitglieder der Bcl-2-Familie interagieren und sich dadurch gegenseitig in ihrer Funktion beeinflussen.

Die Interaktion wird grundsätzlich durch die evolutionär konservierten BH-Domänen vermittelt (Chao & Korsmeyer 1998). Strukturanalysen zeigen, dass die Homologiedomänen BH1-BH3 eine hydrophobe Tasche bilden, an welche die amphipathische-α-Helix der BH3-Domäne binden kann (Muchmore *et al.* 1996; Sattler *et al.* 1997; Petros *et al.* 2001). Die Bindung der BH3-α-Helix an diese Tasche ist ursächlich für alle Homo- bzw. Heterodimerisierungen innerhalb der Bcl-2 Homologen. Grund hierfür ist die gegenseitige Beeinflussung der Bcl-2 Familienmitglieder in ihrer Funktion, sodass die Balance zwischen pro- und antiapoptotisch wirkenden Proteinen letztendlich das Überleben der Zelle bestimmt (Adams & Cory 1998; Chao & Korsmeyer 1998; Wong & Puthalakath 2008). Aus diesem Grund wurden niedermolekulare Antagonisten der antiapoptotischen Proteine, sog. BH3-Mimetiker entwickelt, um das Gleichgewicht zu Gunsten der proapoptotischen Mediatoren zu verschieben und dadurch die Apoptose in Tumorzellen zu induzieren (Vogler *et al.* 2009).

Für die Permeabilisierung der äußeren Mitochondrienmembran und der Freisetzung pro-apoptotischer Faktoren aus den Mitochondrien ist die Aktivierung des Proteins Bax und dessen Homolog Bak entscheidend. Diese These geht aus Studien mit Bax/Bak doppelt defizienten Zellen hervor, die gegenüber unterschiedlicher Zelltodstimuli geschützt wurden (Chipuk *et al.* 2006; Danial 2007; Brenner & Mak 2009). Beide Proteine werden vornehmlich

durch die BH3-only Proteine, die als Sensoren und Mediatoren apoptotischer Stimuli stromaufwärts der Mitochondrien agieren, aktiviert. Derzeit werden u.a. zwei widersprüchliche Modelle der Bax/Bak Aktivierung postuliert:

Das (1) Modell der direkten Aktivierung postuliert, dass bestimmte BH3-only Proteine, die sog. Aktivatoren wie z.B. tBid, Bim und möglicherweise Puma, durch direkte Bindung an Bax und Bak diese aktivieren, während die restlichen BH3-only Proteine, die als *sensitizer* bezeichnet werden, mit antiapoptotischen Proteinen interagieren und dadurch die sequestrierten BH3-only Aktivatoren freisetzen.

Das (2) indirekte Aktivierungsmodell (oder auch Verdrängungsmodell) postuliert im Gegensatz zum direkten, die Hemmung der proapoptotischen Aktivität von Bax und Bak durch deren Bindung an antiapoptotische Bcl-2 Proteine. Die BH3-only Proteine binden nach ihrer Aktivierung selektiv an antiapoptotische Bcl-2 Proteine und setzen dabei die sequestrierten Bax bzw. Bak Proteine frei, die daraufhin die intrinsische Apoptose auslösen. Die Hauptfunktion der antiapoptotischen Bcl-2 Proteine ist dabei die Bax bzw. Bak Wirkung zu antagonisieren und nicht die BH3-only Aktivatoren zu sequestrieren, wie im direkten Aktivierungsmodell angenommen wird (Giam *et al.* 2008; Brenner & Mak 2009).

Im Hinblick auf die oben beschriebenen äußerst stark umstrittenen Aktivierungsmodelle, favorisieren viele Arbeitsgruppen derzeit noch ein sog. *embedded together* Modell, das einige Aspekte von dem direkten und indirektem Aktivierungsmodell einbezieht (Leber *et al.* 2007). Entsprechend diesem Modell können die BH3-only Proteine sowohl Bax/Bak aktivieren als auch antiapoptotische Proteine sequestrieren. Obwohl die Modelle gegenwärtig kontrovers diskutiert werden, scheint die Bax/Bak Aktivierung allen Modellen gemeinsam zu sein.

Die Permeabilisierung der äußeren Mitochondrienmembran definiert den Punkt der unumkehrbaren Apoptoseinduktion (Kroemer *et al.* 2007). Der zugrundeliegende Mechanismus der Bax/Bak-vermittelten Permeabilisierung der äußeren Mitochondrien-membran ist noch unklar. Es gibt jedoch experimentelle Hinweise, dass die Bildung von Kanälen insbesondere durch Bax/Bax-Oligomere bzw. Bak einen möglichen Mechanismus für den Ausstrom proapoptotischer Faktoren aus dem Intermembranraum der Mitochondrien darstellt. Neben der Freisetzung mitochondrialer Apoptose-induzierender Proteine durch Bax/Bak gebildete Poren wird zusätzlich postuliert, dass diese Moleküle auf bereits bestehende mitochondriale Kanäle einwirken können. Hierzu zählt die Permeabilitäts-Transitionspore der Mitochondrien (PTP *permeability transition pore*). Diese Pore besteht z.B. aus spannungsabhängigen Anionenkanälen, sogenannten VDACs (*voltage-dependent anion channel*) in der äußeren und Adenin-Nukleotid-Transportern (ANT) in der inneren

mitochondrialen Membran, sowie Cyclophilin D in der Matrix. Der geöffnete Kanal erlaubt hier den Durchtritt von Molekülen bis zu einer Größe von 1,5 kDa, wobei der Protonengradient reduziert und die oxidative Phosphorylierung entkoppelt wird. Infolgedessen kommt es zum Zusammenbruch des mitochondrialen Membranpotentials $\Delta\Psi m$ und zum osmotischen Wassereinstrom, der zum Anschwellen und zur Ruptur der Membran führt. Dabei werden Cytochrom c sowie andere Apoptose-induzierende Faktoren wie AIF und Smac/DIABLO freigesetzt. Zahlreiche Studien haben ergeben, dass die Aktivitäten der VDACs sowohl durch anti- als auch durch proapototische Proteine reguliert werden und dass einige Bcl-2 Familienmitglieder wie z.B. Bcl-2/Bcl-X_L, Bax und Bak direkt mit VDACs interagieren (Tsujimoto & Shimizu 2000b; Kroemer *et al.* 2007; Brenner & Mak 2009).

1.4 Reaktive Sauerstoff- und Stickstoffspezies

Die durch endogene und exogene Faktoren eingeleitete Zerstörung der β-Zellen im Verlauf der Typ 1 Diabetes Pathogenese, führt initial zu zellulärem Stress der so attackierten β-Zellen. Während dieses Prozesses werden in der Zelle überwiegend reaktive Sauerstoffspezies (ROS: *reactive oxygen species*) sowie reaktive Stickstoffspezies (RNS: *reactive nitrogen species*) generiert. Zu den ROS werden einerseits freie Radikale wie Superoxid- ($O_2^{\bullet-}$), Peroxyl- ($RO_2^{\bullet}$), Alkoxyl- ($RO^{\bullet}$) und Hydroxylradikale ($HO^{\bullet}$) als auch nicht radikalische Verbindungen wie Wasserstoffperoxid (H_2O_2) und Singulett-Sauerstoff ($^1O_2$) gezählt. Von den RNS ist neben dem Sticktoffmonoxid ($NO^{\bullet}$) das Peroxynitrit ($ONOO^-$) von Bedeutung (Droge 2002; Halliwell & Gutteride 2007).

Das **Superoxidradikalanion ($O2^{\bullet-}$)** wird durch die Aufnahme eines Elektrons in eines der beiden freien π*-Orbitale des molekularen O_2 gebildet. Diese Ein-Elektronen Reduktion des O_2 erfolgt vornehmlich in der mitochondrialen Atmungskette und ist aufgrund des hohen Reduktionspotentials in diesem Organell thermodynamisch begünstigt (Orrenius *et al.* 2007; Forkink *et al.* 2010). So wird geschätzt, dass die *steady state* $O_2^{\bullet-}$-Konzentration im Mitochondrium 5-10fach höher ist als in anderen Zellkompartimenten, sodass die Mitochondrien als intrazelluläre Hauptquelle der ROS Generierung angesehen werden (Cadenas & Davies 2000). Gegenwärtig werden ca. 10 mitochondriale ROS Bildungsorte beschrieben, wobei die Atmungskettenkomplexe I und III die Hauptentstehungsorte darstellen. Zudem sind noch die Citratzykluskomplexe, wie Pyruvat- und α-Ketoglutarat-Dehydrogenase, zu nennen (Circu & Aw 2010; Forkink *et al.* 2010). Das Superoxidanion besitzt eine große Polarität und eine niedrige Halbwertzeit (10^{-6} s), sodass seine Reichweite und Membranpermeablität stark eingeschränkt sind. Es unterliegt unter physiologischen

Bedingungen einer spontanen Dismutation zu H_2O_2 und molekularem O_2, dessen Disproportionierung durch das Enzym Superoxiddismutase (SOD) um den Faktor 9 beschleunigt werden kann (Fridovich 1983; Fridovich 1997; Liochev & Fridovich 1999). $O_2^{\bullet-}$ selbst ist ein relativ schwach oxidierendes Agens und kann keine direkten Schäden an Makromolekülen, wie DNA und Lipiden hervorrufen (Wiseman & Halliwell 1996; Turrens 2003). Es gibt jedoch experimentelle Hinweise, dass das $O_2^{\bullet-}$ Fe-S–haltige Proteine wie z.B. Aconitase und NADH-Dehydrogenase direkt oxidiert und dadurch das Übergangsmetallion Fe^{2+} freisetzt. Das Fe^{2+} kann H_2O_2 in der sog. Fentonreaktion zum stärksten, in biologischen Systemen auftretende Oxidans, Hydroxylradikal ($HO^{\bullet}$) reduzieren. Des Weiteren kann $O_2^{\bullet-}$ direkt mit $NO^{\bullet}$ zum hoch toxischen Peroxynitrit ($ONOO^-$) reagieren (Turrens 2003; Orrenius *et al.* 2007).

Das **Wasserstoffperoxid (H_2O_2)**, das Produkt der $O_2^{\bullet-}$ Dismutation, ist ein nicht polares und eines der wenigen membranpermeablen ROS (Turrens 2003; Giorgio *et al.* 2007). Es wird durch Dismutation von Superoxidanionen, aber auch von $p66^{Shc}$, ein Enzym des mitochondrialen Intermembranraums (Migliaccio *et al.* 2006) sowie durch Monoaminoxidase (MAO), die in der äußeren Mitochondrienmembran lokalisiert ist, gebildet (Andreyev *et al.* 2005). H_2O_2 selbst ist ein reaktionsträges Agens und kann keine direkten Oxidationsschäden an Makromolekülen hervorrufen. In Anwesenheit von Metallionen (Fe^{2+} oder Cu^+) allerdings reagiert es sehr rasch zu dem hochreaktiven Hydroxylradikal.

Das **Hydroxylradikal ($HO^{\bullet}$)** stellt die aggressivste ROS in biologischen Systemen dar. Es kann mit nahezu allen biologischen Molekülen wie Lipiden, Proteinen, Nukleinsäuren und Kohlenhydraten im Organismus reagieren. Infolge seiner hohen Reaktivität und seiner extrem kurzen Halbwertzeit (10^{-9} s) reagiert es unmittelbar mit Molekülen in direkter Umgebung seiner Entstehung. Endogen entsteht das $HO^{\bullet}$ durch die metallkatalysierte Disproportionierung von H_2O_2 in der sog. Fenton- bzw. Haber-Weiss-Reaktion (Halliwell & Gutteride 2007).

Das **Stickstoffmonoxid ($NO^{\bullet}$)** ist ein ungeladenes und membranpermeables Radikal, welches intrazellulär durch die Oxidation von L-Arginin zum Citrullin gebildet wird. Dieser Prozess wird von NADPH-abhängigen NO-Synthasen katalysiert. Davon gibt es zwei konstitutiv exprimierte Formen, die neuronale (nNOS) und endotheliale (eNOS) sowie eine induzierbare (iNOS) Form. Die iNOS ist durch pro-inflammatorische Zytokine reguliert, welche in der Lage ist, große Mengen an $NO^{\bullet}$ zu produzieren (Pacher *et al.* 2007; Lenzen 2008). Die Reaktion von $NO^{\bullet}$ mit Biomolekülen beschränkt sich auf wenige spezifische Modifikationen, z.B. die Bindung an Häm-oder Thiolgruppen. Wichtig für die Toxizität des $NO^{\bullet}$ ist jedoch die

extrem schnelle Reaktion mit $O_2^{\bullet-}$ zum $ONOO^-$. Mit einer Reaktionskonstante von 3-4 x 10^9 $M^{-1}s^{-1}$ ist die Bildung von Peroxynitrit viel schneller als die Reaktion von $O_2^{\bullet-}$ mit SOD (Pacher *et al.* 2007).

Peroxynitrit ($ONOO^-$) ist ein starkes Oxidans und entsteht durch die Diffusions-kontrollierte Reaktion zwischen $NO^{\bullet}$ und $O_2^{\bullet-}$. Es oxidiert effizient Häm-haltige Proteine, Fe-S-Zentren, Zinkfingermotive und Thiolgruppen. Ferner inaktiviert es die Häm-haltige iNOS und unterliegt somit einer negativen Feedback-Kontrolle unter inflammatorischen Bedingungen (Pacher *et al.* 2007).

1.4.1 Antioxidative Abwehrmechanismen

Zur Abwehr oxidativer Angriffe verfügt der Organismus über zahlreiche Schutzsysteme. Diese Abwehrmechanismen sind unterschiedlicher Natur. Es können nicht-enzymatische antioxidativ wirkende Substanzen (z.B. Vitamin A, C, E und reduziertes Glutathion) oder enzymatische Antioxidanzien sein. Bei den Letzten handelt es sich vornehmlich um Superoxiddismutasen (SOD), Katalasen und Glutathionperoxidasen.

Superoxiddismutasen sind ubiquitär vorkommende Enzyme und erleichtern die Disproportionierung von Superoxidanionen zu H_2O_2 und O_2.

$$2O_2^{\bullet-} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

Es existieren drei Isoformen: die zytoplasmatische CuZnSOD, die mitochondriale MnSOD und die extrazelluläre EC-SOD. Die CuZnSOD (Homodimer) und die extrazelluläre Isoform (Homotetramer) enthalten je ein Kupfer- und Zinkion pro Untereinheit, während die MnSOD (Homotetramer) ein Manganion enthält (Fridovich 1997).

Die Katalase ist in allen aeroben Zellen allgegenwärtig; sie ist ein tetrameres Hämprotein mit jeweils einer Fe(III) Protoporphyringruppe im aktiven Zentrum (Ursini *et al.* 1982). Die Katalase kommt hauptsächlich in Peroxisomen vor, tritt aber auch im Zytosol und mikrosomalen Fraktionen der Zelle auf. Die Hauptfunktion der Katalase ist die Detoxifizierung von H_2O_2 zu H_2O und molekularem O_2.

$$2H_2O_2 \xrightarrow{Katalase} 2H_2O + O_2$$

Die Glutathionperoxidasen (GPx) sind tetramere Enzyme, bestehend aus vier identischen 22 kDa Monomeren, wobei jede Untereinheit ein Selenocystein im aktiven Zentrum enthält. Sie befinden sich im Zytosol und in den Mitochondrien und katalysieren die Glutathion-abhängige Reduktion von H_2O_2 zu H_2O und von organischen Peroxiden zu entsprechenden Alkoholen (Halliwell & Gutteride 2007).

$$2GSH + H_2O_2 \xrightarrow{GPx} GSSG + 2H_2O$$

Anders als gegen ROS verfügen eukaryotische Zellen über keine direkten enzymatischen Abwehrmechanismen gegen RNS. Ein indirekter Schutz kann jedoch durch die Nitrosylierung von Hämoglubin oder die Bildung von S-Nitrosothiolen erreicht werden. Dadurch wird $NO^{\bullet}$ abgefangen und die Bildung von $ONOO^-$ verhindert.

1.5 Freie Radikale in der Pathogenese der β-Zellzerstörung

Reaktive Sauerstoff- und Stickstoffspezies spielen in der Pathogenese der β-Zellzerstörung eine entscheidende Rolle (Lenzen 2008). Diese zellschädigenden Verbindungen werden einerseits extrazellulär durch aktivierte mononukleäre Zellen freigesetzt, andererseits entstehen sie im Signalprozess von freigesetzten Entzündungsmediatoren in der β-Zelle. Zytokine wie IL-1β, IFN-γ und der TNF-α wirken als typische Vertreter der Entzündungsmediatoren zytostatisch oder gar zytotoxisch auf β-Zellen (Mandrup-Poulsen 2001). Sie wirken über spezifische Rezeptoren und induzieren die Aktivierung verschiedener Transkriptionsfaktoren wie z.B. NF-κB und STAT-1 (*signal transducer and activator of transcription 1*) (Pirot *et al.* 2008). Die Aktivierung dieser Transkriptionsfaktoren führt zur Expression der iNOS und in direkter Folge zur intrazellulären Bildung von $NO^{\bullet}$. Durch die gesteigerte NO-Freisetzung kommt es zur Inaktivierung unterschiedlicher enzymatischer Systeme und infolgedessen zur vermehrten ROS Generierung. Des Weiteren wird die Expression von MnSOD durch IL-1β stark induziert, sodass die Dismutationsrate von $O_2^{\bullet-}$ zu H_2O_2 stark ansteigt. Dieser Effekt wird durch die synergistische Wirkung von IL-1β, IFN-γ und der TNF-α zusätzlich verstärkt (Lortz *et al.* 2005). Der β-Zelltod ist dabei die Folge eines labilen Gleichgewichts aus der Menge von ROS und den antioxidativen Enzymen. Die β-Zellen sind durch ein extrem niedriges Expressionsniveau zytoprotektiver Enzyme, insbesondere der H_2O_2-inaktivierenden Enzyme Katalase und Glutathionperoxidase gekennzeichnet (Lenzen *et al.* 1996). Das Expressionsniveau dieser Enzyme entspricht lediglich ca. 5 % der Expressionsrate in Hepatozyten, wohingegen das Expressionsniveau der Superoxiddismutasen ca. 30 % beträgt (Tiedge *et al.* 1997). Dieses Missverhältnis begünstigt die Akkumulation von H_2O_2 und anschließend die Generierung von toxischem $HO^{\bullet}$.

Die Aufklärung des enzymatischen Ungleichgewichts und der β-zelltoxischen Wirkung freier Radikale führte zur Strategie des antioxidativen oder zytoprotektiven Schutzes. Dafür wurde die Expression entsprechender enzymatischer Schutzsysteme wie der Katalase, GPx oder SOD in insulinproduzierenden RINm5F-Zellen mittels effizienter Expressionssysteme erhöht. Durch die Überexpression der Katalase und GPx konnte ein effektiver Schutz gegenüber

β-zelltoxischen Zytokinen erreicht werden (Tiedge *et al.* 1997; Tiedge *et al.* 1998; Tiedge *et al.* 1999; Lortz *et al.* 2000). Dieser Schutz wird durch die zellkompartiment gerichtete Überexpression der Katalase in Mitochondrien signifikant verbessert (Gurgul *et al.* 2004). Dieses deutet einerseits auf den mitochondrialen Ursprung der schädigenden Radikale nach Zytokinstimulation und andererseits auf das in Mitochondrien von insulinproduzierenden Zellen bestehende Ungleichgewicht zwischen Superoxidradikal- und Wasserstoffperoxid-inaktivierenden Enzymsystemen hin. In Übereinstimmung mit diesen Ergebnissen konnte in weiteren Studien gezeigt werden, dass durch Überexpression der mitochondrial lokalisierten MnSOD die Toxizität von mitochondrial generierten Superoxidradikalen und Zytokinen gesteigert wird, während durch Suppression mittels *antisense* Technik die Toxizität reduziert wird (Lortz *et al.* 2005).

1.6 Fragestellung

Beim Typ 1 Diabetes mellitus kommt es durch eine Autoimmunreaktion zur selektiven Zerstörung der β-Zellen des Pankreas und somit zu einem absoluten Insulinmangel. Die Zerstörung der pankreatischen β-Zellen ist ein komplexer Vorgang, der durch zelluläre Elemente des Immunsystems sowie durch proinflammatorische Zytokine vermittelt wird. Obwohl die Bedeutung der proinflammatorischen Zytokine für die Zerstörung der insulinproduzierenden β-Zellen durch zahlreiche *in vitro* und *in vivo* Studien belegt werden konnte, sind die molekularen Mechanismen, über die proinflammatorische Zytokine zum Zelltod der β-Zellen führen, trotz intensiver Forschung noch nicht vollständig geklärt. Zum besseren Verständnis der pathobiochemischen Vorgänge während der Manifestation des Typ 1 Diabetes mellitus, sollten folgende Fragestellungen bearbeitet werden:

1. Quantifizierung der Bcl-2 Familienmitglieder in primären Inselzellen und in insulinproduzierenden RINm5F Gewebekulturzellen vor und nach der Exposition mit β-zelltoxischen Zytokinen.
2. Charakterisierung des Einflusses des antioxidativen Abwehrstatus auf das Expressionsprofil der Bcl-2 Familienmitglieder vor und nach Zytokinexposition. Des Weiteren sollte die zytokinvermittelte Freisetzung proapoptotischer Faktoren aus dem Mitochondrium sowie die kompartimentspezifische Caspase-Aktivierung in nicht transfizierten und stabil transfizierten RINm5F Zellen untersucht werden.
3. Untersuchung des klassischen und alternativen NF-κB Signalweges in primären Inselzellen und dessen Bedeutung für die zytokinvermittelte β-Zellzerstörung.
4. Kompartimentspezifische Quantifizierung von reaktiven Sauerstoffspezies in primären Inselzellen und in insulinproduzierenden RINm5F Zellen nach der Exposition mit β-zelltoxischen Zytokinen. Darüber hinaus sollte deren schädigende Wirkung an zellulären Strukturen charakterisiert werden.

2 Publikationen

Titel:

Modulation of Bcl-2-related protein expression in pancreatic beta cells by pro-inflammatory cytokines and its dependence on the antioxidative defense status

Zeitschrift:

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Titel:

Overexpression of the Nuclear Factor-{kappa} B subunit c-Rel protects against human islet cell death in vitro

Zeitschrift:

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Am J Physiol Endocrinol Metab, volume 297, page E1067-E1077, figure no (s) 1-8, year 2009, DOI 10.1152/ajpemdo. 00212.2009

Titel:

Cytokine toxicity in insulin-producing cells is mediated by nitrooxidative stress-induced hydroxyl radical formation in mitochondria

Zeitschrift:

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Induction of the intrinsic apoptosis pathway in insulin-secreting cells is dependent on oxidative damage of mitochondria but independent of caspase-12 activation

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Modulation of Bcl-2-related protein expression in pancreatic beta cells by pro-inflammatory cytokines and its dependence on the antioxidative defense status

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ABSTRACT

Pro-inflammatory cytokines are key mediators in the selective and progressive destruction of insulin-producing beta cells during type 1 diabetes development. However, the mechanisms of cytokine-induced beta cell apoptosis are not fully understood.

This study demonstrates that pro-inflammatory cytokines strongly modified the expression of the anti-apoptotic protein Bcl-2 and the pro-apoptotic BH3-only proteins Bad, Bim, and Bid in primary rat islets and insulin-producing RINm5F cells. Overexpression of mitochondrially located catalase (MitoCatalase) specifically increased basal Bcl-2 and decreased basal Bax expression, suppressed cytokine-mediated reduction of Bcl-2, and thereby prevented the release of cytochrome c, Smac/DIABLO and the activation of caspase-9 and -3. Thus, cytokine-mediated decrease of Bcl-2 expression and the sequentially changed Bax/Bcl-2 ratio are responsible for the release of pro-apoptotic mitochondrial factors, activation of caspase-9, and ultimately caspase-3.

These results indicate that activation of the intrinsic/mitochondrial apoptosis pathway is essential for cytokine-induced beta cell death and the mitochondrial generation of reactive oxygen species, in particular mitochondrial hydrogen peroxide, differentially regulates the Bax/Bcl-2 ratio.

1. Introduction

Pancreatic beta cell apoptosis plays a crucial role in the pathogenesis of Type 1 Diabetes mellitus (T1DM) (Atkinson, 2005; Eizirik and Mandrup-Poulsen, 2001; Kim and Lee, 2009). This process is initiated by two main pathways: the "extrinsic" or death receptor and the "intrinsic" or mitochondrial apoptosis pathway (Huerta et al., 2007; Millan and Huerta, 2009). The intrinsic death pathway involves loss of mitochondrial homeostasis, particularly of the outer mitochondrial membrane integrity, and subsequently the release of mitochondrial pro-apoptotic factors including cytochrome c. Once released, cytochrome c promotes the assembly of the so-called apoptosome, consisting of cytochrome c, apoptosis-protease activating factor-1 (Apaf-1), ATP/dATP, and pro-caspase-9. Upon formation of this complex, activated caspase-9 triggers the processing and activation of effector caspases, which ultimately culminates in apoptotic cell death (Danial and Korsmeyer, 2004; Kroemer et al., 2007; Susnow et al., 2009). Permeablization of the mitochondrial outer membrane and the consequent release of cytochrome c are tightly regulated by a group of proteins known as the Bcl-2 protein family (Adams and Cory, 1998). This family is composed of pro- and anti-apoptotic proteins that share up to four conserved regions known as Bcl-2 homology (BH) domains. Anti-apoptotic members such as Bcl-2 and Bcl-X_L contain all four subtypes of BH domains and promote cell survival by inhibiting the function of the pro-apoptotic Bcl-2 family members. The pro-apoptotic members can be further subdivided into two subfamilies: multidomain proteins (e.g. Bax and Bak), containing BH1-3, or the BH3-only proteins (e.g. Bad, Bim, Bid, Noxa, and Puma) that contain only the BH3 domain critical for the induction of apoptosis (Danial and Korsmeyer, 2004; Strasser, 2005). Although the precise mechanisms by which these proteins regulate cell death are not fully understood, it appears that the interaction and the ratio between anti- and pro-apoptotic Bcl-2 family proteins determine the fate of cells exposed to apoptotic stimuli (Adams and Cory, 1998; Wong and Puthalakath, 2008).

Abbreviations: Apaf-1, apoptosis-protease activating factor-1; BH, Bcl-2 homology domain; CREB, cAMP response element-binding protein; Ct, cycle threshold; GPX1, glutathione peroxidise-1; H_2O_2, hydrogen peroxide; IAP, inhibitor of apoptosis protein; IFN-γ, interferon-γ; IL-1β, interleukin-1β; MAPK, mitogen-activated protein kinase; NFκB, nuclear factor kappa B; PI-3K, phosphatidylinositol 3-kinase; ROS, reactive oxygen species; Smac/DIABLO, second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI; T1DM, Type 1 Diabetes mellitus; TNF-α, tumor necrosis factor-α; XIAP, X-linked inhibitor of apoptosis protein.

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Studies with non-insulin-secreting cells suggested that reactive oxygen species (ROS), in particular hydrogen peroxide (H_2O_2), decrease the expression of Bcl-2 and increase that of pro-apoptotic proteins, e.g. Bax, thereby changing the Bax/Bcl-2 ratio which determines the susceptibility of cells to apoptosis (Korsmeyer et al., 1993; Oltvai et al., 1993; Raisova et al., 2001). In addition, the pro-inflammatory cytokines interleukin 1β (IL-1β), tumor necrosis factor α (TNF-α), and interferon γ (IFN-γ) down-regulated the expression of the anti-apoptotic protein Bcl-2 in pancreatic islets (Piro et al., 2001; Trincavelli et al., 2002; Van de Casteele et al., 2002); whereas, the overexpression of Bcl-2 in beta cells protected against cytokine-induced cell death (Barbu et al., 2002; Rabinovitch et al., 1999; Tran et al., 2003). Furthermore, inhibition of Bax mitochondrial translocation by the potential Bax suppressor protein V5 (also known as Ku70) resulted in a higher resistance against apoptotic stimuli in HEK293T and HeLa cells (Sawada et al., 2003) and also against cytokine-induced toxicity in human pancreatic beta cells (Grunnet et al., 2009).

The relation between ROS and the pro-inflammatory beta cell toxic cytokines IL-1β, TNF-α, and IFN-γ in their influence on the expression of anti- (Bcl-2 and Bcl-X_L) and pro-apoptotic (Bax, Bad, Bim, and Bid) proteins is still unknown. Therefore, the expression pattern of these proteins and its relation to the toxicity of pro-inflammatory cytokines, as well as its dependence on the antioxidative defense status, was analyzed in primary rat islets and in insulin-producing RINm5F cells overexpressing antioxidative enzymes.

2. Materials and methods

2.1. Tissue culture of RINm5F cells

Insulin-producing RINm5F tissue culture cells were cultured as described earlier in RPMI-1640 medium supplemented with 10 mM glucose, 10% (v/v) fetal calf serum (FCS), penicillin, and streptomycin in a humidified atmosphere at 37 °C and 5% CO_2 (Lortz et al., 2000). The RINm5F cells overexpressing catalase in the cytosol (CytoCatalase) or in mitochondria (MitoCatalase) were generated as described before in detail (Gurgul et al., 2004). Briefly, for overexpression of catalase in cytosol or mitochondria, human catalase cDNA was subcloned into the pcDNA3 (cytosolic overexpression) or pCMV/myc/mito-plasmid (mitochondrial overexpression) and the stable clones were selected through resistance against G418. Cells transfected with the pCMV/myc/mito vector lacking insert were used as control cells. Expression of antioxidative enzymes in the cells was analyzed by catalase enzyme activity measurement (Gurgul et al., 2004). The catalase enzyme activities were as follows in the control cells with an empty vector and in cells overexpressing CytoCatalase or MitoCatalase, respectively (in U/mg total protein): control cells, 31 ± 0.2; CytoCatalase, 346 ± 2.8; MitoCatalase, 594 ± 18.8.

2.2. Rat islet isolation

Pancreatic islets were isolated from 250 to 300 g adult male Lewis rats by collagenase digestion, separated by Ficoll gradient, and handpicked under a stereo microscope (Tiedge et al., 1997). Isolated islets were cultured overnight in RPMI-1640 medium supplemented with 5 mM glucose, 10% FCS, penicillin, and streptomycin at 37 °C in a humidified atmosphere of 5% CO_2.

2.3. Exposure to cytokines

Control and transfected RINm5F cells were seeded at different concentrations depending on the further experimentation and allowed to attach for a period of 24 h. For islet incubations, 100–150 uniformly sized handpicked, precultured islets were used. Cells were exposed to 600 U/ml human IL-1β or a combination of cytokines (cytokine mixture) consisting of 60 U/ml IL-1β, 185 U/ml human TNF-α, and 14 U/ml IFN-γ (PromoCell, Heidelberg, Germany) for a subsequent 24 h incubation. The indicated cytokine concentrations were selected based upon the results of previous studies (Lortz et al., 2000; Nerup et al., 1994; Nielsen et al., 2004). IL-1β is the most potent beta cell toxic cytokine and strongly contributes to beta cell death by activation of the NFκB and MAPK transduction pathways. TNF-α and IFN-γ alone are significantly weaker in this respect; however in synergy with IL-1β they potentiate its deleterious action through induction of additional signaling pathways (Eizirik and Mandrup-Poulsen, 2001). Therefore, the used cytokine mixture contained 10-fold less IL-1β than the IL-1β solution alone, in order to achieve a comparable toxicity (Souza et al., 2008).

2.4. Real-time quantitative RT-PCR

Total RNA from insulin-producing RINm5F cells was isolated using the Chomczynski protocol, while the RNA from incubated islets was isolated with NucleoSpin RNA/Protein columns (Macherey-Nagel, Düren, Germany). RNA was quantified and analyzed by the Experion automated electrophoresis system (BioRad Laboratories, Hercules, CA, USA). For cDNA synthesis, random hexamers were used to prime the reaction of the RevertAid H^- M-MuLV reverse transcriptase (Fermentas, St. Leon-Rot, Germany). QuantiTect SYBR Green technology (Qiagen, Hilden, Germany), which uses a fluorescent dye that binds only double-stranded DNA, was employed. The reactions were performed using the DNA Engine Opticon Sequence Detection System (BioRad Laboratories, Hercules, CA, USA). Samples were first denatured at 94 °C for 3 min followed by 40 PCR cycles comprised of a melting step at 94 °C for 30 s, an annealing step at 60 °C for 30 s, and an extension step at 72 °C for 30 s. Primers for qRT-PCR were used at an annealing temperature of 60 °C and gave an amplicon of 79–216 base pairs (Table 1). The optimal parameters for the PCR reactions were empirically defined and the purity and specificity of the amplified PCR product in each experiment was verified by melting curves. All analyzed transcripts showed Ct-values, which were at least 10 Ct-values lower than the blank values. Each PCR amplification was performed in triplicate. Data are expressed as relative gene expression after normalization to the beta actin housekeeping gene using the *Qgene96* and *LineRegPCR* software.

2.5. Tissue fractionation

Trypsinized RINm5F cells were collected by centrifugation at 700 × *g* for 5 min, washed twice with ice-cold PBS, and centrifuged at 700 × *g* for 5 min at 4 °C. The cell pellets were resuspended in 500 μl of ice-cold H-medium (70 mM sucrose, 210 mM mannitol, 20 mM HEPES, 150 mM KCl, and 0.5 mM EGTA, pH 7.4) supplemented with complete protease inhibitor cocktail (Roche Diagnostics, Manheim, Germany), and homogenized on ice with 25–30 strokes in a Potter-Elvehjem homogenizer. Fractions were obtained through differential centrifugation. Cell homogenates were centrifuged at 500 × *g* for 10 min at 4 °C to discard nuclei and unbroken cells. The mitochondrial fraction was obtained through centrifugation for 10 min at 10 000 × *g* at 4 °C. The supernatant was then centrifuged for another 60 min at 100 000 × *g* at 4 °C, to separate the microsomal and the cytoplasmic fractions (Lenzen et al., 1985).

2.6. Western blot analyses

RINm5F whole cell extracts (for detection of Bcl-2 family members) or subcellular fractions (for cytochrome c and Smac/DIABLO detection) were sonified in ice-cold PBS on ice for 15 s at 60 W with a Braun-Sonic 125 sonifier. Protein content was determined by the BCA assay (Pierce, Rockford, IL, USA) or the Bradford assay. Whole islet cell proteins were obtained through a combined RNA/protein isolation procedure (NucleoSpin RNA/Protein columns, Macherey-Nagel, Düren, Germany) and prepared for Western blot analyses in accordance with the manufacturer's protocol. RINm5F cell protein (20–30 μg) or the protein fraction of 100–150 islets per lane were separated by a 12.5% SDS-PAGE and transferred to polyvinylidene fluoride membranes. Nonspecific binding sites of the membranes were blocked with 5% non-fat dry milk for either 1 h at room temperature or overnight at 4 °C. Then, the membranes were incubated with specific primary antibodies for either 4 h at room temperature or overnight at 4 °C. The following antibodies were used: Bcl-2 (sc-7382, diluted 1:500), Bax (sc-7480, 1:250), Smac/DIABLO (sc-12683, 1:250), cytochrome c (sc-7159, 1:250), and actin (sc-1615, 1:250) (Santa Cruz Biotechnology, Santa Cruz, CA, USA); Bcl-X_L (610211, 1:1000) (BD Biosciences, Franklin Lakes, NJ, USA); phospho-Ser^{136} Bad, (44-524, 1:500) (Biosource, Camarillo, CA, USA); Bid (1:500), (R&D Systems, Minneapolis, MN, USA); and Bim (ap-330, 1:1000) (Stressgen, Victoria, BC, Canada). The excess of primary antibody was removed by three washes with wash buffer (PBS, 0.1% Tween 20, 0.1% BSA). Subsequently, the membranes were incubated with peroxidase-labeled secondary antibodies at a dilution of 1:20 000 at room temperature for 1 h. The protein bands were visualized by chemiluminescence using the ECL detection system (Amersham Bioscience, Freiburg, Germany). The intensity of the bands was quantified with respect to β-actin bands through densitometry with the Gel-Pro Analyzer 6.0 program (Media Cybernetics, Silver Spring, MD, USA).

2.7. Flow cytometric quantification of caspase-9 and caspase-3 activity

Caspase-9 and -3 activities were determined with the CaspGLOW staining kits (Promocell, Heidelberg, Germany). Control and overexpressing RINm5F cells were seeded at a density of 1×10^6 cells per well and allowed to attach for a period of 24 h at 37 °C before incubation with the indicated cytokines. After 24 h exposure to IL-1β or the cytokine mixture, the cells were trypsinized and collected by centrifugation at 700 × *g* for 5 min. Cell pellets were resuspended in 1 ml medium and 300 μl of each sample were transferred to Eppendorf tubes. According to the manufacturer's protocol, FITC-LEHD-FMK (caspase-9) or Red-DEVD-FMK (caspase-3) was added to these cells and incubated for 45 min at 37 °C followed by washing twice with wash buffer. The cells were resuspended in 1 ml of washing buffer and 20 000 cells of each sample were analyzed by flow cytometry (CyFlow ML, Partec, Münster,

Table 1
Sequences and amplification parameters for all primers used for quantitative *Real Time* PCR analysis.

Gene	Primer sequence	Product size	Annealing-temperature
Bcl-2	fw.: ACTTCTCTCGTCGCTACCGTCGC rv.: AGAGCGATGTTGTCCACCAGGG	216 bp	60 °C
Bcl-X_L	fw.: GAGACCCCCAGTGCCATCAATG rv.: CTCATCGCCAGCCTCTCTCAGC	152 bp	60 °C
Bad	fw.: CCAGAGTTTGAGCCGAGTGAGCA rv.: CTGTTATTGGCTGCCTGTCCCG	151 bp	60 °C
Bid	fw.: TCTGAGGTCAGCAATGGCTCAGG rv.: TTCTTCCATGATAGAAGGAGCGGCT	210 bp	60 °C
Bim	fw.: CGGCACCCATGAGTTGTGACAA rv.: TGCAAACGCCCTCCTCGTGTA	199 bp	60 °C
Bax	fw.: CCAGGACGCATCCACCAAGAAG rv.: CCCAGTTGAAGTTGCCGTCTGC	169 bp	60 °C
β-Actin	fw: GAACACGGCATTGTAACCAACTGG rv: GGCCACACGCAGCTCATTGTA	79 bp	60 °C

Germany) using the FL-1 channel (488 nm/527 nm) for caspase-9 or FL-2 channel (488 nm/575 nm) for caspase-3.

2.8. Data analysis

The data are expressed as means ± SEM. Statistical analyses were performed by ANOVA followed by Dunnett's test for multiple comparisons using the Prism analysis program (Graphpad, San Diego, CA, USA).

3. Results

3.1. Expression of Bcl-2-related proteins in primary rat islets and insulin-producing cells

Mitochondrial integrity and stability are dependent upon the expression and interaction of pro- and anti-apoptotic Bcl-2 proteins. Therefore, the expression of anti- and pro-apoptotic Bcl-2 family members in primary rat islets and insulin-producing RINm5F tissue culture cells was characterized by qRT-PCR. Gene expression of the anti-apoptotic (Bcl-2 and Bcl-X_L) and pro-apoptotic (Bax, Bad, Bim, and Bid) Bcl-2 proteins could be detected in primary rat islets and RINm5F cells. The pro-apoptotic protein Bax showed the highest gene expression level of all investigated Bcl-2 proteins and its expression level was 5-fold higher in primary rat islets and 8.5-fold higher in RINm5F cells, compared with the anti-apoptotic protein Bcl-2. Moreover, Bcl-X_L and Bad revealed a 2-fold higher gene expression than Bcl-2 in primary islets and 4-fold higher in RINm5F cells; whereas the relative gene expression of the BH3-only proteins Bim and Bid was comparable with that of Bcl-2 (Fig. 1a and b). Overall, the expression pattern of all investigated Bcl-2-related proteins was comparable in primary rat islets and RINm5F cells.

3.2. Effects of pro-inflammatory cytokines on the expression of Bcl-2-related proteins in primary rat islets and insulin-producing cells

Exposure of primary islets and insulin-producing cells to IL-1β alone resulted in alteration of multiple genes, cellular function impairment, and apoptosis. In combination with TNF-α and/or IFN-γ these phenomena could be synergistically potentiated (Eizirik and Mandrup-Poulsen, 2001). To characterize the influence of these beta cell toxic cytokines on the gene expression pattern of Bcl-2 related proteins, primary rat islets and RINm5F cells were incubated with IL-1β alone or with a pro-inflammatory cytokine mixture consisting of IL-1β, TNF-α, and IFN-γ. A 24 h exposure of primary rat islets to IL-1β or the cytokine mixture caused a significant reduction of Bcl-2 ($P<0.001$) and Bad (IL-1β: $P<0.05$; cytokine mixture: $P<0.01$) gene expression. In contrast, the gene expression of Bcl-X_L ($P<0.01$) and Bid (IL-1β: $P<0.01$; cytokine mixture: $P<0.001$) was significantly increased under the same treatment conditions, whereas that of Bim was only slightly but not significantly upregulated. The gene expression of Bax remained unchanged after cytokine treatment (Fig. 2a). The changes in expression patterns, induced by cytokines in primary rat islets, were also observed in RINm5F tissue culture cells (Fig. 2c). Again, expression of Bcl-2 was significantly reduced by IL-1β alone and the cytokine mixture ($P<0.001$). The Bad gene expression was only marginally affected by exposure to IL-1β alone, while the cytokine mixture induced a significant 25% decrease of Bad ($P<0.05$). The Bcl-X_L gene expression was significantly increased by 38% only after cytokine mixture treatment ($P<0.01$). However, in contrast

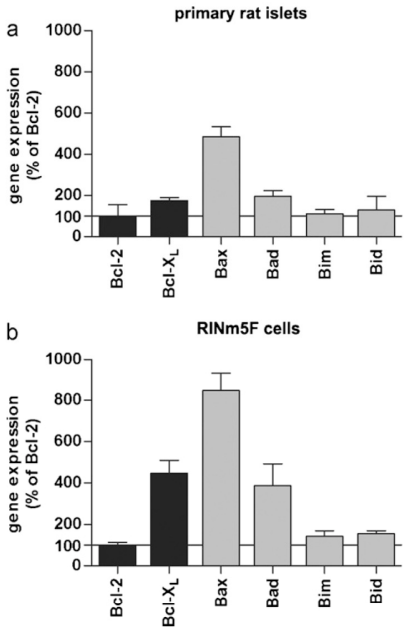

Fig. 1. Expression of Bcl-2-related proteins in primary rat islets and insulin-producing RINm5F cells. (a) Gene expression of anti-apoptotic and pro-apoptotic Bcl-2-related proteins in primary rat islets under control conditions. (b) Gene expression of anti-apoptotic and pro-apoptotic Bcl-2-related proteins in insulin-producing RINm5F cells under control conditions. Total RNA was isolated 24 h after islet isolation or cell seeding and analyzed by qRT-PCR with gene specific primer sets. The relative expression levels were normalized to the housekeeping gene β-actin. The gene expression of Bcl-2 in each cell type was set as 100%. Depicted values are means ± SEM, $n = 6–8$.

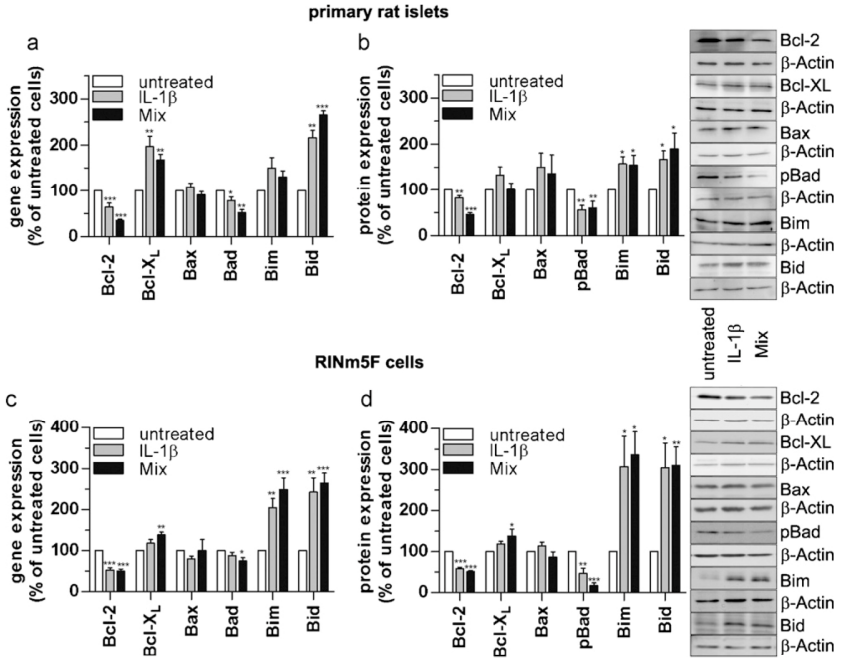

Fig. 2. Effects of pro-inflammatory cytokines on gene and protein expression of Bcl-2-related proteins in primary rat islets and insulin-producing RINm5F cells. (a) and (c) Isolated rat islets and RINm5F cells were incubated under control conditions, with IL-1β (600 units/ml), or with a cytokine mixture (60 units/ml IL-1β, 185 units/ml TNF-α, and 14 units/ml IFN-γ). After 24 h, RNA was isolated and relative gene expression was analyzed by qRT-PCR and normalized to the housekeeping gene β-actin. (b) and (d) Isolated rat islets and RINm5F cells were incubated as in (a) and (c) and protein expression was quantified by Western blot analysis and normalized to the housekeeping protein β-actin. A representative blot is shown for each Bcl-2 related protein. The expression of each gene or protein under control conditions was set as 100%. Depicted values are means ± SEM, $n = 6–8$ (mRNA expression) or $n = 4$ (protein expression). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ *vs.* untreated cells.

to primary rat islets, the Bim gene expression in RINm5F culture cells was significantly induced by IL-1β and the cytokine mixture (IL-1β: $P < 0.01$; cytokine mixture: $P < 0.001$), whereas the Bid gene expression was similarly enhanced as observed in primary islets (IL-1β: $P < 0.01$; cytokine mixture: $P < 0.001$, Fig. 2c). As in primary rat islets, the gene expression of Bax was not influenced by IL-1β or the cytokine mixture.

To verify the gene expression data obtained by qRT-PCR, Western blot analyses were performed. As shown in Fig. 2b and d, the protein expression of all investigated Bcl-2-related proteins in rat islets and RINm5F cells showed a very high correlation with the gene expression data. Nevertheless, the observed cytokine-induced increase of Bcl-X_L gene expression in primary islets could not be verified on protein level, whereas the Bim protein expression was significantly induced after treatment with IL-1β alone or the cytokine mixture ($P < 0.05$). Since the BH3-only protein Bad remains under control conditions in an inactive phosphorylated state and is sequestered in the cytosol by a 14-3-3 molecule (Zha et al., 1996), we examined not only the total Bad protein expression, but also the cytokine-mediated dephosphorylation of Bad at Ser^{136}. In rat islets and in RINm5F cells, IL-1β alone and the cytokine mixture induced Bad Ser^{136} dephosphorylation (Fig. 2b and d), without affecting total Bad expression (data not shown).

Taken together, these findings demonstrate that pro-inflammatory cytokines modify the expression of most of the investigated Bcl-2-related proteins, indicating a complex regulation and a possible participation of these proteins in cytokine-induced beta cell toxicity, both in primary rat islets and in insulin-producing RINm5F cells. Among the investigated Bcl-2 proteins, Bcl-2, Bim, and Bid expression revealed the highest alterations in response to pro-inflammatory cytokines.

3.3. Effects of antioxidative enzymes on the expression of Bcl-2-related proteins in insulin-producing cells

In beta cells and insulin-producing cell lines the H_2O_2 inactivating capacity in comparison to other tissues is extremely low (Lenzen et al., 1996; Tiedge et al., 1997). Catalase overexpression in insulin-producing RINm5F cells resulted in a higher resistance against different chemical ROS donors. However, stable overexpression of catalase in the mitochondrial compartment protected these cells more efficiently against cytokine-induced toxicity than the overexpression of catalase in the cytosolic compartment (Gurgul et al., 2004; Lortz et al., 2000). Therefore, these RINm5F cells stably overexpressing the H_2O_2 detoxifying enzyme catalase in the cytosol (CytoCatalase) or mitochondria (MitoCatalase) were used to examine the influence of antioxidative enzymes on the expression of Bcl-2-related proteins.

RINm5F cells overexpressing CytoCatalase showed significantly lower Bcl-2 gene expression ($P < 0.01$), whereas the expression level of Bax was only slightly increased (Bcl-2: 32% and Bax: 141% of the control cells). In accordance with this, protein expression analysis also revealed a significantly lower Bcl-2 ($P < 0.001$) and up-regulated Bax expression (Bcl-2: 10% and Bax: 135% of the control cells). Thus, the calculated Bax to Bcl-2 ratio of the CytoCatalase cells increased 4.4-fold at the mRNA level and 13.5-fold at the protein level, compared with control cells (Fig. 3a and b).

In contrast, Bcl-2 gene expression in MitoCatalase cells was significantly higher and that of Bax significantly lower than in control cells ($P < 0.05$, Bcl-2: 204% and Bax: 48% of the control cells). Western blot analysis also revealed elevated Bcl-2 and significantly decreased Bax ($P < 0.05$) protein levels (Bcl-2: 206% and Bax: 46% of the control cells, Fig. 3b). Based on this observation, the ratio of

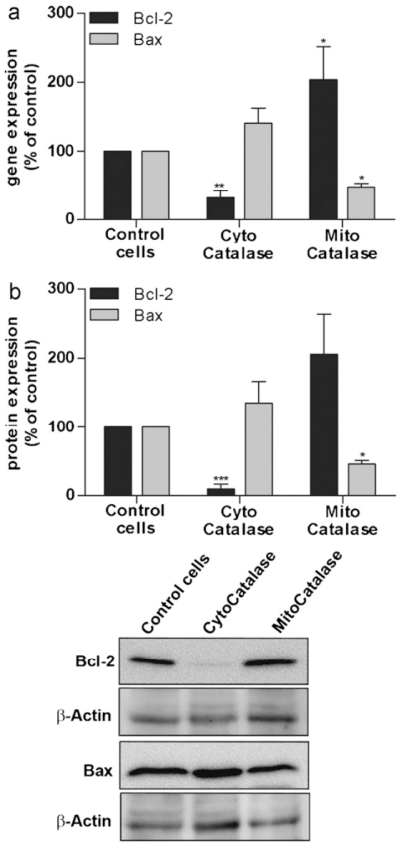

Fig. 3. Effects of cytoplasmic and mitochondrial catalase overexpression on the expression of the anti-apoptotic protein Bcl-2 and the pro-apoptotic protein Bax in insulin-producing RINm5F cells. (a) Gene expression of Bcl-2 and Bax in RINm5F control cells and cells overexpressing catalase in the cytoplasm (CytoCatalase) or in mitochondria (MitoCatalase) under control conditions. Total RNA was isolated 24 h after cell seeding, qRT-PCR was performed with gene specific primer sets, and the relative expression levels were normalized to the housekeeping gene β-actin. (b) Protein expression of Bcl-2 and Bax in RINm5F control cells and cells overexpressing catalase in the cytoplasm (CytoCatalase) or in mitochondria (MitoCatalase) under control conditions was quantified by Western blot analysis and normalized to the housekeeping protein β-actin. Gene or protein expression of RINm5F control cells was set as 100%. A representative blot is shown for Bcl-2 and Bax protein expression. Depicted values are means ± SEM, $n = 6$ mRNA expression or $n = 4$ protein expression. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ *vs.* control cells.

Bax to Bcl-2 decreased 4.3-fold at the mRNA level and 3.3-fold at the protein level, compared with control cells. The expression levels of Bcl-X_L, Bad, Bim, and Bid did not differ significantly between control cells and cells overexpressing CytoCatalase or MitoCatalase (data not shown). Therefore, only the expression of Bcl-2 and Bax in response to cytokines was characterized in these catalase overexpressing RINm5F cells.

3.4. Effects of pro-inflammatory cytokines on the expression of Bcl-2 and Bax in insulin-producing cells overexpressing antioxidative enzymes

Next, we examined the effects of pro-inflammatory cytokines on the expression of Bcl-2 and Bax in insulin-producing RINm5F cells stably overexpressing CytoCatalase or MitoCatalase. Control and CytoCatalase cells, incubated for 24 h with IL-1β alone or in combination with TNF-α and IFN-γ, resulted in a significant reduction of Bcl-2 gene expression (control cells: IL-1β 52% ($P < 0.001$) and cytokine mixture 50% ($P < 0.001$) of untreated cells; CytoCatalase cells: IL-1β 67% ($P < 0.01$) and cytokine mixture 55% ($P < 0.001$) of untreated cells). On the other hand, MitoCatalase cells showed no significant reduction of Bcl-2 gene expression after cytokine treatment (Fig. 4a). These gene expression results were confirmed by Western blot analyses. As shown in Fig. 4b, Bcl-2 protein expression in control cells was reduced to a similar extent as the gene expression in response to cytokines (IL-1β: 59% and cytokine mixture: 51% of untreated cells, $P < 0.001$). However, the reduction of Bcl-2 protein expression in cells overexpressing CytoCatalase was markedly greater after cytokine incubation (IL-1β: 28% and cytokine mixture: 23% of untreated cells, $P < 0.001$); whereas, Bcl-2 protein expression remained unchanged in cells overexpressing MitoCatalase (IL-1β: 115% and cytokine mixture: 109% of untreated cells). Thus, MitoCatalase overexpression completely prevented cytokine-induced Bcl-2 down-regulation.

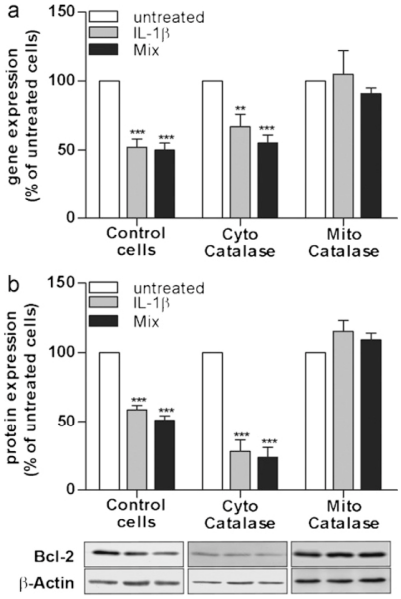

Fig. 4. Effects of pro-inflammatory cytokines on Bcl-2 gene and protein expression in insulin-producing RINm5F control cells and cells overexpressing CytoCatalase or MitoCatalase. (a) Gene expression of Bcl-2 in RINm5F control cells and cells overexpressing catalase in the cytoplasm (CytoCatalase) or in mitochondria (MitoCatalase) after incubation under control conditions, with IL-1β (600 units/ml), or with a cytokine mixture (60 units/ml IL-1β, 185 units/ml TNF-α, and 14 units/ml IFN-γ). Total RNA was isolated after 24 h incubation, qRT-PCR was performed with a Bcl-2 specific primer set, and the relative expression levels were normalized to the housekeeping gene β-actin. (b) Cell clones were treated as in (a) and Bcl-2 protein expression was quantified by Western blot analysis and normalized to the housekeeping protein β-actin. The Bcl-2 gene or protein expression in each cell clone under control conditions was set as 100%. A representative blot is shown for Bcl-2 protein expression. Depicted values are means ± SEM, $n = 6$ mRNA expression or $n = 4$ protein expression. $^{**}P < 0.01$; $^{***}P < 0.001$ *vs.* untreated cells.

In contrast, Bax gene expression was not affected by cytokine treatment in control cells and cells overexpressing CytoCatalase or MitoCatalase (Fig. 5a). At the protein level, similar expression profiles of Bax were observed in control and CytoCatalase cells. Conversely, Bax protein expression was surprisingly decreased in CytoCatalase cells after incubation with the cytokine mixture (48% of untreated cells, $P < 0.01$, Fig. 5b). In MitoCatalase cells Bax protein expression was only moderately but not significantly increased after cytokine treatment (IL-1β: 128% and cytokine mixture: 151% of untreated cells, Fig. 5b).

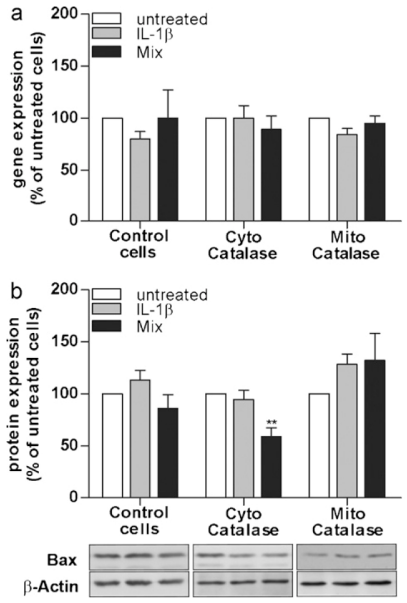

Fig. 5. Effects of pro-inflammatory cytokines on Bax gene and protein expression in insulin-producing RINm5F control cells and cells overexpressing CytoCatalase or MitoCatalase. (a) Gene expression of Bax in RINm5F control cells and cells overexpressing catalase in the cytoplasm (CytoCatalase) or in mitochondria (MitoCatalase) after incubation under control conditions, with IL-1β (600 units/ml), or with a cytokine mixture (60 units/ml IL-1β, 185 units/ml TNF-α, and 14 units/ml IFN-γ). Total RNA was isolated after 24 h incubation, qRT-PCR was performed with a Bax specific primer set, and the relative expression levels were normalized to the housekeeping gene β-actin. (b) Cell clones were treated as in (a) and Bax protein expression was quantified by Western blot analysis and normalized to the housekeeping protein β-actin. The Bax gene or protein expression in each cell clone under control conditions was set as 100%. A representative blot is shown for Bax protein expression. Depicted values are means ± SEM, $n = 6$ mRNA expression or $n = 4$ protein expression. $^{**}P < 0.01$ *vs.* untreated cells.

3.5. Cytokine-induced release of cytochrome c from mitochondria and activation of caspase-9 and -3 in insulin-producing cells

To characterize the influence of the variant Bax/Bcl-2 ratios detected in the control, MitoCatalase, and CytoCatalase cells on mitochondrial stability after incubation with IL-1β or the cytokine mixture, the cytokine-induced release of cytochrome c from mitochondria into the cytosol was studied by Western blot analysis. A 24 h treatment of control and CytoCatalase overexpressing cells with IL-1β or the cytokine mixture induced an augmented cytochrome c release into the cytosol (cytokine mixture: $P < 0.05$, Fig. 6a). On the other hand, the overexpression of MitoCatalase fully prevented this cytokine-induced cytochrome c release into the cytosol (Fig. 6a). In addition to the cytochrome c release data, the subcellular distribution of the intramitochondrial pro-apoptotic protein, second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI (Smac/DIABLO), a protein that antagonizes the inhibitory effects of the inhibitor of apoptosis protein (IAP) family, in particular of X-linked IAP, was investigated (Fig. 6b). After cytokine treatment, Smac/DIABLO was released into the cytosol in control ($P < 0.01$) and CytoCatalase overexpressing cells (IL-1β: $P < 0.05$; cytokine mixture: $P < 0.01$). The extent of cytokine-mediated Smac/DIABLO release into the cytosol was comparable to the release of cytochrome c. However, in MitoCatalase overexpressing cells, no significant release of Smac/DIABLO from the mitochondria into the cytosol could be detected (Fig. 6b).

Since cytosolic cytochrome c is part of the apoptosome, executing the processing of pro-caspase-9 to activated caspase-9, the activity of this caspase was quantified in these cell clones. Control cells treated with the indicated cytokines resulted in a significant activation of caspase-9 (IL-1β: 190%, cytokine mixture: 208% compared with untreated cells, $P < 0.01$). This cytokine-mediated activation of caspase-9 was also observed in CytoCatalase overexpressing cells (IL-1β: 235% ($P < 0.05$), cytokine mixture: 271% ($P < 0.01$) compared with untreated cells). Nonetheless, the overexpression of MitoCatalase suppressed this cytokine-mediated caspase-9 activation nearly completely (IL-1β: 127%, cytokine mixture: 120% compared with untreated cells, Fig. 7a). In addition, the cytokine-induced activation of the executioner caspase-3 was assessed as a characteristic hallmark of apoptotic cell death. As shown in Fig. 7b the exposure of control cells to IL-1β alone or the cytokine mixture resulted in a significant activation of caspase-3 (IL-1β: 213%, cytokine mixture: 202% compared with untreated cells, $P < 0.05$). CytoCatalase overexpressing cells showed also increased caspase-3 activation after cytokine treatment. However, only cells treated with the cytokine mixture exhibited a statistically significant activation of caspase-3 (IL-1β: 158%, cytokine mixture: 179% ($P < 0.05$) compared with untreated cells). In accordance with the prevention of caspase-9 activation in MitoCatalase cells, cytokine-induced caspase-3 activation was also completely abolished by MitoCatalase (IL-1β: 98%, cytokine mixture: 104% compared with untreated cells, Fig. 7b).

Taken together, these results on cytochrome c release and caspase-9 activation indicate mitochondria are a major target of pro-inflammatory cytokines finally leading to caspase-3 activation and induction of apoptosis. Prevention of this intrinsic apoptosis pathway by MitoCatalase overexpression suggests mitochondrially generated H_2O_2 is involved in this process.

4. Discussion

In the present study we show that the pro-apoptotic protein Bax is expressed at significantly higher levels than the anti-apoptotic protein Bcl-2, in both primary rat islets and insulin-producing RINm5F cells. Exposure to cytotoxic cytokines strongly increased the Bax/Bcl-2 ratio in favor of the pro-apoptotic Bax protein. However, overexpression of mitochondrially located H_2O_2 detoxifying catalase (MitoCatalase) decreased the Bax expression and increased expression of Bcl-2 in insulin-producing cells. In addition, cells overexpressing MitoCatalase prevented the cytokine-induced release of the mitochondrial pro-apoptotic factors cytochrome c and Smac/DIABLO and consequently the activation of caspase-9 and the downstream executioner caspase-3. Taken together, these data indicate that ROS-mediated loss of mitochondrial integrity and an increased Bax/Bcl-2 ratio are crucial for cytokine-induced beta cell apoptosis.

Previously, it was suggested that Bcl-2 family members are key regulators of apoptosis and that the stoichiometry of pro- and anti-apoptotic molecules decides the fate of the cell (Youle and Strasser, 2008). High Bax activity, induced through various apoptotic stimuli, has been considered a critical event in the process of beta cell apoptosis (Grunnet et al., 2009; Tonnesen et al., 2009). Interestingly, in the present study the pro-apoptotic protein Bax showed the highest expression level of all investigated Bcl-2-related proteins in primary rat islets and insulin-producing RINm5F cells; whereas its antagonist Bcl-2 showed the lowest expression. This imbalance between pro- and anti-apoptotic proteins contributes to the particular vulnerability of insulin-producing cells to apoptosis. These results are consistent with previous studies performed on isolated human islets, showing that Bax is hyperexpressed compared with Bcl-2; it was postulated that this high Bax expression was caused by the stressful islet isolation procedure (Thomas et

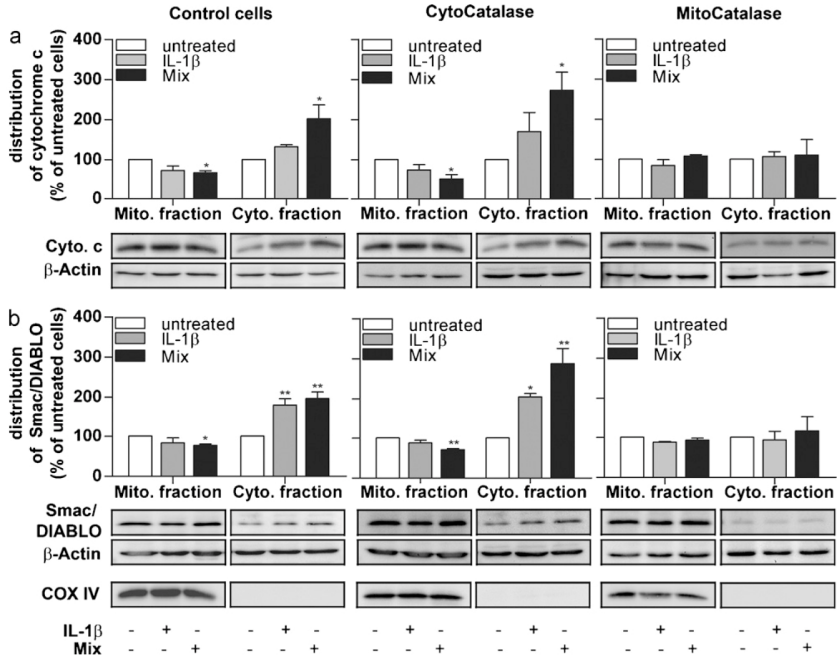

Fig. 6. Cytokine-induced release of cytochrome c and Smac/DIABLO from mitochondria of insulin-producing RINm5F control cells and cells overexpressing CytoCatalase or MitoCatalase. Western blot analysis of cytochrome c (a) and Smac/DIABLO (b) release of RINm5F control cells and cells overexpressing catalase in the cytoplasm (CytoCatalase) or in mitochondria (MitoCatalase) after 24 h incubation with IL-1β (600 units/ml) or with a cytokine mixture (60 units/ml IL-1β, 185 units/ml TNF-α, and 14 units/ml IFN-γ). Thereafter, the cells were lysed and fractionated into cytoplasmic and mitochondrial fractions by differential centrifugation. Samples were separated by SDS-PAGE, followed by immunoblotting. All blots were re-probed using a specific COX IV and β-actin antibody to ensure correct separation of mitochondrial and cytosolic fractions and correct sample loading. The cytochrome c or Smac/DIABLO content in each cellular fraction of untreated cells was set as 100%. A representative blot of both proteins is shown. Depicted values are means ± SEM, $n = 3$. $^{*}P < 0.05$; $^{**}P < 0.01$ *vs.* untreated cells.

al., 2002). However, our findings in both insulin-producing tissue culture cells and isolated primary rat islets strongly suggest that this imbalance is a characteristic of pancreatic beta cells and can be further enhanced by pro-apoptotic stimuli.

Exposure of pancreatic beta cells to pro-inflammatory cytokines has been previously shown to modulate the expression of Bcl-2 family members. In particular, Bcl-2 expression was down-regulated in mouse and human pancreatic islets upon cytokine exposure (Piro et al., 2001; Trincavelli et al., 2002; Van de Casteele et al., 2002); whereas, overexpression of Bcl-2 protected beta cells against cytokine-mediated cell death (Barbu et al., 2002; Rabinovitch et al., 1999; Tran et al., 2003). In the present study with primary islets and insulin-producing RINm5F cells, Bcl-2 expression was also significantly decreased upon exposure to IL-1β and a cytokine mixture, whereas the expression of Bax remained unchanged. This cytokine-mediated modulation of Bcl-2 expression resulted in a markedly elevated Bax/Bcl-2 ratio, which may determine the apoptotic potential of primary islets and RINm5F cells under stress conditions.

Pro-inflammatory cytokines induced expression of the BH3-only proteins Bim and Bid and activated Bad, through Ser^{136} dephosphorylation, in primary rat islets and insulin-producing RINm5F cells. Bim gene and protein expression were shown to be inducible especially by ER stress inducing compounds, such as thapsigargin. Interestingly, the Bim induction was more profound on the protein than on the mRNA level, possibly due to post-translational modifications resulting in higher protein stability (Puthalakath et al., 2007). In agreement with this study, in primary rat islets and RINm5F cells, Bim protein expression was more pronounced than gene expression in response to cytokines, suggesting that ER stress is also triggered by the cytokines, as previously shown (Eizirik et al., 2008). Bid, proteolytically cleaved by death receptor-activated caspase-8, and calcineurin-mediated Bad Ser^{136} dephosphorylation have been considered to be required for beta cell apoptosis (Grunnet et al., 2009; McKenzie et al., 2008). These BH3-only proteins antagonize not only the anti-apoptotic activity of Bcl-2 and Bcl-X_L, but Bid and Bim can also bind Bax with high affinity, thereby inducing its conformational change and translocation to mitochondria (Strasser, 2005; Youle and Strasser, 2008). This results in an amplification of the caspase cascade through engagement of the intrinsic apoptosis pathway. However, neither CytoCatalase nor MitoCatalase had significant effects on basal or cytokine-induced Bim, Bid, and Bad expression, indicating that the detoxification of H_2O_2 in both cellular compartments is not involved in BH3-only protein regulation. Despite cytokine-mediated activation of BH3-only proteins in all cell clones, the MitoCatalase cells prevented the cytokine-induced beta cell apoptosis, most likely through decreasing Bax and increasing Bcl-2 expression.

The expression of Bcl-2-related proteins is precisely regulated on the transcriptional and post-translational level and modified by various stimuli including ROS. Studies with non-insulin-secreting cells suggested that ROS, in particular H_2O_2, decrease the expression of Bcl-2 and increase that of pro-apoptotic proteins, e.g. Bax (Korsmeyer et al., 1993; Oltvai et al., 1993; Raisova et al., 2001). On the other hand, the reduction of oxidative stress by ROS scavengers and H_2O_2 inactivating enzymes could prevent down-regulation of Bcl-2 expression, resulting in higher resistance to apoptotic stimuli in other cell types (Faucher et al., 2005; Hildeman et al., 2003; Pugazhenthi et al., 2003; Wang et al., 2008). While beta cells and insulin-producing cell lines show a moderate even though overall appropriate expression of the superoxide-radical-inactivating enzyme in mitochondria, the H_2O_2

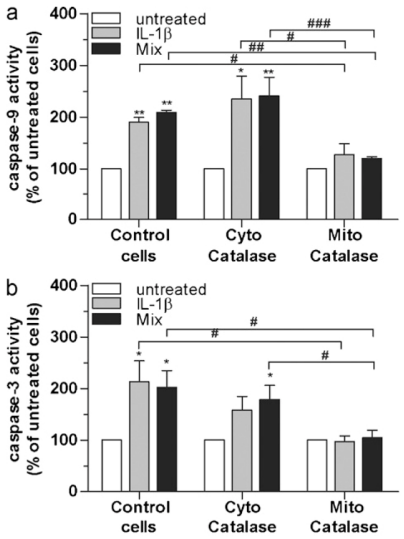

Fig. 7. Effects of pro-inflammatory cytokines on caspase-9 and caspase-3 activation in insulin-producing RINm5F control cells and cells overexpressing CytoCatalase or MitoCatalase. Quantification caspase-9 (a) and caspase-3 (b) activation of RINm5F control cells and cells overexpressing catalase in the cytoplasm (CytoCatalase) or in mitochondria (MitoCatalase) after 24 h incubation with IL-1β (600 units/ml) or with a cytokine mixture (60 units/ml IL-1β, 185 units/ml TNF-α, and 14 units/ml IFN-γ). Thereafter the cells were trypsinized, incubated for 45 min with the FAM-LEDH-FMK (caspase-9) or Red-DEVD-FMK (caspase-3), and the intracellular fluorescence intensity was measured by flow cytometry. Results are means ± SEM, $n = 4$. $^{*}P < 0.05$; $^{**}P < 0.01$ *vs.* cells incubated under control conditions; $^{\#}P < 0.05$; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$ MitoCatalase cells *vs.* RINm5F control and CytoCatalase cells under the same conditions.

inactivating capacity is extremely low compared with other tissues (Lenzen et al., 1996; Tiedge et al., 1997; Lortz et al., 2005). This imbalance is further accentuated by the cytokine-induced expression of MnSOD leading to an aggravation of an already limited antioxidative defense capacity. Thus, these cells highly efficiently dismutate superoxide radicals, but they are prone to accumulate H_2O_2 and exhibit a high susceptibility to ROS, especially under inflammatory conditions (Lenzen, 2008). Quantification of intracellular H_2O_2 formation in insulin-producing RINm5F cells exposed to pro-inflammatory cytokines by using the specific H_2O_2 sensor HyPer revealed a significant accumulation of H_2O_2 within the mitochondria (Lenzen et al., 2009). ROS dissipation through overexpression of catalase resulted in an increased resistance against chemical ROS donors and also cytokine-induced toxicity of these cells. Importantly, catalase overexpression did not affect typical cytokine signaling events such as NFκB activation and iNOS induction (Gurgul et al., 2004; Lortz et al., 2000). ROS formation, induced by pro-inflammatory cytokines, primarily targets mitochondria, causing beta cell damage and death (Lenzen, 2008).

Overexpression of MitoCatalase prevented mitochondrial H_2O_2 accumulation (Lenzen et al., 2009) and completely abolished the cytokine-mediated down-regulation of Bcl-2, which resulted in increased basal Bcl-2 and decreased Bax expression. These results are consistent with previous reports showing that overexpression of GPX1 and treatment with the synthetic antioxidant MnTBAP, also mitochondrially located, increased Bcl-2 expression and protected against ROS-mediated Bcl-2 down-regulation (Faucher et al., 2005; Orrenius et al., 2007; Wang et al., 2008).

Furthermore, MitoCatalase overexpression successfully suppressed cytokine-mediated release of cytochrome c and consequently the activation of the caspase cascade, as shown by the inhibition of caspase-9 and activation of caspase-3. On the other hand, the overexpression of cytosolically located catalase failed to prevent cytokine-induced Bcl-2 down-regulation, to reverse the imbalanced Bax/Bcl-2 ratio, and to suppress the release of mitochondrial apoptotic factors resulting in the caspase cascade activation. However, the cytokine-induced caspase-3 activation was slightly attenuated in these cells, suggesting a possible involvement of cytosolic H_2O_2 in cytokine-mediated beta cell death. This is consistent with the earlier observed partial protection against pro-inflammatory cytokine toxicity in CytoCatalase overexpressing cells (Gurgul et al., 2004; Lortz et al., 2000). These findings indicate that H_2O_2 of mitochondrial origin induced by pro-inflammatory cytokines is the major oxidative species responsible for Bcl-2 down-regulation and activation of downstream apoptotic events in insulin-producing cells.

Pro-inflammatory cytokines, especially TNF-α, are known to increase mitochondrial ROS production in many cell types (Yang et al., 2007). Thus, mitochondrial ROS are crucial for full toxicity of cytokines. The precise mechanism involving mitochondrial ROS signaling in cytokine-induced apoptosis is still unknown, but it has been postulated that the direct damaging effects of mitochondrial ROS include cardiolipin peroxidation, facilitation of mitochondrial permeability transition, and inhibition of mitochondrial metabolism (Orrenius, 2007). Besides these direct damaging effects of mitochondrial ROS, the effects of MitoCatalase overexpression on Bcl-2 gene and protein expression suggest a crosstalk between mitochondrial ROS generation and Bcl-2 expression. The Bcl-2 expression is predominantly regulated by the phosphorylated transcription factor, cAMP response element-binding protein (CREB), which is up-regulated by the PI-3K/Akt pathway and down-regulated by Ca^{2+} dependent phosphatases, including calcineurin (Pugazhenthi et al., 2000; See and Loeffler, 2001). Bcl-2 promoter activity has been impaired through cytokine-induced down-regulation of phosphorylated CREB in MIN6 cells, whereas the overexpression of wild-type CREB protected against cytokine-induced toxicity in these cells (Jambal et al., 2003). Indeed, we also observed a cytokine-induced reduction of CREB dependent Bcl-2 promoter activity in RINm5F cells, which was diminished through overexpression of MitoCatalase (unpublished observation). Thus, it is likely that CREB is the major link between mitochondrial H_2O_2 and the decrease in Bcl-2 gene expression in beta cells. Alternatively, as recently reported, Bcl-2 promoter activity can be modulated by the p53 pathway (Bourgarel-Rey et al., 2009), which can be initiated by mitochondrial ROS (Niizuma et al., 2009; Soberanes et al., 2006). Further experiments are needed to distinguish the impact of CREB, p53, and possibly other transcription factors on the Bcl-2 promoter level and to determine the influence of mitochondrial ROS on their activity in beta cells.

Overall, the present results show that ROS of mitochondrial origin are major mediators in the regulation of cytokine-induced beta cell apoptosis. Moreover, we showed that MitoCatalase overexpression can compensate for the cytokine-mediated increase of the Bax/Bcl-2 ratio and prevent cytochrome c release and the activation of caspase-9 and -3. The efficient detoxification of mitochondrial H_2O_2 protects against cytokine-mediated beta cell apoptosis and provides insights into the action of mitochondrial ROS and their redox signaling during beta cell apoptosis.

Conflict of interest

The authors declare that they have no conflict of interest.

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Overexpression of the nuclear factor-κB subunit c-Rel protects against human islet cell death in vitro

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Overexpression of the nuclear factor-κB subunit c-Rel protects against human islet cell death in vitro

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[1]*Department of Medical Cell Biology, Uppsala University, Uppsala, Sweden;* [2]*Hannover Medical School, Institute of Clinical Biochemistry, Hannover, Germany; and* [3]*Institut de Biologie de Lille, Centre National de la Recherche Scientifique UMR 8161, Lille, France*

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Mokhtari D, Barbu A, Mehmeti I, Vercamer C, Welsh N. Overexpression of the nuclear factor-κB subunit c-Rel protects against human islet cell death in vitro. *Am J Physiol Endocrinol Metab* 297: E1067–E1077, 2009. First published August 25, 2009; doi:10.1152/ajpendo.00212.2009.—The transcription factor nuclear factor (NF)-κB is known to modulate rates of apoptosis and may therefore play a role in the increased β-cell death that occurs in type 1 and type 2 diabetes. The aim of the present investigation was to study the expression of NF-κB subunits in human islet cells and whether overexpression of the NF-κB subunit c-Rel affects islet cell survival. We detected expression of p65, Rel-B, p50, p105, p52, and the ribosomal protein S3 (rpS3) in human islet cells. Among these, only p65 and rpS3 were translocated from the cytosolic to the nuclear fraction in response to cytokines. Interestingly, rpS3 participated in p65 binding to the κB-element in gel shift analysis experiments. We observed cytoplasmic c-Rel expression in vivo in 6J mice, and signs of nuclear translocation in β-cells of infiltrated nonobese diabetic islets. Human islet cells were also dispersed by trypsin treatment and transduced with a c-Rel adenoviral vector. This resulted in increased expression of c-Rel and inhibitory factor κB, increased κB-binding activity, and augmented protein levels of Bcl-$X_{L,}$ c-IAP2, and heat shock protein 72. c-Rel expression in human islet cells protected against cytokine-induced caspase 3 activation and cell death. c-Rel protected also against streptozotocin- and H_2O_2-induced cell death, in both intact rat islets and human islet cells. We conclude that rpS3 participates in NF-κB signaling and that a genetic increase in the activity of the NF-κB subunit c-Rel results in protection against cell death in human islets.

β-cell apoptosis; ribosomal protein S3; Bcl-X_L; heat shock protein 72; caspase 3

IT IS LIKELY THAT the β-cell, by converting external death signal to internal apoptotic events, participates actively in its own destruction in type 1 diabetes. Inflammatory cytokines, particularly the combination of interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ, are known to induce β-cell apoptosis and necrosis (2, 32, 38). Thus it is conceivable that these cytokines not only modulate the activity of islet-infiltrating immune cells, but also exert direct noxious effects on the β-cell in the pathogenesis of type 1 diabetes. It appears that stimulation of β-cells with IL-1β leads to multiple signaling events, including activation of protein kinases [protein kinase C, p38, c-jun NH_2-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), mitogen- and stress-activated protein kinase 1], lipases (phospholipase C, phospholipase D, sphingomyelinase), cyclooxygenase, and transcription factors [nuclear factor (NF)-κB, activating transcription factor-2, c-jun, Elk-1, cAMP response element-binding protein, CCAAT-enhancer-binding protein-β, interferon response factor-1, signal transducers and activators of transcription-1] (13). These events are followed by induction of inducible nitric oxide synthase (iNOS) and stress-related proteins such as heat shock protein (HSP) 72, heme oxygenase, Mn-superoxide dismutase, IL-1-converting enzyme, and others (8, 9, 12, 22, 24, 42, 48, 49).

The transcription factor NF-κB is activated by different mitogenic stimuli, stress signals, or inflammatory cytokines in a broad range of cell types (36, 51). The NF-κB pathways include the classical and alternative pathways using NF-κB precursor proteins p105 (NF-κB-1) and p100 (NF-κB-2), respectively (25, 35). These proteins are processed to the mature p50 NF-κB-1 and p52 NF-κB-2 proteins that preferentially heterodimerize with other members of the NF-κB family, i.e., p65/Rel-A, c-Rel, or Rel-B. Activation of the classical pathway involves release of p50/p65 from inhibitory factor κB (IκB), as a result of phosphorylation by IκB kinase and degradation of the IκB by the proteasome. Activation of the alternative pathway involves cleavage of p100 and dimerization of the mature p52 product with Rel-B. In both cases, the mature dimeric NF-κB proteins translocate to the nucleus and activate genes involved in anti-apoptotic function, the modulation of immune and inflammatory response, cell proliferation, adhesion, and angiogenesis (17).

Although the anti-apoptotic function of NF-κB in most nonislet cells is well documented, the role of NF-κB in islet cells is far from clear and straightforward. In the 1990s, we observed that the NF-κB was rapidly translocated from the cytosol to the nucleus upon stimulation with IL-1β, and that inhibition of this event resulted in protection against IL-1β-induced β-cell dysfunction and death. More recently, it has been demonstrated that NF-κB activation in β-cells leads to induction of the anti-apoptotic gene A20, which results in protection against cell death (31). Furthermore, NF-κB appears to maintain the highly differentiated β-cell phenotype with a high insulin production capacity (19, 33). The finding that a β-cell-specific genetic loss of NF-κB accelerated diabetes in nonobese diabetic (NOD) mice lends also support to a prosurvival role of NF-κB (27). On the other hand, cytokine-induced activation of NF-κB in rodent islet cells promoted increased cell death (34), and genetic ablation of NF-κB activity in mice treated with multiple streptozotocin (STZ) injections resulted in protection against diabetes (15), both suggesting a proapoptotic role of NF-κB. Thus the role and biological significance of NF-κB for human β-cell function and destruction appears to be complex and controversial. Because NF-κB subunit expres-

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sion and cytokine-induced translocation have hitherto not been studied in human islet cells, our first aim was to document activation of classical and alternative NF-κB pathways. Second, in an attempt to mimic an isolated activation of the classical pathway, we overexpressed the NF-κB subunit c-Rel in human islet cells. We report here for the first time that the novel NF-κB subunit ribosomal protein S3 (rpS3) participates in cytokine-induced NF-κB signaling and that c-Rel promotes human islet cell survival.

METHODS

Islet isolation. Rat pancreatic islets were isolated from 3-mo-old Sprague-Dawley rats (Skanbur, Solna, Sweden) by a collagenase digestion procedure (3). Human pancreatic islets were kindly provided by O. Korsgren (Dept. of Clinical Immunology, Uppsala, Sweden). β-Cell percentage was routinely assessed using Newport green staining and fluorescence microscopy and was found to be between 30 and 60%. The insulin release was stimulated 7.5 ± 2.2-fold by a high glucose concentration (16.7 mM) in 13 shipments of human islets.

Preparation of cytoplasmic and nuclear fractions. Human islets (150/group) were either left untreated or treated with the cytokines IL-1β (50 U/ml) and TNF-α (1,000 U/ml) for 30 min. Following cytokine treatment, the islets were incubated for 20 min on ice in *solution A* (40) containing proteinase inhibitor cocktail (Sigma). The islets were lysed with an electric homogenizer and centrifuged for 5 min at 3,200 rpm at 4°C, and proteins in the supernatant fraction were precipitated using aceton (70%). The precipitated proteins were resuspended in SDS-sample buffer. The pelleted fraction of the lysates was resuspended in *solution A* and centrifuged a second time for 5 min at 3,200 rpm at 4°C and resuspended in SDS-sample buffer.

Immunoblotting. Hybond-P membranes (GE Healthcare) were blocked in 5% BSA for 1 h, after which they were probed with c-Rel, p65, Rel-B, p105/p50, p52, Bcl-2, Bcl-X_L, iNOS, polypyrimidine tract-binding protein, X-inhibitor of apoptosis (IAP), c-IAP-2, HSP72, tubulin, ERK (Santa Cruz), rpS3, uncleaved caspase-3, cleaved caspase 3 (Cell Signaling), and c-IAP1 (Abcam) antibodies. Stripping and immunodetection was performed as previously described (38).

Confocal microscopy. The imaging and analysis of intact isolated islets were performed by confocal laser scanning microscopy. Intact islets were cytospinned (1,200 rpm for 3 min) to polylysine-coated slides and fixed in 4% paraformaldehyde for 5 min. The islets were permeabilized in 0.1% Triton X-100 and then incubated for 60 min at 37°C with guinea pig insulin antibodies, rabbit or mouse p65, Rel-B, p50, or p52 antibodies. The Alexa Fluor 488 goat anti-guinea pig (Invitrogen), the Alexa Fluor 568 goat anti-mouse (Invitrogen), and the Cy3 donkey anti-rabbit (Jackson Immunoresearch) IgGs were used as secondary antibodies. Samples were subjected to optical sectioning using a Nikon D-Eclipse C1 confocal laser scanner connected to a Nikon Eclipse C1-2000U inverted microscope. Fluorescence was excited at 488 and 543 nm, and emitted light was collected between 535 and 650 nm.

Adenoviruses. We used serotype 5, E1-deleted/E3-deficient adenoviral vectors derived from the widely used AdEasy system, which express either nothing (control virus), green fluorescent protein (GFP) under the control of the cytomegalovirus (CMV) promoter (GFP virus), and human c-Rel under control of the CMV promotor (c-Rel virus). Virus stocks were purified by cesium chloride density-gradient centrifugation (L-80 ultracentrifuge; Beckman Coulter, Fullerton, CA) and plaque titered by serial dilution and agar overlay on human embryonic kidney 293 cell line. Typical titers were 10^{10} plaque-forming units (PFU)/ml or higher, representing 1–5% of the total viral particles as determined by readings of the optical densities.

In vitro transduction procedure. For the adenoviral-mediated transduction, rat or human islet cells, which had been dispersed in free islet cell suspension by trypsin treatment, were incubated at 37°C for 1 h in a volume of 0.4 ml RPMI-1640 supplemented with 2% FCS and containing 1 or 5 PFU/cell of the adenoviral vector. Transduced islet cells were washed and further cultured for 3 days in complete CMRL 1066 medium. During this subsequent culture period, islet cells reaggregate and form new cell clusters that resemble nondispersed islets (50).

Extraction of nuclear proteins and electromobility shift assay. Islet cells were treated with IL-1β (50 U/ml) for 30 min, after which proteins were extracted for electromobility shift assay (EMSA), which was performed as previously described (40). p65 and p50 or rpS3 antibodies (0.2 μg) were used for supershift, and c-Rel antibody (0.2 μg) was used to block c-Rel binding to the κB-probe.

Determination of cell viability. For the study of the effects of c-Rel expression on cell viability after exposure to a nitric oxide donor, STZ or hydrogen peroxide (H_2O_2), dispersed and reaggregated rat and human pancreatic islet cells were vital stained and analyzed by fluorescence microscopy 3 days after the islet cell trypsination and adenoviral transduction procedure. Before analysis of human islet cells (18 h), 2.0 mM 2,2′-(hydroxynitrosohydrazono)bis-ethanamine (DETA/NO; Cayman Chemical), 15 mM STZ, or 100 μM H_2O_2 was added to the cultures. Rat islet cell cultures were supplemented with 0.5 mM DETA/NO or 50–60 μM H_2O_2 also 18 h before analysis. Cell clusters were vital stained by incubation in medium containing 5 μg/ml bisbenzimide and 10 μg/ml propidium iodide (PI) during the last 15 min. The islet cells were then washed and examined by fluorescence microscopy using a triple-pass filter and a Leitz DMRB microscope (Leica) as previously described (38). Red fluorescence (PI) and blue fluorescence (bisbenzimide) was quantified in the same pictures using Adobe Photoshop software, and the ratio of red over blue was calculated as a relative measure of cell viability.

For 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) analysis of human islet cell viability, cells were transduced with viral vectors and exposed to DETA/NO, H_2O_2, and STZ as described above. After addition of cytotoxic agents (18 h), cells were incubated for 4 h with MTT solution (Cell Proliferation Kit 1; Roche Diagnostics, Mannheim, Germany) followed by overnight incubation with solubilization solution, according to the instructions of the manufacturer. Absorbance was then analyzed at 560 nm using a 96-well plate reader.

For analysis of the c-Rel effect on cytokine-induce human islet cell death, intact human islets were precultured in the presence of cytokines (50 U/ml IL-1β, 1,000 U/ml TNF-α, and 1,000 U/ml IFN-γ) for 4–5 days. The islets were then dispersed and transduced with adenoviral vectors. Transduced and reaggregated cells were cultured for another 3 days in the presence or absence of cytokines before vital staining and fluorescence microscopy as described above. The human islets were precultured for 4–5 days before transduction procedure because human islet cell death occurs not earlier than after 7 days of cytokine exposure (14) and because 7–8 days of c-Rel overexpression could result in confounding secondary effects.

For analysis of the c-Rel effect on cytokine-induce rat islet cell death, rat islets were dispersed and transduced with adenoviral vectors. After transduction (2 days), reaggregated cells were exposed to 50 U/ml human IL-1β and 1,000 U/ml murine IFN-γ. The next day, cells were vital stained, photographed, and analyzed as described above.

Pancreas perfusion transduction. Sprague-Dawley rats were used for pancreas perfusion experiments as previously described (5). The pancreas perfusion buffer contained 0.5×10^9 PFU/pancreas of the GFP-expressing adenoviral vector and 1×10^9 PFU/pancreas of the c-Rel adenoviral vector, which corresponds to 20–50 PFU/islet cell. The capillary endothelium was disrupted before administering the viral vector by preperfusion for 40 s with medium containing 0.1% Triton X-100, followed by a 10-min wash with Krebs-Ringer-bicarbonate-HEPES buffer only. Following transduction, islets were isolated and cultured for 2 days. After the culture period, GFP-positive

islets were identified and separated from GFP-negative islets under a fluorescence stereomicroscope.

RESULTS

Expression of NF-κB subunits in islet cells. We exposed human islets to IL-1β or TNF-α for 30 min and then performed a subcellular fractionation to generate the cytoplasmic and nuclear fractions. Imunnoblotting using antibodies against the known NF-κB subunits p65, Rel-B, c-Rel, p50/p105, and p52/p100 plus the recently discovered NF-κB subunit rpS3 (45) revealed that p65, Rel-B, p50, p105, p52, and rpS3 immunoreactivity could be detected (Fig. 1). p65 was translocated to the nuclei in response to cytokine stimulation (Fig. 1). Somewhat surprisingly, there was only a trend to p50 translocation in response to the 30-min cytokine exposure. Instead, we observed relatively high levels of p50 and p105 in nuclear fractions of control islet cells (Fig. 1). On the other hand, rpS3 was translocated to the nuclei of human islet cells (Fig. 1). The components of the alternative NF-κB pathway, p52/p100 and Rel-B, were not translocated in response to cytokine stimulation (Fig. 1). Confocal analysis of NF-κB subunit subcellular localization confirmed the immunoblot analysis results in that cytokine-induced p65 translocation was observed, both in insulin-positive and insulin-negative cells (Fig. 2*A*). The p50 + p105 signal was strong in both cytosol and nuclei of islet cells with no signs of a cytokine-induced translocation. The Rel-B and p52 signals were weak and evenly distributed throughout the islet cells. We could not observe Rel-B or p52 translocation in response to cytokines (Fig. 2*A*).

Because we could not detect c-Rel expression at the basal condition using the Santa Cruz antibody B-6 c-Rel antibody, we also analyzed paraffin-embedded pancreas sections from control C57BL/6J and female NOD mice using the R&D systems anti-mouse c-Rel antibody. With the use of this approach, c-Rel immunoreactivity was observed in insulin-positive cells (Fig. 2*B*). This immunoreactivity was localized to the cytosol in control 6J mice, resulting in a clear difference between the cytosolic and nuclear c-Rel signal (Fig. 2*B*). However, in NOD islets, which displayed prominent peri-insulitis, some c-Rel immunoreactivity was also observed in nuclei of β-cells (Fig. 2*B*), indicating that c-Rel activation occurs in NOD islet β-cells surrounded by a peri-insular infiltrate.

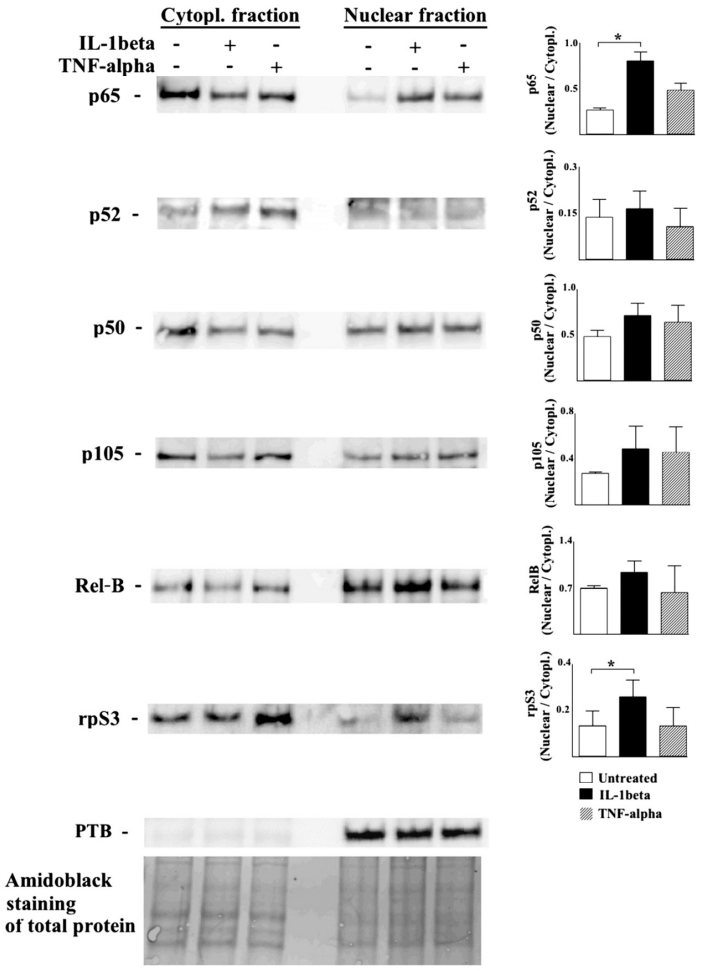


Fig. 1. Immunoblot analysis of expression and sub-cellular localization of nuclear factor (NF)-κB subunits in human islet cells. Human islets were exposed to the cytokines interleukin (IL)-1β (50 U/ml) or tumor necrosis factor (TNF)-α (1,000 U/ml) for 30 min as indicated. The islets were washed, lysed, and fractionated into cytoplasmic and nuclear fractions. Samples were then boiled in SDS sample buffer for immunoblotting analysis using antibodies against the different NF-κB subunits as indicated. The filters were also analyzed using antibodies against the nuclear protein polypyrimidine tract-binding protein to visualize efficiency of subcellular fractionation. Islets from three different donors were analyzed in separate experiments, and results are expressed as the ratio between nuclear fraction and the cytosolic fraction, both corrected for total protein loading (amidoblack staining). Results are means ± SE. $^*P < 0.05$ using Student's *t*-test. Filters were stripped between each antibody incubation.

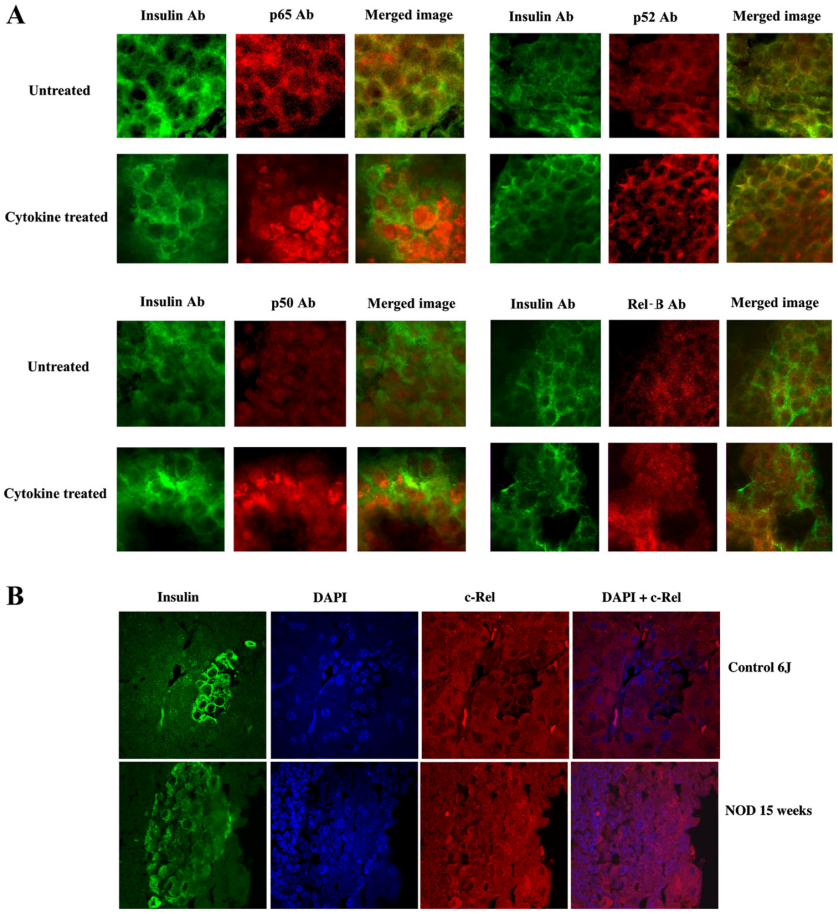


Fig. 2. Confocal imaging of expression and subcellular localization of insulin and NF-κB subunits in human islet cells (*A*) and in mouse pancreatic sections (*B*). *A*: human islets were exposed to IL-1β (50 U/ml) for 30 min as indicated. The islets were then immunostained and analyzed by confocal microscopy for insulin and NF-κB subunit expression and localization. The images shown are representative for three donors. *B*: pancreatic sections from normoglycemic control C57BL/6J and female NOD mice of 15 wk of age were deparaffinized and pressure boiled for antigen retrieval. c-Rel staining was obtained using the R&D Systems anti-mouse biotinylated c-Rel antibody. A secondary layer of Alexa 568-streptavidin conjugate was used. Insulin was visualized using a fluorescein isothiocyanate-conjugated donkey anti-guinea pig antibody. Nuclei were stained with 4′,6-diamino-2-phenylindole dihydrochloride.

To verify that rpS3, upon cytokine stimulation, forms a complex with NF-κB in human islet cells, we performed gel shift analysis of nuclear extracts from islet cells exposed to IL-1β for 30 min. We observed that the κB-binding activity was clearly enhanced by IL-1β in nuclear extracts (Fig. 3). The κB-binding complex was supershifted by both p65 and rpS3 antibodies (Fig. 3). No clear shift could be observed using p50 antibodies. This agrees well with the finding that p50 was not translocated in response to IL-1β or TNF-α (Fig. 1).

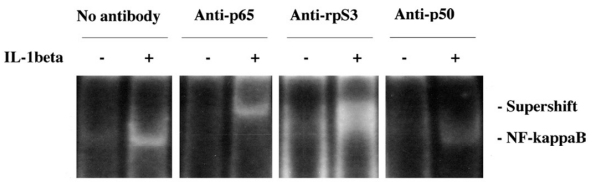


Fig. 3. The κB-binding complex is supershifted by ribosomal protein S3 (rpS3) antibodies. Human islets were exposed to IL-1β (50 U/ml) for 30 min and then used for preparation of nuclear extracts. The extracts were then incubated with a radiolabeled κB probe with or without antibodies against p65, rpS3, and p50. The samples were analyzed by electromobility shift assay (EMSA) and visualized by radiography. Data are representative for experiments with two human islet donors.

Adenoviral-induced expression of c-Rel in human and rat islet cells. Previous studies have established that adenoviral vectors only reach superficially located cells of human islets (3). To reach all islet cells, we dispersed the islet cells by trypsination, a procedure that leads to transfection efficiencies of >90%, of both insulin-positive and insulin-negative cells (4). Using this approach, we observed a very weak c-Rel band with a multiplicity of infection (MOI) of one and a clear band at an MOI of five (Fig. 4*A*). The increased expression of c-Rel did not promote expression of iNOS (Fig. 4*A*). This is in line with previous studies indicating that an isolated increase in NF-κB is not sufficient to iNOS gene expression (39). To verify that transgene-mediated expression of c-Rel increased NF-κB translocation to nuclei, we performed gel shift analysis using the κB-element as probe. Indeed, the κB-binding activity in nuclear extracts was higher in c-Rel-expressing human islet cells than in control-transformed cells, and it was further augmented by cytokines (Fig. 4*B*). It should be noted that c-Rel and p65 migrate to the same position in EMSA gels (20). Addition of a p65 antibody supershifted the entire NF-κB-band, indicating that p65 is present in most NF-κB hetero- or homodimers, also in cells overexpressing c-Rel. Blocking the c-Rel DNA-binding domain with a c-Rel antibody resulted in

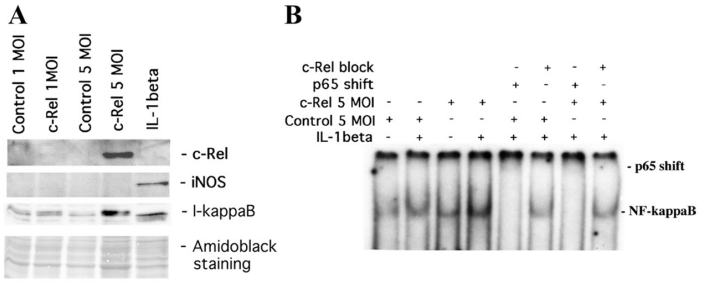


Fig. 4. Expression of c-Rel (*A*) and NF-κB (*B*) activity in human islet cells transduced with a c-Rel adenoviral vector. *A*: human islet cells were dispersed by trypsin treatment and transduced with 1 or 5 multiplicity of infection (MOI) of control or c-Rel virus. After transduction (1 day), IL-1β (50 U/ml) was added to some of the control virus-exposed cells after which cells were cultured for another 24 h. The cells were then washed and lysed in SDS-sample buffer for immunoblot analysis of c-Rel, inducible nitric oxide synthesis (iNOS), and inhibitory factor κB (IκB) expression. *B*: cells treated as in *A* were also used for EMSA. In this case, the IL-1β was added only 30 min before harvest of cells. Data are representative for experiments using three donors.

blunting of the effect of the c-Rel adenoviral transduction, leaving only NF-κB complexes consisting of p65 (Fig. 4*B*). The c-Rel-induced increase in κB-binding activity was paralleled by an increased expression of IκB, a well-known NF-κB target gene (Fig. 4*A*). c-Rel overexpression did not affect p65 and p50 protein levels (results not shown).

Effect of c-Rel on expression of apoptosis-regulating proteins. Dispersed human islet cells transduced with control or c-Rel adenoviral vectors at 5 MOI were studied by immunoblot analysis for expression of apoptosis-regulating proteins known to be transcriptionally induced by NF-κB. We were unable to detect immunoreactivity for FLICE-inhibitory protein (FLIP), c-IAP1, and A20 (results not shown). Specific immunoreactivity for Bcl-X_L, Bcl-2, X-IAP, and HSP72 was, however, obtained (Fig. 5). Expression of c-Rel in human islet cells did not affect Bcl-2 and X-IAP, but increased the contents of Bcl-X_L and HSP72 (Fig. 5). In addition, c-Rel induced increased expression of a 30-kDa band exhibiting specific c-IAP-2 immunoreactivity (Fig. 5). The full-length c-IAP-2 migrates as a 65- to 70-kDa protein, suggesting that the 30-kDa band represents a proteolytic fragment of the c-IAP-2 protein. Full-length c-IAP-2 immunoreactivity was weak and not affected by c-Rel overexpression (results not shown).

Effect of c-Rel on human and rat islet cell viability in response to cytokines, H_2O_2, DETA/NO, or STZ. Dispersed and reaggregated human islet cells, transduced with control or c-Rel adenoviral vectors at 5 MOI, were exposed to the cytokines IL-1β, TNF-α, and IFN-γ, H_2O_2, DETA/NO, or STZ, and relative cell viability was assessed by calculating the ratio between total PI and bisbenzimide (Hoechst) fluorescence. At least 10 photographs, each containing 30–200 reaggregated islet cells, from each culture condition were used

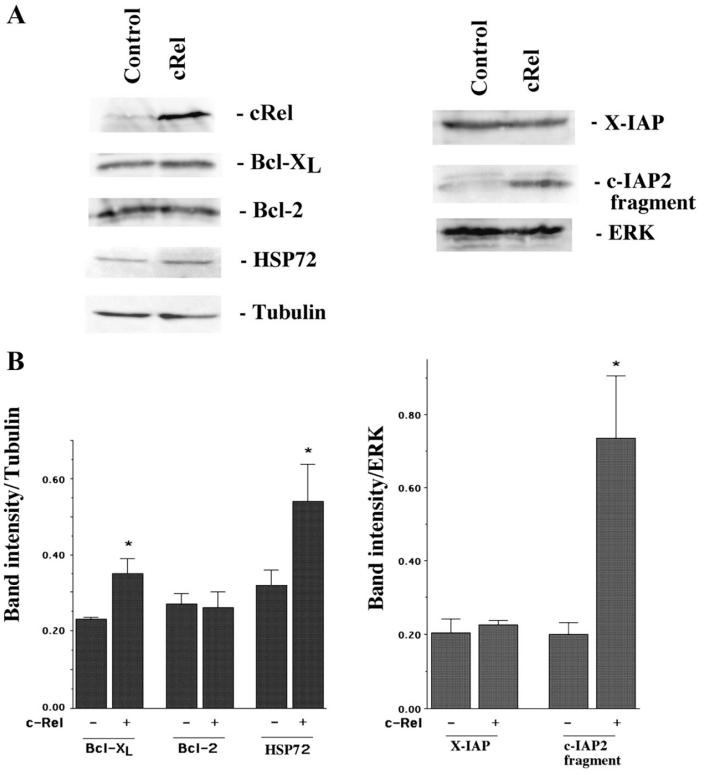


Fig. 5. Effect of c-Rel on human islet cell expression of Bcl-X_L, heat shock protein (HSP)72, and c-IAP2. Human islet cells were dispersed by trypsin treatment and transduced with 5 MOI of control or c-Rel virus. Later (2 days), the cells were washed and lysed in SDS-sample buffer for immunoblot analysis of c-Rel, Bcl-X_L, Bcl-2, HSP72, X-IAP, and c-IAP2 expression. The same filters were stripped between the reblottings, and total protein loading was visualized using tubulin and total extracellular signal-regulated kinase (ERK). *A*: immunoblots for one human islet donor. *B*: results from densitometric scannings of three experiments. Results are means ± SE. $*P < 0.05$ using Student's *t*-test.

for cell viability calculations. The PI-to-Hoechst ratios of the different experimental groups are either given without any further calculations (Fig. 6, *B* and *D*) or expressed as percentages of the control group (Fig. 6*A*). We observed a c-Rel-induced partial protection against cytokines (Fig. 6*A*), STZ, and H_2O_2 (Fig. 6*B*). The viability in cells exposed to DETA/NO was quite variable, and there was only a trend toward c-Rel-induced protection (Fig. 6*B*). However, when human islet cells were analyzed using the MTT assay, we observed a significantly improved viability in response to c-Rel expression when challenged with DETA/NO (Fig. 6*C*). Also in dispersed rat islet cells there was partial protection against H_2O_2, but not against DETA/NO (Fig. 6*D*). Rat islet cell death was lower in c-Rel-expressing cells compared with control cells when challenged with cytokines (Fig. 6*D*). To verify that β-cells, and not only non-β-cells, were protected when overexpressing c-Rel, we costained for insulin and cleaved caspase 3 (Fig. 6*E*). The percentage of β-cells positive for cleaved caspase 3 was 12 ± 2.9 in the control group and 32 ± 3.2 in the cytokine group ($n = 3$). The corresponding percentages in cells overexpressing c-Rel were 6.3 ± 3.5 and 13 ± 3.0, respectively. We also costained for insulin and c-Rel to verify efficient transduction of β-cells (Fig. 6*F*). Furthermore, the c-Rel-mediated increase in viability, following a cytokine exposure, was paralleled by less pronounced activation of caspase 3, as evidenced by weaker bands corresponding to the two forms of cleaved caspase 3 (17 and 19 kDa) (Fig. 7).

Effect of c-Rel on the viability of intact rat islets. To avoid dispersion of islet cells by trypsination, we used the pancreas perfusion technique that yields efficient whole islet transduction in situ (5). The transduced islets were isolated and cultured for 2 days before visual inspection under a fluorescence stereo microscope. Typically, two-thirds of the islets were GFP-positive and one-third GFP-negative, which probably results from incomplete perfusion of the entire pancreas with the viral vector-containing solution. The GFP-negative islets were separated from the GFP-positive islets by hand-picking and used as c-Rel-negative control islets. Overnight exposure to H_2O_2, DETA/NO, or STZ resulted in a significantly increased cell death of the control islets (Fig. 8). Cell death of GFP-positive islets was less pronounced and did not reach statistical significance in response to any of the treatments. In addition, the cell death in response to STZ was significantly lower in c-Rel-positive islets than corresponding c-Rel-negative islets (Fig. 8).

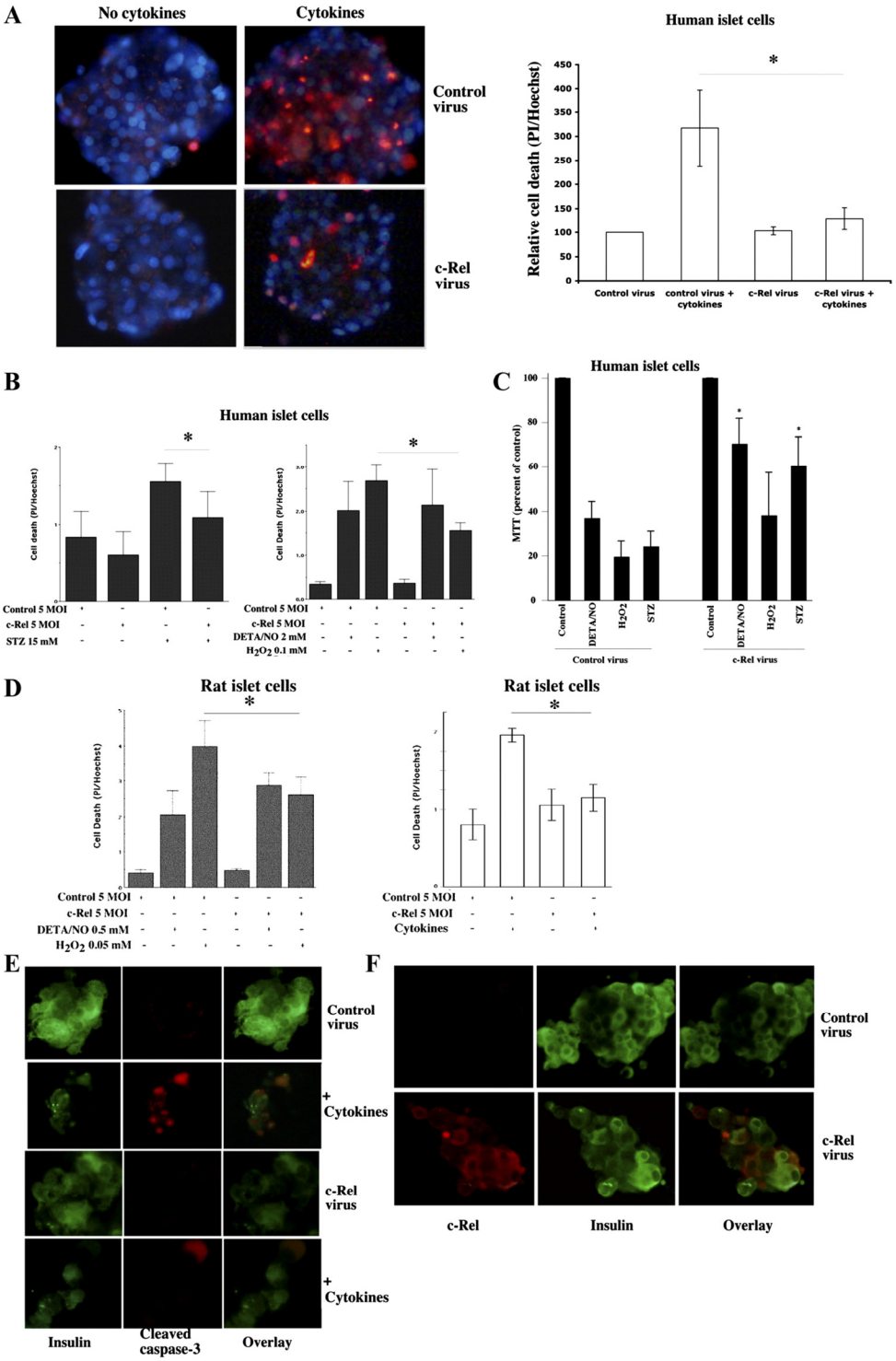
DISCUSSION

We have shown in this study human islet expression of the NF-κB subunits p65, Rel-B, p50, p105, p52, and rpS3, but not of p100 and c-Rel. The lack of p100 and c-Rel expression may have resulted from a poor sensitivity of the antibodies, and it is therefore not possible to exclude a low level of expression of these proteins. Indeed, using a different antibody and paraffin-embedded pancreatic sections, cytosolic c-Rel expression was observed in mouse β-cells. Interestingly, because some nuclear c-Rel signal was observed in NOD islet cells, it is possible that c-Rel is activated in vivo as a response to islet-infiltrating immune cells and therefore participates in inflammatory signaling and the defense against β-cell death. Also, a low, but not absent, level of p100 is likely to be present in islet cells, since this is the precursor to p52. In other types of cells than islet cells, p100 is only slowly converted to p52 (52). Our finding that p52, but not p100, is readily detected might indicate that the alternative NF-κB pathway is differentially regulated in human islets and that mature islet cells rely on a high and constitutive p52/Rel-B activity. This notion is also supported by our observation that cytokine treatment failed to induce nuclear translocation of p52 and Rel-B. Activation of the alternative NF-κB pathway has been reported to occur in islet cells in response to enhanced Fas/FLIP signaling, an event associated with increased β-cell survival, function, and proliferation (41).

In concert with previous studies in nonislet cells (36, 51), cytokine treatment promoted p65 nuclear translocation. The failure of TNF-α to induce significant p65 translocation, even though a trend to an increase in nuclear p65 was observed, supports the notion that IL-β is the most potent NF-κB activator of the two. In INS-1E cells, p65 homodimers were translocated early after IL-1β stimulation, whereas the p50 homodimers became translocated later on (34). In the present investigation, we observed a possible trend to p50 translocation after 30 min of cytokine exposure. Thus it may be that the p50 homodimers translocate also in human islet cells, but with slower kinetics than the p65 homodimers. However, the processing of p105 in p50 was unaffected by cytokine treatment and does not seem to be a crucial event in human islets.

Interestingly, the rpS3 protein was also translocated in response to IL-1β. The rpS3 protein is normally a component of the 40S ribosome and has a key role in protein translation. However, in a recent study, rpS3 was identified as a non-Rel subunit of p65 homo- or p65/p50 heterodimers (45). Moreover, rpS3 was found to be important for the specific binding of p65 to regulatory sites within DNA and essential for induction of NF-κB-controlled genes (45). It has also been reported that rpS3 has distinctive roles in DNA repair and apoptosis (23). In this context, it can be speculated that protective effects of rpS3 against apoptosis (10, 21) originate from its participation in

Fig. 6. Effect of c-Rel expression on islet cell viability in response to cytokines, streptozotocin (STZ), 2,2′-(hydroxynitrosohydrazono)bis-ethanamine (DETA/NO), or H_2O_2. Dispersed human (*A*, *B*, *C*, *E*, and *F*) and rat (*D*) islet cells were transduced with 5 MOI of control or c-Rel adenoviral vectors. Cytokine-treated human islet cells (*A*) were exposed to 50 U/ml of IL-1β, 1,000 U/ml of TNF-α, and 1,000 U/ml of interferon (IFN)-γ for 4–5 days before the transduction procedure and 3 days after transduction. After the transduction procedure (2 days), rat islet cells (*D*) were exposed to murine IFN-γ (1,000 U/ml) and human IL-1β for 24 h. STZ (*B*), DETA/NO (*C*), or H_2O_2 (*D*) were added to the cell cultures 2 days after the transduction with the adenoviral vectors. The 3rd day after the transduction procedure, islet cells were either stained with propidium iodide (PI) and bisbenzimide (Hoechst) (*A*, *B*, and *D*), and photographed in a fluorescence microscope using a triple band filter, or analyzed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (*C*). *A*, *left*: representative cell aggregates in which blue nuclei (Hoechst) represent living cells and red nuclei (PI) dead cells. Red and blue fluorescence was also quantified using Adobe Photoshop, and ratios were calculated. Ratios between red and blue fluorescence were expressed as percent control. *B* and *D*: results are not percentages but instead ratios between red and blue mean fluorescence. Results are means ± SE for 4 (human islet cells) and 4–6 (rat islet cells) separate experiments. *$P < 0.05$ vs. the corresponding control virus group using Student's *t*-test. *E* and *F*: human islet cells were dispersed by trypsin treatment and transduced with 5 MOI of control or c-Rel virus. Some of the islets had been precultured with cytokines for 4 days before the transduction procedure as given above. Later (3 days), islet cell aggregates were stained for cleaved caspase 3 (red) and insulin (green) (*E*) or for c-Rel (red) and insulin (green) (*F*).


A
No cytokines
Cytokines
Control virus
c-Rel virus
Human islet cells
Relative cell death (PI/Hoechst)
Control virus
control virus + cytokines
c-Rel virus
c-Rel virus + cytokines
B
Human islet cells
Cell death (PI/Hoechst)
Control 5 MOI
c-Rel 5 MOI
STZ 15 mM
DETA/NO 2 mM
H2O2 0.1 mM
C
Human islet cells
MTT (percent of control)
Control
DETA/NO
H2O2
STZ
Control virus
c-Rel virus
D
Rat islet cells
Cell Death (PI/Hoechst)
Control 5 MOI
c-Rel 5 MOI
DETA/NO 0.5 mM
H2O2 0.05 mM
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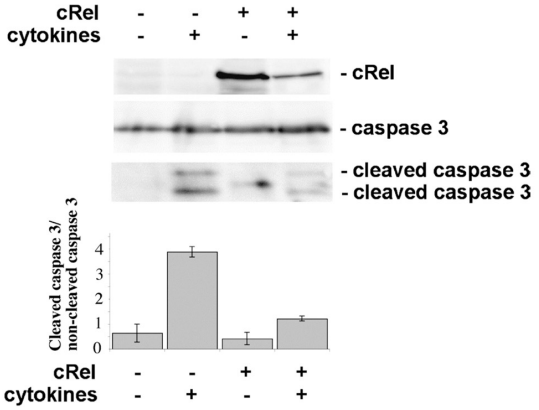

Fig. 7. Cytokine-induced activation of caspase 3 in c-Rel-transduced human islet cells. Dispersed human islet cells were transduced with 5 MOI of c-Rel adenoviral vectors. Cytokine-treated islet cells had been exposed to cytokines (50 U/ml IL-1β, 1,000 U/ml TNF-α, and 1,000 U/ml IFN-γ) for 4–5 days before the transduction procedure. After the transduction (3 days), cells were harvested for immunoblot analysis of c-Rel and noncleaved and cleaved caspase 3. The ratios between cleaved and noncleaved caspase 3, quantified on the same filters, were calculated and are depicted at *bottom*. Results are means ± SE for three separate experiments using islets from three donors.

NF-κB-induced gene transcription. Further investigations of rpS3 and its role in the control of NF-κB-induced genes in islet cells are clearly warranted.

To mimic an isolated activation of the classical NF-κB pathway, we have presently used an adenoviral vector that expresses c-Rel under the control of the CMV promoter. We chose to express c-Rel in human islet cell because it is known to perform similar functions as its family member p65 (29). In addition, because human islets did not express detectable levels of the c-Rel subunit at basal conditions, the risk of disturbing endogenous c-Rel protein levels/function, when expressing this particular NF-κB subunit, is probably low. Consistent with the anti-apoptotic role of NF-κB in other cell types (25), dispersed human islet cells overexpressing c-Rel showed decreased rates of cell death in response to cytokines, STZ and H_2O_2. Assuming that c-Rel and p65 exert similar actions in human islet cells, it is possible that not only enhanced c-Rel activity, but also p65 activity, which is promoted by cytokine exposure, mediates protection against human islet cell death.

To transduce intact islets, we used the recently developed whole pancreas perfusion technique by which vectors are delivered via the vascular system, resulting in transduction of ~70% of the islet cells (5). c-Rel transduction of intact rat islets using this novel technique protected the islets from STZ-induced cell death. However, c-Rel transduction failed to protect rat islets from DETA/NO-induced cell death. In this context, it should be noted that NO may induce β-cell death by inhibiting aconitase, decreasing the mitochondrial membrane potential and ATP production, leading to necrosis rather than apoptosis (6, 46). Thus it may be that c-Rel protects mainly against apoptosis and not against necrosis.

It has been shown in other cell types that NF-κB influences the expression of pro- and anti-apoptotic proteins and that this affects both the intrinsic (mitochondrial) and the extrinsic (death factor receptor) pathways of apoptosis (25). First, NF-κB promotes transcription of the Bcl-2 family member genes, such as Bcl-2, Bfl-1, and Bcl-X_L, that block activation of the intrinsic pathway. In our experimental setup, c-Rel expression resulted in increased Bcl-X_L, but not Bcl-2, expression. It is not clear why Bcl-2 protein levels were not increased, but it may be that a putative increase in Bcl-2 mRNA was counteracted by micro-RNA-mediated translational arrest, as has been observed in other cell types (11). Second, NF-κB induces also the IAP and FLIP proteins, which both counteract the extrinsic caspase 8 pathway. We could not detect FLIP and c-IAP1 immunoreactivity, possibly because of low antibody sensitivity. On the other hand, we observed a distinct increase in a c-IAP2 fragment. This finding is consistent with the notion that c-IAP2 is induced by NF-κB and that it is a ubiquitinating enzyme that controls its own levels by self-ubiquitination and degradation (44). Third, NF-κB is also known to upregulate the expression of X-IAP, a protein that inhibits effector caspases. However, this event appears to not occur in islet cells, since we could not observe an increase in X-IAP, nor was X-IAP mRNA induced by cytokines in INS-1 cells (34). Fourth, NF-κB activates transcription of other anti-apoptotic genes, such as TNFAIP3/A20 and HSPs. Cytokines were observed to induce the TNFAIP3/A20 mRNA 20-fold in INS-1 cells (34), and it is therefore possible that the presently observed lack of TNFAIP3/A20 immunoreactivity resulted from a low sensitivity of the antibody. Furthermore, the present finding of an increase level of HSP72 is in line with reports demonstrating regulatory NF-κB binding sites in the promoters of different heat shock genes (1, 28, 30). Taken together, our results support a protective role for c-Rel-mediated NF-κB activity in human islets, mediated by an enhanced expression of the anti-apoptotic proteins Bcl-X_L, c-IAP2, and HSP72.

In summary, we report that the ribosomal protein rpS3, upon stimulation with IL-1β, becomes translocated together with p65 to islet cell nuclei and participates in binding to the κB-element, and that c-Rel overexpression promotes anti-apoptotic effects in human islet cells. Both anti-apoptotic (18, 19, 27, 31, 33, 41, 43) and pro-apoptotic (15, 34) effects of NF-κB have been observed in other islet cell studies. There are several possible explanations for the seemingly contradictory pro- and anti-apoptotic effects of NF-κB in islet cells. It might be that a specific mode of NF-κB activation, depending on subunit composition and kinetics, results in protection against cell death whereas others do not. In addition, the possibility exists that the pro-apoptotic activity of NF-κB is a rodent specific phenomenon. Because NF-κB activation is required for cytokine-induced iNOS induction (40), and because increased NO production is the main cause for cytokine-induced β-cell death in rodents, inhibition of NF-κB efficiently ameliorates cell death in this particular case. On the other hand, human islet cells are not highly sensitive to nitric oxide (14, 27). Thus it may be that the anti-apoptotic actions of NF-κB, presently observed in human islet cells, are overwhelmed by the induction of iNOS in rodent β-cells. Finally, the possibility also exists that the activity of NF-κB is modulated by other cytokine-induced signaling pathways so that a more pro-apoptotic set of genes is induced in response to cytokines compared with an isolated NF-κB activation. For example, it has been

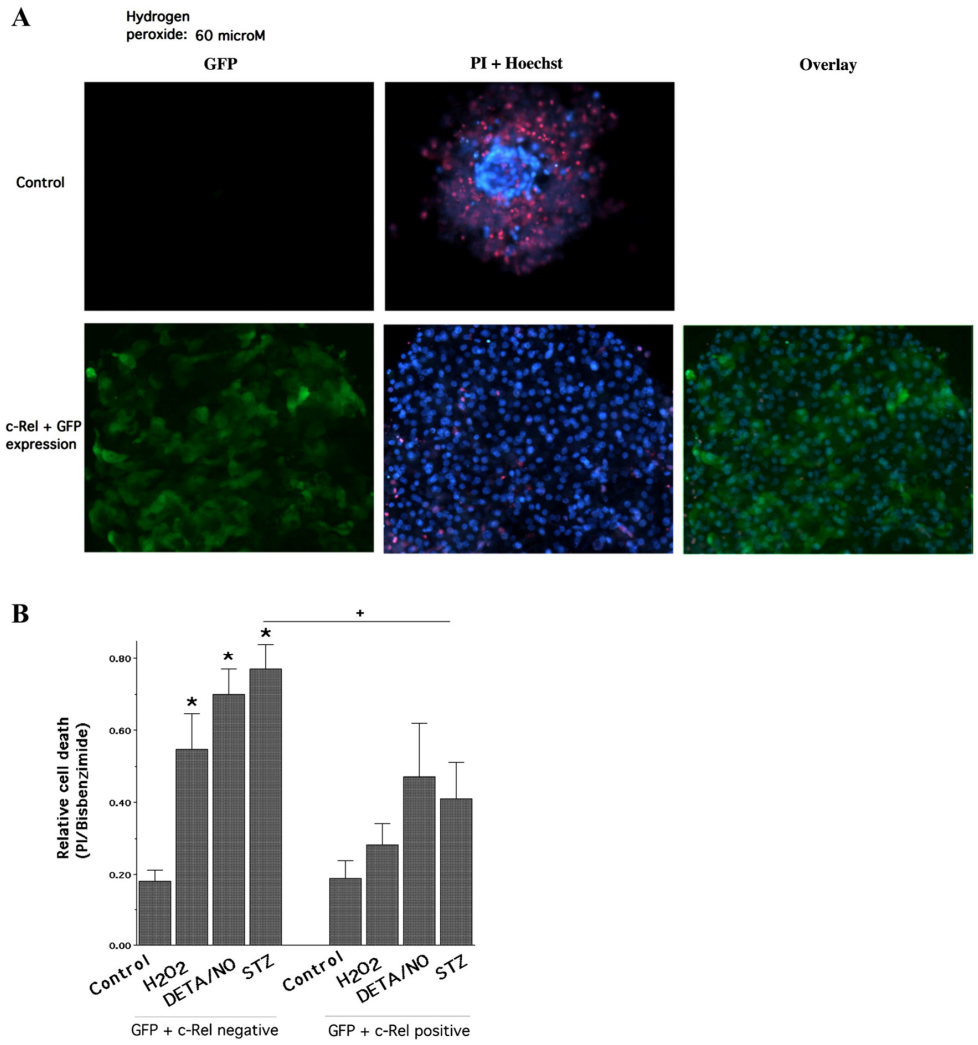

Fig. 8. PI and Hoechst fluorescence in green fluorescent protein (GFP)-negative (control) and GFP-positive (c-Rel + GFP expression) intact rat islets. Rat islets were transduced in situ using the pancreas perfusion technique. After viral perfusion of the pancreases, the islets were isolated and cultured for 2 days. GFP-negative islets were then separated from GFP-positive islets. The two groups of islets were exposed to control conditions or 60 μM H_2O_2 and stained the following day with PI and Hoechst for fluorescence microscope analysis. *A*: fluorescence microscopy pictures of GFP and PI + Hoechst fluorescence in a GFP-negative islet and a GFP-positive islet. *B*: red and blue fluorescence was quantified using Adobe Photoshop, and ratios were calculated. Results are means ± SE for 4 separate experiments. $P < 0.05$ vs. corresponding control by Student's *t*-test (*) and vs. corresponding control islets using 2-way ANOVA and Student's *t*-test (+).

observed that JNK and HSPs exert reciprocal effects upon the anti-apoptotic action of NF-κB (7, 26).

During revision of this manuscript, a study by Sakar et al. (37) was published, in which it was observed that several anti-apoptotic genes were induced in human islets in response to cytokine stimulation and NF-κB activation. One of the induced genes was c-IAP2, which is in good agreement with the present findings, and induction of anti-apoptotic genes was paralleled by no increase in islet cell apoptosis (37). Although the Sakar study did not analyze the effects of an isolated increase in NF-κB, this recent report further strengthens our present conclusion that NF-κB has important anti-apoptotic activities in islet cells. In light of such a scenario, strategies that aim at restoring or enhancing NF-κB anti-apoptotic activity, but not NF-κB pro-inflammatory activity, in β-cells might prove useful in the future treatment of diabetes.

GRANTS

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Cytokine toxicity in insulin-producing cells is mediated by nitrooxidative stress-induced hydroxyl radical formation in mitochondria

Running head: Nitrooxidative stress in insulin-producing cells

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Abstract

Although nitric oxide (NO) and oxidative stress both contribute to proinflammatory cytokine toxicity in pancreatic β-cells during type 1 diabetes mellitus (T1DM) development, the interactions between NO and reactive oxygen species (ROS) in cytokine-mediated β-cell death have not been clarified. Exposure of insulin-producing RINm5F cells to IL-1β generated NO, while exposure to a combination of IL-1β, TNFα and IFNγ, which simulates T1DM conditions, generated both NO and ROS. In theory, two reactions between NO and ROS are possible, one with the superoxide radical yielding peroxynitrite, and the other with hydrogen peroxide (H_2O_2) yielding hydroxyl radicals. Results of the present work exclude peroxynitrite involvement in cytokine toxicity to β-cells, because its generation did not correlate with the toxic action of cytokines. On the other hand, we show that hydrogen peroxide (H_2O_2), produced upon exposure of insulin-producing cell clones and primary rat islet cells to cytokines almost exclusively in the mitochondria, reacted in the presence of trace metal (Fe^{++}) with NO forming highly toxic hydroxyl radicals, thus explaining the severe toxicity that causes apoptotic β-cell death. Expression of the H_2O_2-inactivating enzyme catalase in mitochondria protected against cytokine toxicity by preventing hydroxyl radical formation. We therefore conclude that proinflammatory cytokine-mediated β-cell death is due to nitrooxidative stress-mediated hydroxyl radical formation in the mitochondria.

Keywords: nitrooxidative stress, insulin-producing cells, cytokines, mitochondria, hydroxyl radical, peroxynitrite.

Introduction

Reactive oxygen and nitrogen species (ROS and RNS) and the concomitant dysregulation in the cellular redox state play a crucial role in the pathogenesis of many inflammatory and autoimmune diseases, in reperfusion injury, and in cancer [1-3]. ROS and nitric oxide (NO) are thought to affect signal cascades and to contribute to cytokine toxicity, which leads to the death of insulin-producing cells [4; 5]. During autoimmune attack, insulin-producing cells and immune cells generate reactive species in response to humoral factors by mechanisms that are currently unknown [5]. The extraordinary sensitivity of pancreatic β-cells to oxidative stress can be explained by a significant expression of superoxide dismutases (cytoplasmic CuZnSOD and mitochondrial MnSOD), which dismutate superoxide radicals into H_2O_2 and concomitantly, a very low expression of the antioxidant enzymes catalase and glutathione peroxidase (GPX), which inactivate H_2O_2 [6]. Proinflammatory cytokines modulate the expression levels and activities of antioxidant enzymes, thereby further promoting the imbalance in the redox status of insulin-producing cells [7-11]. Proinflammatory cytokines also stimulate inducible NO synthase (iNOS) expression, promoting NO formation [11-13]. Thus, both NO and ROS are crucial elements in cytokine-mediated β-cell destruction [4; 5].

A number of studies have shown that improving the antioxidative defense status in insulin-producing cells promotes β-cell survival not only upon exposure to ROS and RNS donors [14-18], or hyperglycemia [19], but also, and importantly, during cytokine attack [20-22]. NO and ROS interaction is thought to potentiate toxicity when they are generated simultaneously [23] via the generation of peroxynitrite from NO and superoxide radicals [24]. Generation of peroxynitrite has been proposed as an explanation for the toxicity of proinflammatory cytokines in pancreatic β-cells and is traditionally considered to be the main cause of cytokine toxicity [25-28]. However, a number of other studies have reported conflicting data. For example, overexpression of MnSOD in insulin-producing cells, which can reduce superoxide radicals and therefore reduce peroxynitrite formation, was found to increase the cell death rate in response to cytokines [9], while suppression of MnSOD expression promoted survival of insulin-producing cells treated with cytokines [9]. Overexpression of the H_2O_2 detoxifying enzymes catalase and GPX also provided protection against cytokine toxicity [21; 22]. On the other hand, NO formation alone is insufficient for cytokine toxicity in insulin-producing cells [13; 29; 30].

In this study, we provide strong and novel evidence that the interaction between NO and H_2O_2, yielding toxic hydroxyl radicals, but not peroxynitrite, is crucial for cytokine

toxicity in insulin-producing cells. Based on our results, we hypothesize that cytokine toxicity is ultimately due to hydroxyl radicals generated by this chemical reaction. We therefore conclude that proinflammatory cytokine-mediated β-cell death is due to nitrooxidative stress-induced hydroxyl radical formation.

Materials and Methods

Reagents

Cytokines were obtained from PromoCell (Heidelberg, Germany). Hybond N nylon membranes, the ECL detection system, and autoradiography films were from Amersham Biosciences (Freiburg, Germany), and Immobilon-P PVDF membranes from Milipore (Bedford, MA, USA). All other reagents were from Sigma Chemicals (München, Germany).

Cell culture and cytokine incubation

Insulin-producing RINm5F cells were cultured as described previously [22] in RPMI medium supplemented with 10 mM glucose, 10 % (v/v) fetal calf serum (FCS), penicillin, and streptomycin in a humidified atmosphere at 37 °C and 5 % CO_2. The cytokine IL-1β was used at 600 U/ml, and the cytokine mixture contained IL-1β (60 U/ml), TNFα (185 U/ml), and IFNγ (14 U/ml).

Rat islet isolation, culture and treatment

Pancreatic islets were isolated from 250-300 g adult male Lewis rats by collagenase digestion and handpicked under a stereo microscope. Isolated islets were cultured on extracellular matrix (ECM)-coated plates (35 mm) (Novamed, Jerusalem, Israel, the ECM being derived from bovine corneal endothelial cells) in RPMI-1640 medium containing 5 mmol/l glucose, 10% FCS, penicillin, and streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. The islets were cultured for 10–12 days on the ECM plates to adhere and spread before they were infected with HyPer-Mito lentivirus or preincubated with HPF. The islets were then treated with 600 U/ml IL-1β or a cytokine mixture for 24 h following fluorescence measurements.

Targeted expression of catalase and MnSOD in insulin-producing RINm5F cells

Insulin-producing RINm5F cells contain the same antioxidant enzyme profile as primary islet cells [16; 31]. Transfection of RINm5F cells with a pcDNA3 vector lacking an insert (as a negative control) did not affect the expression of any of the antioxidant enzymes. The pCMV/myc/mito-plasmid was used to overexpress mitochondrially-targeted human catalase cDNA, as described previously [21]. The pcDNA3 vector containing human MnSOD in the sense or antisense orientation was used to overexpress or suppress MnSOD, as described previously [9].

MTT cell viability assay

In all experiments, cell viability was determined 72 h after incubation with cytokines in the presence or absence of the iNOS blocker L-NARG (L-nitroarginine; 5 mM) using a microplate-based MTT assay as described previously [21].

Caspase-3 activity assay

Activation of caspase-3 was quantified in cells after 72 h exposure to cytokines using a Green Caspase-3 staining Kits (PromoCell, Heidelberg, Germany) according to the manual instruction. After staining and washing, cell suspensions were promptly read in the CyFlow ML cytometer (Partec, Münster, Germany). A total of 20,000 events were acquired. Non-labelled cells were used as a negative control and for the determination of the gates. Data were analyzed by FlowJo software (Tree Star, Ashland, OR). The data are expressed as % of the caspase-3 positive cells without exposure to cytokines.

Western blotting for catalase, CuZnSOD, GPX, iNOS, and MnSOD

Cells were homogenized in ice-cold PBS using short bursts (Braun-Sonic 125 Homogenizer, Quigley-Rochester, Rochester, NY). Protein content was determined by the BCA assay (Pierce). For Western blotting, 20 µg (for catalase, CuZnSOD, GPX and MnSOD) or 40 µg (for iNOS) of total protein was resolved by SDS polyacrylamide gel electrophoresis and then electroblotted onto membranes. Immunodetection was performed using specific primary antibodies against Cat (rabbit polyclonal, Rockland, Gilbertsville, PA), CuZnSOD, or MnSOD, or GPX (rabbit polyclonal, kind gifts from Dr. K. Dobashi, Japan), or iNOS (NOS2 rabbit polyclonal IgG, Santa Cruz Biotechnology, Heidelberg, Germany) followed by exposure to secondary peroxidase-conjugated AffiniPure donkey anti-rabbit IgG (H+L) (Dianova, Hamburg, Germany). Hybridized antibodies were visualized through chemiluminescence using the ECL detection system after short exposure (2-3 min) to autoradiography film.

Nitric oxide determination

Nitrite accumulation after 72 h of cytokine exposure was determined spectrophotometrically at 562 nm by the Griess reaction as described previously [17].

Determination of oxidative stress using DCFDA-H_2

To detect overall oxidative and nitrosative stress, 5×10^3 cells were seeded onto 96-well black plates and cultured for 24 h. The cells were then pre-incubated with 10 µM dichlorodihydrofluorescein diacetate DCFDA-H_2 (Invitrogen, Karlsruhe, Germany) for 40 min at 37 °C. The medium containing the chemical was discarded, and fresh medium was

added with or without cytokines in the presence or absence of the iNOS inhibitor L-NARG. After incubation for 72 h, the plates were analyzed at 480/520 nm excitation/emission using the fluorescence reader Victor^2 1420 Multilabel Counter (Perkin Elmer, Fremont, CA). Each condition was measured at least in duplicate. The data were expressed as a % of untreated cells.

Analysis of H_2O_2 levels using HyPer vector.

The pHyPer-Mito and pHyPer-Cyto eukaryotic expression vectors (Evrogen, Moscow, Russia) were used for mitochondrial and cytoplasmic expression, respectively, of the fluorescent H_2O_2 sensor protein HyPer. 24 h after 5×10^4 cells were seeded onto 6 cm dishes, cells were stably transfected with 2 µg DNA (RIN-HyPerMito or RIN-HyPerCyto clones) using Lipofectamine (Invitrogen) or were infected with the lentiviral construct pLenti-HyperMito (diluted 1:400, kind gift of Dr. M. Elsner, Hannover, Germany) (Mito-Cat-HyPerMito and MnSOD-HyPerMito clones). Positive clones were selected by resistance to G418 (250 µg/ml) (RIN-HyPerCyto and RIN-HyPerMito) or to blasticin (10 µg/ml) (Invitrogen) (Mito-Cat-HyPerMito, MnSOD-HyPerMito and MnSODantisense-HyPerMito) and verified by fluorescence measurements. HyPer oxidation was analyzed after a 72 h incubation with cytokines. For ratiometrical quantification of H_2O_2, 2×10^4 cells were seeded onto 96-well black plates. Plates were analyzed at 475/427 nm excitation and 520 nm emission using the Victor^2 1420 Multilabel Counter fluorescence reader (Perkin Elmer). The data were expressed as mean values of the F475/F427 ratio. For imaging, 5×10^4 cells were seeded onto either black 24-well plates with glass bottoms (Greiner, Frickenhausen, Germany) or onto Mat-Tek dishes (MatTek, Ashland, MA). A Cell^R/Olympus IX81 inverted microscope system equipped with a Cellcubator was used (UPLSAPO 60 x 1.35 numerical aperture oil-immersion objective, CFP-YFP dual filter (427 nm and 504 nm excitation, and 520 nm emission), fluorescence intensity of 12.5 %) (Olympus, Hamburg, Germany).

Analysis of hydroxyl radical-induced oxidative damage to DNA

The level of oxidative DNA damage after incubation for 72 h with cytokines was measured using the Biotrin OxyDNA assay according to the manufacturer's instruction (Biotrin OxyDNA, Biotrin, Dublin, Ireland).

Hydroxyl radical formation estimation

The level of hydroxyl radical formation after incubation for 72 h with cytokines was estimated using the fluorescent dye 3'-(*p*-hydroxyphenyl) fluorescein (HPF) (Invitrogen) according to the manufacturer's instruction.

Nitrotyrosine ELISA

The concentration of nitrotyrosine after incubation for 72 h with cytokines was measured using the Oxiselect Nitrotyrosine ELISA (BioCat GmbH, Heidelberg, Germany) according to the manufacturer's instruction.

Confirmation of mitochondrial localization of the HyPerMito protein

Insulin-producing RINm5F-HyPerMito cells were seeded onto Mat-Tek dishes (MatTek) 24 h before loading with MitoTracker Deep Red (Invitrogen) for 15 minutes at 37°C. Images were captured and analyzed using a Cell^{R}/Olympus IX81 inverted microscope system equipped with a Cellcubator (UPLSAPO 60 x 1.35 numerical aperture oil-immersion objective, GFP/DsRed filter, fluorescence intensity of 3 %) (Olympus, Hamburg, Germany).

Data analysis

All data are expressed as means ± SEM. Statistical analyses were performed using the Prism analysis program (Graphpad, San Diego, CA); p-values of < 0.05 were considered significant.

Results

Characterization of insulin-producing RINm5F cell clones

Stable transfection of insulin-producing cells was verified by Western blotting (Fig. S1). The level of catalase protein was very low in control cells (set as 100 %) and in clones that overexpressed or suppressed MnSOD (102 ± 13 %, 93 ± 7 %, n=4). In contrast, the Mito-Cat clone expressed approximately 9-times more catalase protein than control cells (869 ± 75 %, n=4). There was an approximately 3-fold increase in MnSOD protein expression in the MnSOD clone cells (340 ± 35 %, n=4) and an approximately 50 % decrease in MnSOD protein expression in the MnSOD antisense clone. CuZnSOD and GPX levels were similar in all clones and were not affected by transfection with the other expression vectors.

Mitochondrial antioxidative status and cytokine-induced toxicity

IL-1β, TNFα, IFNγ. In control insulin-producing RINm5F cells 60 U/ml IL-1β caused approximately 15 % cell death, while 185 U/ml TNFα led to 30 % of cell loss and 14 U/ml IFNγ had no toxic effects (Table S1). Mito-Cat cells were protected against toxicity of all three proinflammatory cytokines. In contrast, MnSOD cells were extraordinarily sensitive to IL-1β toxicity (Table S1). MnSODantisense cells were slightly better protected against TNFα toxicity (Table S1). Incubation of control cells with a higher concentration of 600 U/ml IL-1β caused a loss of cell viability in the MTT assay of around 35 % (Table 1). In cells expressing Mito-Cat, IL-1β was significantly less toxic than in control cells (Table 1). In contrast, mitochondrial overexpression of superoxide dismutase in the MnSOD cells significantly increased IL-1β toxicity compared to the control clone (Table 1). There was no protective effect of MnSOD suppression (Table 1). The iNOS inhibitor L-nitroarginine (L-NARG; 5 mM) slightly improved the viability of control cells treated with IL-1β; this effect was very significant in MnSOD cells (Table 1). L-NARG had no protective effect on Mito-Cat and MnSODantisense cells (Table 1).

Cytokine mixture. Exposure of insulin-producing RINm5F control cells to a combination of the cytokines IL-1β (60 U/ml), TNFα (185 U/ml), and IFNγ (14 U/ml) caused a 70 % decrease in cell viability in the MTT assay (Table 1). Mitochondrial overexpression of catalase in the Mito-Cat clone provided the strongest protection against toxicity, with only a 30 % decrease in cell viability (Table 1). In contrast, overexpression of MnSOD significantly increased cell death compared to control, with a 90 % cell loss upon exposure to the cytokine mix (Table 1). In contrast, the MnSODantisense cells were protected against cytokine toxicity. This protection was, however, not as prominent as that in the Mito-Cat clone. As was

the case of IL-1β, L-NARG significantly improved also the viability of all cells treated with the cytokine mixture (Table 1). Importantly, the protective effect of L-NARG was stronger in control cells treated with the mixture than in control cells treated with IL-1β alone, suggesting higher NO production after exposure to a mixture of cytokines (Table 1). On the other hand, even in the presence of L-NARG, there was still a ~30 % cell death rate, clearly indicating that NO is not the only factor involved in cell death after cytokine exposure. Interestingly, L-NARG did not significantly affect cell viability in the Mito-Cat clone, but the contribution of NO to cytokine-mediated cell death was strong in MnSOD cells (Table 1). Incubation of MnSODantisense cells with L-NARG resulted only in a minor additional protective effect.

As shown in Fig. 1a both IL-1β and the cytokine mixture significantly induced caspase-3 activation in insulin-producing control cells. Overexpression of MnSOD pronounced cytokine effects towards caspase-3 activation (Fig.1a). In contrast, reduction of intramitochondrial hydrogen peroxide concentration in the Mito-Cat and MnSODantisense clones led to a significant inhibition of cytokine-induced caspase-3 activation (Fig. 1a). These findings confirm the MTT data.

Cytokine-induced DCFDA-H_2 oxidation

IL-1β, TNFα, IFNγ. DCFDA-H_2 as a fluorescent indicator of overall oxidative and nitrosative stress. In the presence of various ROS as well as of NO, DCFDA-H_2 is rapidly oxidized into DCF. In control insulin-producing RINm5F cells 60 U/ml IL-1β increased DCFDA-H_2 oxidation by approximately 37 % compared (Table S2). TNFα (185 U/ml) led to a 35 % increase while 14 U/ml IFNγ had no stimulatory effect on DCFDA-H_2 oxidation (Table S2). Mito-Cat cells were protected against cytokine-induced DCFDA-H_2 oxidation. In contrast, IL-1β and TNFα caused in MnSOD cells a huge, significantly higher increase of DCFDA-H_2 oxidation when compared to control cells (2.4-fold induction after IL-1β, and over 2-fold induction after TNFα; Table S2). Interestingly, in the MnSOD cells the incubation with IFNγ also accelerated DCFDA-H_2 oxidation up to 70 % over untreated cells (Table S2). MnSODantisense cells were slightly more prone to cytokine-induced DCFDA-H_2 oxidation when compared with control cells (Table S2).

Incubation of control cells with a higher concentration of 600 U/ml IL-1β induced oxidation of DCFDA-H_2 to a comparable extent as at the low concentration (100 % basal vs. 138 % after 600 U/ml IL-1β; Table 2). To distinguish the extent to which ROS or NO contributed to IL-1β-induced DCFDA-H_2 oxidation and cytokine toxicity, a series of experiments was performed using L-NARG. In control cells treated with IL-1β and L-NARG, the oxidation

rate of DCFDA-H_2 was about 40 % lower than in the cells incubated with IL-1β alone (Table 2). In cells overexpressing Mito-Cat, the oxidation rate of DCFDA-H_2 was minimal upon incubation with IL-1β, as it was in the MnSODantisense clone (Table 2). In contrast, the oxidation rate of DCFDA-H_2 was significantly increased in the MnSOD clone (Table 2). In all clones, the increase in DCFDA-H_2 oxidation was prevented by simultaneous addition of L-NARG (Table 2).

Cytokine mixture. Exposure of insulin-producing RINm5F control cells, MnSOD-overexpressing or suppressing cells to the cytokines IL-1β (60 U/ml), TNFα (185 U/ml), and IFNγ (14 U/ml) induced DCFDA-H_2 oxidation to a significantly greater extent than exposure to IL-1β alone (Table 2). The cytokine mixture significantly induced DCFDA-H_2 oxidation also in the Mito-Cat clone (Table 2). In control cells, cytokine-induced oxidation of DCFDA-H_2 was reduced by 50 % in the presence of L-NARG; however, a 30 % difference in fluorescence was still observed compared to untreated cells, indicating that this difference was due to ROS (Table 2). L-NARG completely prevented oxidation of DCFDA-H_2 in the Mito-Cat clone (Table 2), suggesting that DCFDA-H_2 oxidation was due to RNS rather than to ROS. In contrast, inhibiting iNOS in the MnSOD clone reduced DCFDA-H_2 oxidation by around 50 % only (Table 2), indicating a significant ROS component. The DCFDA-H_2 oxidation in the MnSODantisense clone incubated with cytokines in the presence of L-NARG was lower than in those exposed only to cytokines, but was not completely abolished (Table 2), indicating a ROS component, however, less so than in MnSOD cells.

Cytokine-stimulated iNOS protein expression, nitrite and peroxynitrite production. No iNOS protein was detected in untreated insulin-producing RINm5F cells (Fig. 1b). In control cells, both 600 U/ml IL-1β and the cytokine mixture induced iNOS expression after incubation for 72 h (Fig. 1b), but not by TNFα and IFNγ (data not shown). Cytokine-stimulated iNOS expression in the Mito-Cat and in both the MnSOD and MnSODantisense clones was similar to that in the control clone (Fig. 1b).

Nitrite production was measured to estimate how much NO was produced in insulin-producing cell clones following exposure to cytokines. In line with the iNOS protein estimation, virtually no nitrite was detectable after incubation under control conditions in the presence or absence of the L-NARG, indicating virtually no expression of any other NOS type under these conditions. In the control clone, both IL-1β alone and the cytokine mixture stimulated nitrite production, and the induction was higher after exposure to the cytokine mixture (Table 3). As expected, the use of L-NARG completely prevented NO formation (Table 3). Nitrite levels measured in the MnSOD clone were much higher than in the control

cells (Table 3), while in the MnSODantisense clone they were lower than in control cells, but higher than in Mito-Cat cells. Interestingly, there was little nitrite production in the Mito-Cat clone exposed to cytokines (Table 3). L-NARG prevented NO formation in the Mito-Cat clone and significantly diminished nitrite formation in the MnSOD and MnSODantisense cell clones (Table 3).

Tyrosine residues are modified to 3-nitrotyrosine and the concentration of nitrotyrosine correlates with the formation of peroxynitrite. The results revealed a significant cytokine-induced production of peroxynitrite in the control clone (Fig. 1c). Cytokine-mediated peroxynitrite formation was slightly attenuated by overexpression of catalase in the mitochondria (Fig. 1c). In the MnSOD clone, overexpressing the enzyme dismutating superoxide radicals, one of the substrates for peroxynitite formation, the concentration of nitrotyrosine after exposure to cytokines was significantly lower than in control cells (Fig. 1c). In contrast, the nitrotyrosine concentration in the MnSODantisense cells treated with the cytokine mixture was significantly higher (Fig. 1c).

Cytokine-stimulated H_2O_2 production

Transfection of cells with vectors that expressed H_2O_2-sensitive and -specific HyPer proteins in the cytoplasmic or mitochondrial compartments allowed the localization (i.e. cytoplasmic vs. mitochondrial) and degree of H_2O_2 formation in insulin-producing cells under cytokine attack. The HyPer protein has two excitation peaks (420 nm and 475 nm) and one emission peak (520 nm). Upon exposure to H_2O_2, the excitation peak at 420 nm decreases proportionally to the increase in the 475 nm peak. Thus, in low-oxidation conditions, the F475/F420 ratio is expected to be lower for the HyPer protein (green fluorescence) than in high-oxidation conditions (red fluorescence).

An analysis of HyPer oxidation revealed a higher basal oxidation rate of HyPerMito than of HyPerCyto (Fig. 2a). Incubation with cytokines did not significantly affect HyPerCyto oxidation (Fig. 2a). Interestingly, the HyPerMito protein was significantly more oxidized in cells incubated with cytokines, indicating an increase in mitochondrial H_2O_2 (Fig. 2a). Importantly, HyPerMito oxidation was higher in cells exposed to a cytokine mixture than to IL-1β alone (Fig. 2a). To study the effects of the antioxidative defense system in the mitochondria, we used insulin-producing cell clones that expressed the HyPerMito protein specifically in mitochondria (Fig. 3) and also overexpressed either Mito-Cat or MnSOD. The basal ratio between red and green fluorescence of the HyPer-Mito protein was lower in cells overexpressing catalase in the mitochondria (predominantly green fluorescence in the cells) and was significantly higher in the MnSOD clone (shift towards red fluorescence) compared

to control cells (Fig. 2b and Fig. 4a, c and e). As expected, a significantly stronger shift of the HyPer-Mito protein oxidation towards red fluorescence as well as a significantly higher H_2O_2 production were detected in the MnSOD clone after 72 h incubation with cytokines, when compared to the control clone (Fig. 2b and Fig. 4b and f). On the other hand, most cells of the Mito-Cat clone remained viable after 72 h of incubation with the cytokine mixture and showed greater HyPer-Mito green fluorescence, indicating that there was no significant H_2O_2 production in their mitochondria under these conditions (Fig. 2b and Fig. 4d).

These observations were confirmed in primary rat islet cells treated with cytokines for 24 h. While incubation of islet cells with IL-1β led to a small induction of intramitochondrial hydrogen peroxide formation (Fig. 5a and b), exposure to the cytokine mixture caused a strong, significant rise in hydrogen peroxide generation (Fig. 5a and b).

Cytokine-induced hydroxyl radical formation

In a last step the generation of the hydroxyl radical ($^{\bullet}OH$) was estimated in insulin-producing cells exposed to cytokines using a fluorescent probe that detects oxidatively-damaged DNA (8-oxyguanine) as a reliable test for the estimation of $^{\bullet}OH$ induced cell toxicity. In control cells incubation with IL-1β for 72 h increased oxidative DNA damage 2-fold and incubation with the cytokine mixture led to a 4-fold increase (Fig. 6a). In contrast, no oxidative damage was detected in the Mito-Cat clone (Fig. 6a). A massive increase of oxidative DNA damage was seen in cells overexpressing MnSOD: a 5-fold increase in the case of IL-1β and an 8-fold increase in the case of the cytokine mixture (Fig. 6a). On the other hand, a lower rate of DNA oxidative damage was observed in the MnSODantisense cells (Fig. 6a). We confirmed these findings using a second, independent method, namely oxidation of the fluorescence probe HPF, which is considered to have high specificity and selectivity towards hydroxyl radicals (Fig. 6b).

Measurements of HPF oxidation in rat islet cells revealed a minimal induction of hydroxyl radical formation after a 24 h incubation with IL-1β (120 ± 7 % vs. untreated 100 %, n=4), and a significant 2.5-fold increase of hydroxyl radical formation after exposure to the cytokine mixture (244 ± 30 %, n=4).

Discussion

Proinflammatory cytokines are major effectors of programmed cell death during the development of type 1 diabetes [4; 32-34], but the mechanism of action is not fully understood. Although it has been suggested that nitrosative [35-37] as well as oxidative [9-11; 15; 16; 21; 22; 38; 39] stress play a role in cytokine toxicity, their interplay and their relative contributions to death of insulin-producing cells is still unclear.

To elucidate the importance of the interaction between nitrosative and oxidative stress in the mechanism of cytokine toxicity, we established an experimental protocol using insulin-producing cell clones with different mitochondrial antioxidative enzyme expression profiles. Cell viability, DCFDA oxidation, nitrite accumulation, peroxynitrite and H_2O_2 production, and hydroxyl radical generation were analyzed in the presence and absence of the iNOS inhibitor L-NARG to define the relative contribution of nitrosative and oxidative stress in cytokine toxicity.

The results indicate that IL-1β toxicity, in contrast to TNFα and IFNγ toxicity, in our experimental system was predominantly NO-mediated, confirming earlier reports [11; 12; 35; 40]. However, the toxicity of a mixture of IL-1β, TNFα, and IFNγ, which simulates type 1 diabetes conditions, is apparently mediated by both RNS and ROS. Cytokine toxicity was related to the production of H_2O_2, because cells that overexpressed catalase in their mitochondria were substantially protected against cytokine toxicity, as shown with different cell death detection methods. The protective effect of the iNOS inhibitor was more pronounced in control cells treated with the cytokine mixture than in cells treated with IL-1β alone. However, induction of the iNOS protein after exposure to IL-1β and the cytokine mixture was of comparable magnitude, indicating that not only the availability of NO but also of ROS determines the rate of nitrite formation in combined exposure.

Measurements of cytokine-induced DCFDA-H_2 oxidation in the presence of the iNOS inhibitor L-NARG indicated that the higher toxicity of the cytokine mixture was due to higher ROS generation. This increased ROS generation originated not only from TNFα-induced electron transport chain changes, as shown in other cell types [41-43], but also from induction of MnSOD expression by TNFα [11; 44]. This was confirmed by measuring intracellular H_2O_2 using HyPer protein oxidation as a sensor. Cytokines did not significantly induce oxidation of the cytoplasmic isoform of the HyPer protein, indicating little cytoplasmic H_2O_2 production. In contrast, cytokines caused a large oxidation of the mitochondrial isoform of the HyPer protein. The incubation with the cytokine mixture caused greater oxidation of the

HyPer-Mito protein than did IL-1β alone. Oxidation of the HyPer-Mito protein indicated that H_2O_2 levels in cells expressing Mito-Cat were significantly lower than in control cells and were not increased by cytokines. The very high HyPer-Mito protein oxidation rate in the MnSOD clone was apparently caused by H_2O_2 produced from superoxide radicals in a reaction catalyzed by high levels of MnSOD. Taken together, these results demonstrate that generation of H_2O_2 in mitochondria of insulin-producing cells exposed to cytokines ultimately resulted in the formation of the highly toxic hydroxyl radical, as proven also by the detection of mitochondrial DNA oxidative damage and hydroxyl radical formation. Importantly, experiments in primary rat islet cells confirmed the concept developed on the basis of the cell line studies, showing a strong induction of intramitochondrial hydrogen peroxide formation and a significant rise of hydroxyl radicals after cytokine exposure.

The analysis of the effects of the antioxidant defense status on cell viability confirmed the protective effect of mitochondrial catalase overexpression [21] and of MnSOD suppression [9] and the deleterious effects of MnSOD overexpression [9]. Inhibition of the iNOS enzyme significantly improved viability in control and, to a greater extent, in MnSOD-overexpressing cells treated with cytokines, but had no effect on the Mito-Cat clone. Although the magnitude of iNOS protein induction by cytokines in the Mito-Cat clone was similar to that in control cells, there was only minor nitrite production in this clone, which was largely protected against cytokine toxicity. It is possible therefore that nitric oxide produced in the Mito-Cat cells undergoes another reaction, namely reacts with superoxide radicals yielding peroxynitrite. Indeed we have observed a significant generation of peroxynitrite in these cells. Thus, overexpression of catalase in the mitochondria prevented not only H_2O_2 but went along also with reduced nitrite formation.

It is important to state that the suppression of MnSOD had opposite effects to its overexpression, but similar, however weaker, effects to the Mito-Cat overexpression, confirming that the effects observed were crucially dependent on the level of H_2O_2 in the mitochondria and its interaction with NO.

Notably we could exclude a crucial involvement of peroxynitrite in cytokine-stimulated toxicity in insulin-producing cells, because the level of cytokine-induced peroxynitrite formation did not correlate with cytokine toxicity. Namely the MnSOD cells, in which we measured the lowest levels of peroxynitrite, what is in line with the observation in other cell types [45], were the most sensitive to cytokine toxicity but, on the other hand, the MnSODantisense cells showed high peroxynitrite production in parallel with protection against cytokines. Interestingly, mitochondrial overexpression of catalase slightly attenuated

cytokine-stimulated peroxynitrite formation. This could be due to peroxynitrite scavenging by catalase, which has been reported recently [46]. Notably, tyrosine residues can be nitrated not only by peroxynitrite, but also by other nitrating agents, like nitrogen dioxide [47]. Therefore the slight reduction in nitrotyrosine formation in the Mito-Cat cells could also be due to scavenging of hydrogen peroxide, thereby lowering the reaction rate of hydrogen peroxide with nitrite in the presence of transition metal and peroxidase [47].

Taken together, based on the results of the present study, the following conclusions can be made. NO plays a dominant role in IL-1β–induced toxicity; however, both RNS and ROS play important roles in toxicity induced by IL-1β, TNFα, and IFNγ. This combination of cytokines simulates type 1 diabetes conditions in vitro [48; 49]. Mitochondria are the primary sites for ROS formation and interaction with NO in insulin-producing cells under cytokine attack. In theory, two possible reactions can occur between NO and ROS in insulin-producing cells after incubation with cytokines:

A. A reaction between the superoxide radical and NO in which peroxynitrite is formed [24-28]:

$$O_2^{\bullet -} + NO^{\bullet} \rightarrow ONOO^- \xleftrightarrow{H+} ONOOH$$

B. A reaction between H_2O_2 and NO in the presence of a trace metal (e.g. Fe^{++}) in which the highly toxic hydroxyl radical and nitrite are generated [50; 51]:

$$H_2O_2 + NO^{\bullet} \xrightarrow[Metal]{Trace} {}^{\bullet}OH + NO_2^- + H^+$$

A relatively high basal expression of MnSOD, which is increased further during exposure to cytokines [9-11; 30; 44], along with minimal catalase expression for H_2O_2 inactivation in insulin-producing cells [6; 31], results in high H_2O_2 production in mitochondria upon cytokine attack. Nitric oxide, produced at a high concentration in the cytoplasm, can diffuse through the mitochondrial membrane and react in this free iron-rich environment with $H_2O_{2.}$, promoting hydroxyl radical formation. Hydroxyl radicals do not travel at all, but instead rapidly interact with virtually any molecule in the place of origin. Therefore the main site of hydroxyl radical formation in beta cells are the mitochondria and they are therefore the primary site of toxic action of ROS, making mitochondrial damage responsible for cell death caused by proinflammatory cytokines.

Our experiments provide strong evidence that elevated levels of the highly toxic hydroxyl radical, but not of peroxynitrite, are primarily responsible for cytokine toxicity in insulin-producing cells (Scheme 1a). The best protection against cytokine toxicity can be achieved by

mitochondrial catalase expression, as in the Mito-Cat clone. As shown in the present study, this prevents the reaction between H_2O_2 and NO that otherwise leads to the formation of the toxic hydroxyl radical (Scheme 1b).

The alternative concept, namely the generation of peroxynitrite [24-28], which can provide an alternative explanation for cytokine toxicity, may be a valid explanation for cytokine toxicity in many other cell types with high expression levels of hydrogen peroxide inactivating enzymes [9-11; 30; 44], but not in insulin-producing cells with their extremely low expression level of hydrogen peroxide inactivating enzymes [9-11; 30; 44].

Therefore hydroxyl radical formation, but not peroxynitrite generation, is likely to be the ultimate effector in cytokine-induced nitrooxidative stress, leading to the dysregulation of antioxidant redox signaling and eventually to pancreatic β-cell death in autoimmune diabetes (Scheme 1). Our observation that increasing the hydrogen-peroxide inactivating enzyme capacity in the mitochondria can successfully block the deleterious cytokine-induced, nitrooxidative stress-mediated hydroxyl radical formation can open new therapeutic perspectives for pancreatic β-cell protective treatment in type 1 diabetes.

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Author Disclosure Statement

There is no conflict of interest.

Abbreviations

Cat, catalase; CuZnSOD, copper-zinc superoxide dismutase; DCFDA, dichlorofluorescein diacetate; ELISA, enzyme-linked immunosorbent assay; GPX, glutathione peroxidase; H_2O_2, hydrogen peroxide; HPF, 3'-(*p*-hydroxyphenyl) fluorescein; IL-1β, interleukin 1β; IFNγ, interferon γ; iNOS, inducible NO synthase; L-NARG, L-nitroarginine; Mito-Cat, mitochondrially targeted catalase; MnSOD, manganese superoxide dismutase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; NO, nitric oxide; $^{\bullet}OH$, hydroxyl radical; RNS, reactive nitrogen species; ROS, reactive oxygen species; TNFα, tumor necrosis factor α; T1DM, type 1 diabetes mellitus

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Tables

Table 1. Effects of mitochondrial antioxidative status and an iNOS blocker on cytokine-induced toxicity in insulin-producing cell clones

RINm5F cell clone	IL-1β	Mix	IL-1β+ iNOS blocker	Mix + iNOS blocker
Control	66 ± 5 (21)*	32 ± 2 (12)*	78 ± 2 (22)*	70 ± 4 (16)§
Mito-Cat	80 ± 3 (12)#	71 ± 2 (12)*#	82 ± 2 (10)	80 ± 2 (12)
MnSOD	49 ± 3 (10)*#	10 ± 1 (10)*#	65 ± 3 (8)*§	48 ± 2 (6)*#§
MnSODantisense	68 ± 3 (12)*	44 ± 1 (10)*#	78 ± 3 (8)	52 ± 2 (6)*#§

Insulin-producing RINm5F cells were incubated for 72 h with 600 U/ml IL-1β or a cytokine mixture (60 U/ml IL-1β, 185 U/ml TNFα, 14 U/ml IFNγ). The viability of the cells was determined by the MTT assay and expressed as % of untreated cells. Data are means ± SEM with the numbers of experiments in parentheses; each measurement in at least 3 repetitions and is reported as the % of untreated cells. *$p < 0.05$ vs. untreated; #$p < 0.05$ vs. control clone treated in the same way; §$p < 0.05$ treated with cytokines vs. treated with cytokines in the presence of an iNOS blocker; ANOVA followed by Bonferroni. Treatment with the iNOS blocker did not affect cell viability (% of untreated cells): control clone, 99 ± 3; Mito-Cat clone, 108 ± 5; MnSOD clone, 93 ± 4; MnSODantisense clone 94 ± 3.

Table 2. Effects of mitochondrial antioxidative status and an iNOS blocker on cytokine-stimulated DCFDA-H_2 oxidation in insulin-producing cell clones

RINm5F cell clone	IL-1β	Mix	IL-1β + iNOS blocker	Mix + iNOS blocker
Control	138 ± 5 (6)*	156 ± 5 (8)*	116 ± 4 (6)	130 ± 3 (8)*
Mito-Cat	113 ± 3 (8)	126 ± 7 (6)*	99 ± 6 (6)	98 ± 4 (6) #§
MnSOD	181 ± 20 (6)*#	221 ± 8 (6)*#	116 ± 4 (6)§	157 ± 6 (8)*#§
MnSODantisense	111 ± 5 (6)	135 ± 6 (6)*	106 ± 5 (6)	121 ± 5 (6)*

Insulin-producing RINm5F cells were incubated for 72 h with 600 U/ml IL-1β or a cytokine mixture (60 U/ml IL-1β, 185 U/ml TNFα, 14 U/ml IFNγ). The overall oxidative and nitrosative stress was estimated by DCFDA-H_2 oxidation assay and expressed as % of untreated cells. Data are means ± SEM with the numbers of experiments in parentheses; each measurement in at least 3 repetitions and is reported as the % of untreated cells, set as 100 %. *$p < 0.05$ vs. untreated; #$p < 0.05$ vs. control clone treated in the same way; §$p < 0.05$ treated with cytokines vs. treated with cytokines in the presence of an iNOS blocker; ANOVA followed by Bonferroni.

Table 3. Effects of mitochondrial antioxidative status and an iNOS blocker on cytokine-stimulated nitrite production in insulin-producing cell clones

RINm5F cell clone	IL-1β	Mix	IL-1β + iNOS blocker	Mix + iNOS blocker
Control	3.9 ± 0.7 (7)*	4.7 ± 0.5 (8)*	0.0 ± 0.4 (6)§	0.1 ± 0.1 (6)§
Mito-Cat	0.6 ± 0.2 (9)#	1.9 ± 0.3 (9)	0.0 ± 0.5 (6)	0.0 ± 0.0 (6)§
MnSOD	6.4 ± 0.8 (9)*#	29 ± 7 (9)*#	2.6 ± 0.5 (5)#§	7.1 ± 1.5 (6)*§
MnSODantisense	0.7 ± 0.2 (9)#	3.7 ± 0.7 (9)*	0.1 ± 0.0 (5)§	0.6 ± 0.2 (6)§

Insulin-producing RINm5F cells were incubated for 72 h with 600 U/ml IL-1β or a cytokine mix (60 U/ml IL-1β, 185 U/ml TNFα, 14 U/ml IFNγ). Data are means (pmoles/µg protein) ± SEM with the numbers of experiments in parentheses; each measurement was repeated at least twice. There was virtually no nitrite in the untreated or in cells incubated with only the iNOS blocker. * $p < 0.05$ vs. untreated; # $p < 0.05$ vs. control clone treated in the same way; § $p < 0.05$ treated with cytokines vs. treated with cytokines in the presence of an iNOS blocker; ANOVA followed by Bonferroni.

Figures

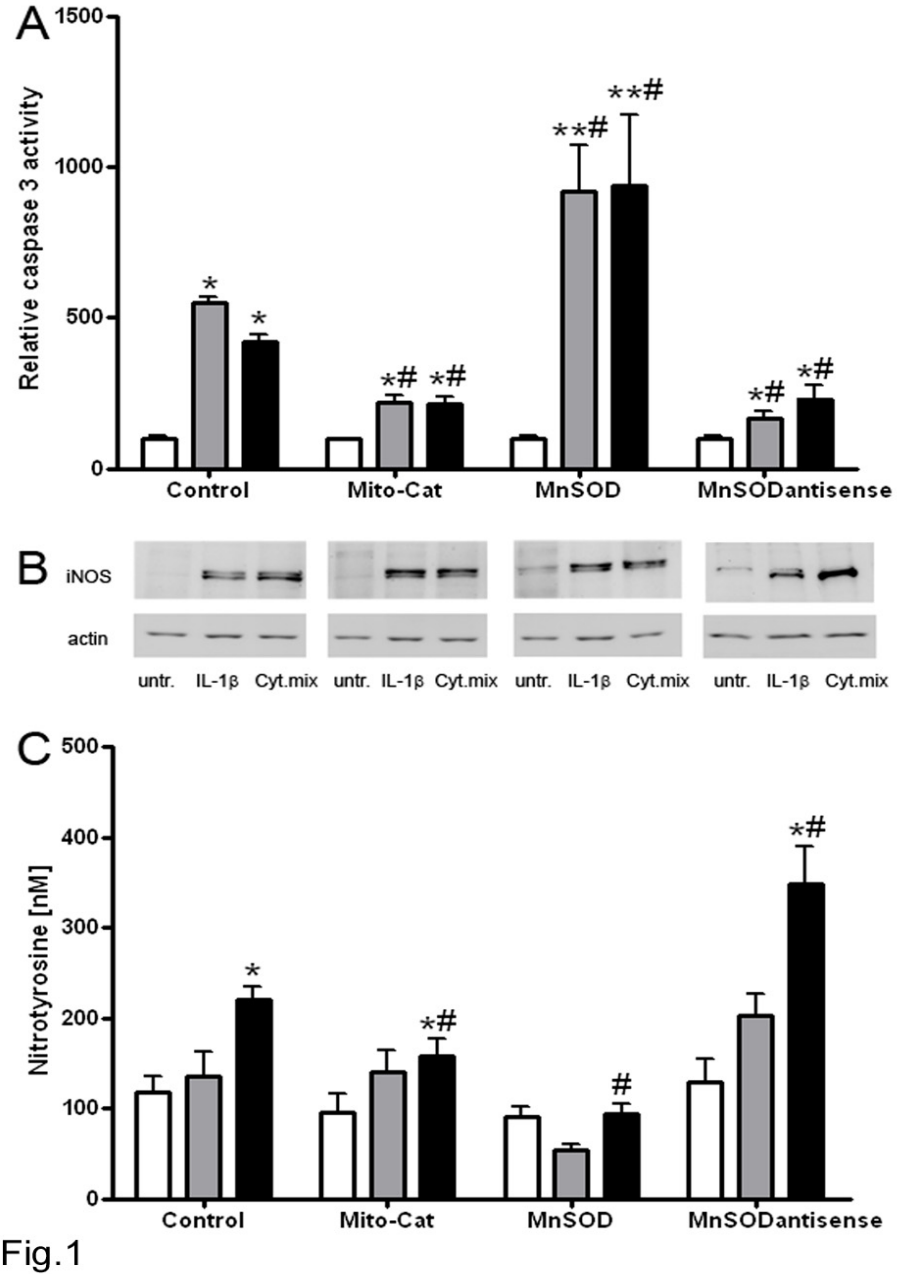


Fig.1

Fig. 1. Caspase-3 activity, iNOS protein expression and nitrotyrosine formation in control, Mito-Cat, and MnSOD insulin-producing cell clones after exposure to cytokines. (**A**) Caspase-3 activation in insulin-producing cells exposed to cytokines. After 72 h incubation with 600 U/ml IL-1β or a cytokine mixture (60 U/ml IL-1β, 185 U/ml TNFα, 14 U/ml IFNγ) cells were trypsinized and thereafter analyzed for caspase-3 activity by flow cytometry. *Open bars*: untreated, *grey bars*: IL-1β, *black bars*: cytokine mixture. Data are means of 6 independent experiments ± SEM. *p < 0.05 vs. untreated, **p < 0.01 vs. untreated, #p < 0.05

vs. control cells treated in the same way. ANOVA followed by Bonferroni. (**B**) A representative blot from 4 independent experiments for iNOS protein expression. Insulin-producing RINm5F cells were incubated for 72 h with 600 U/ml IL-1β or a cytokine mixture (60 U/ml IL-1β, 185 U/ml TNFα, 14 U/ml IFNγ). Total cellular proteins were isolated, and Western blotting was used to analyze the expression of the iNOS protein in control, Mito-Cat and MnSOD and MnSODantisense cells. The blots were thereafter stripped and re-loaded with beta actin antibodies for loading control. (**C**) Nitrotyrosine in insulin-producing cells exposed to cytokines. After 72 h incubation with 600 U/ml IL-1β or a cytokine mixture (60 U/ml IL-1β, 185 U/ml TNFα, 14 U/ml IFNγ) cell extracts were collected and nitrotyrosine concentration was estimated by ELISA. *Open bars*: untreated, *grey bars*: IL-1β, *black bars*: cytokine mixture. Data are means of 6 independent experiments ± SEM. *$p < 0.05$ vs. untreated, #$p < 0.05$ vs. control cells treated in the same way. ANOVA followed by Bonferroni.

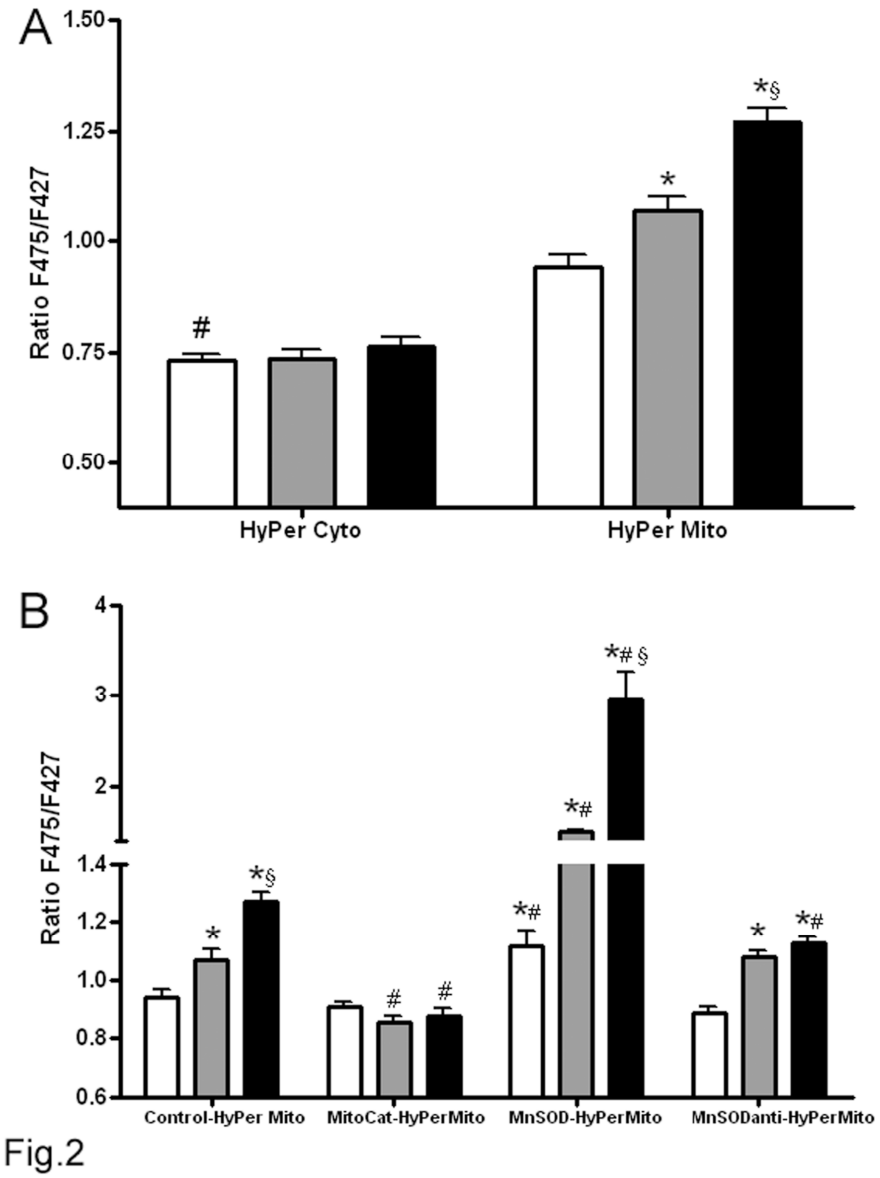


Fig.2

Fig. 2. Ratiometric measurement of HyPer protein oxidation in insulin-producing cells after incubation with cytokines for 72 h. (**A**) HyPer oxidation in the subcellular compartments of insulin-producing RINm5F control cells. (**B**) HyPer-Mito oxidation in different insulin-producing cell clones. Insulin-producing RINm5F cells were stably transfected with the HyPer-Cyto or HyPer-Mito vector. 24 h after seeding, the basal fluorescence was measured and IL-1β (600 U/ml) or a cytokine mixture (60 U/ml IL-1β, 185 U/ml TNFα, 14 U/ml IFNγ) were added. After 72 h, fluorescence was measured again. *Open bars*: untreated, *grey bars*: IL-1β, *black bars*: cytokine mixture. Each bar represents the mean values from 4 independent experiments ± SEM. *$p < 0.05$ vs. untreated, #$p < 0.05$ vs. control-HyPerMito. §$p < 0.05$ vs. IL-1β. ANOVA followed by Bonferroni.

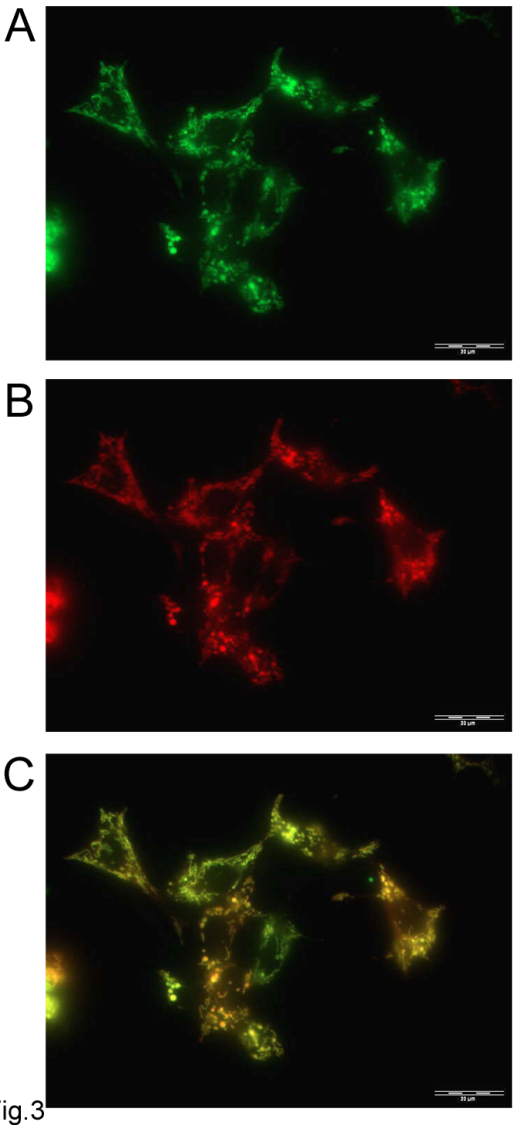


Fig.3

Fig. 3. Hyper-Mito protein intracellular localization in insulin-producing cells.

Insulin-producing RINm5F cells were transfected with HyPer-Mito vector and loaded with MitoTracker Deep Red for 15 minutes at 37°C. Images were captured and analyzed using an Olympus Fluorescence Microscope using a 60x oil objective.

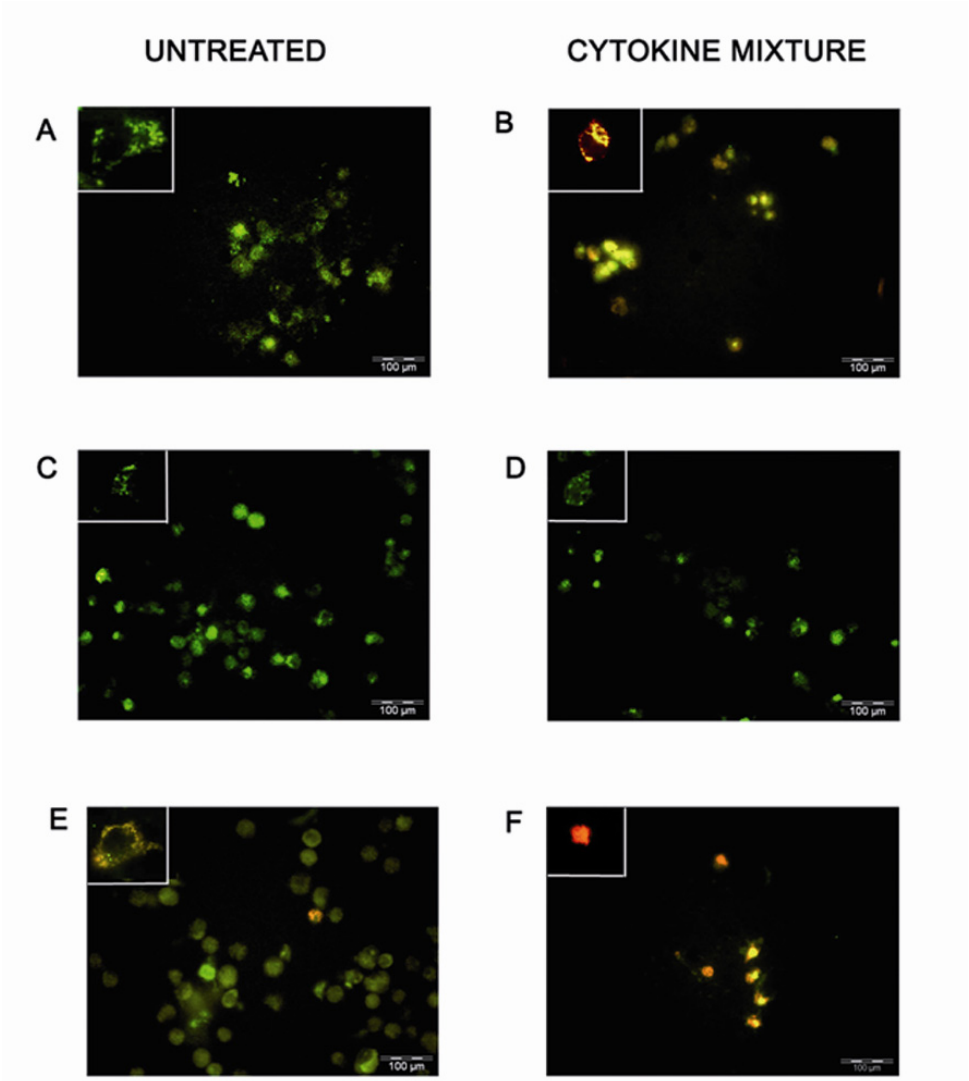


FIG. 4

Fig. 4. HyPer-Mito protein oxidation in control, Mito-Cat, and MnSOD insulin-producing cell clones after incubation with cytokines. Insulin-producing RINm5F cells were transfected with the HyPer-Mito vector and treated with a cytokine mixture (60 U/ml IL-1β, 185 U/ml TNFα, 14 U/ml IFNγ) for 72 h. A, C, and E untreated; B, D, and F cytokine mixture. A and B control cells, C and D Mito-Cat cells, and E and F MnSOD cells. Images were captured and analyzed using an Olympus Fluorescence Microscope. Panel images were obtained using a 20x air objective; insert images, which show representative cells at a higher magnification, were obtained using a 60x oil objective.

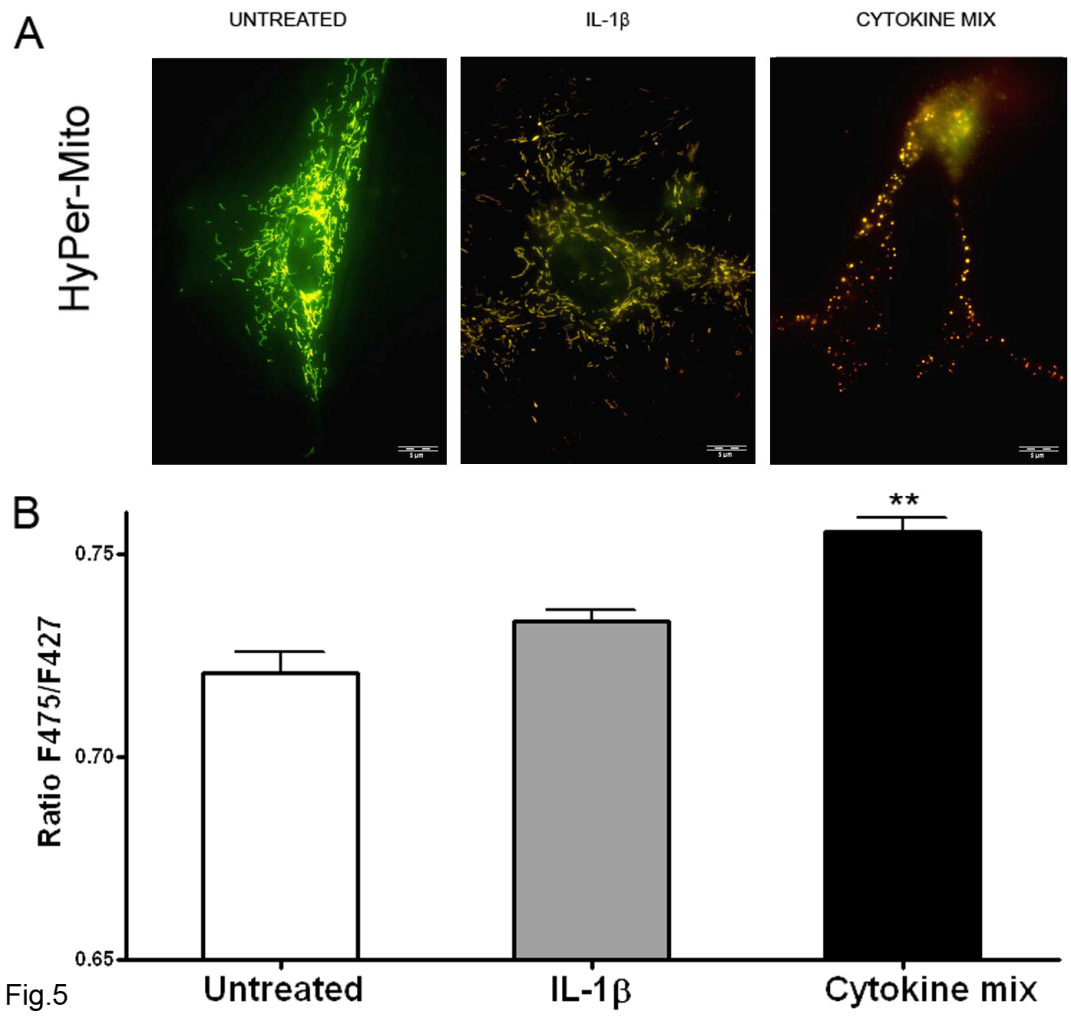


Fig. 5. Hyper-Mito protein oxidation in primary rat islet cells after incubation with cytokines. Rat islets were isolated from Lewis rats using collagenase digestion and cultured in groups of 100 islets onto ECM-coated plates for 12 days. Once the monolayer was formed the cells were infected with pLenti HyPer-Mito. Cytokines were added for 24 h and then the fluorescence was analyzed. (**A**) Representative pictures of primary rat islet cells expressing HyPer-Mito protein. From left to right: untreated, treated with 600 U/ml IL-1β and exposed to a cytokine mixture (60 U/ml IL-1β, 185 U/ml TNFα, 14 U/ml IFNγ). (**B**) quantitative, ratiometric measurement of HyPer-Mito protein oxidation in primary rat islet cells. *Open bars*: untreated, *grey bars*: IL-1β, *black bars*: cytokine mixture. Each bar represents the mean value from 4-6 rat islet preparations ± SEM. **p < 0.01 vs. untreated, ANOVA followed by Bonferroni.

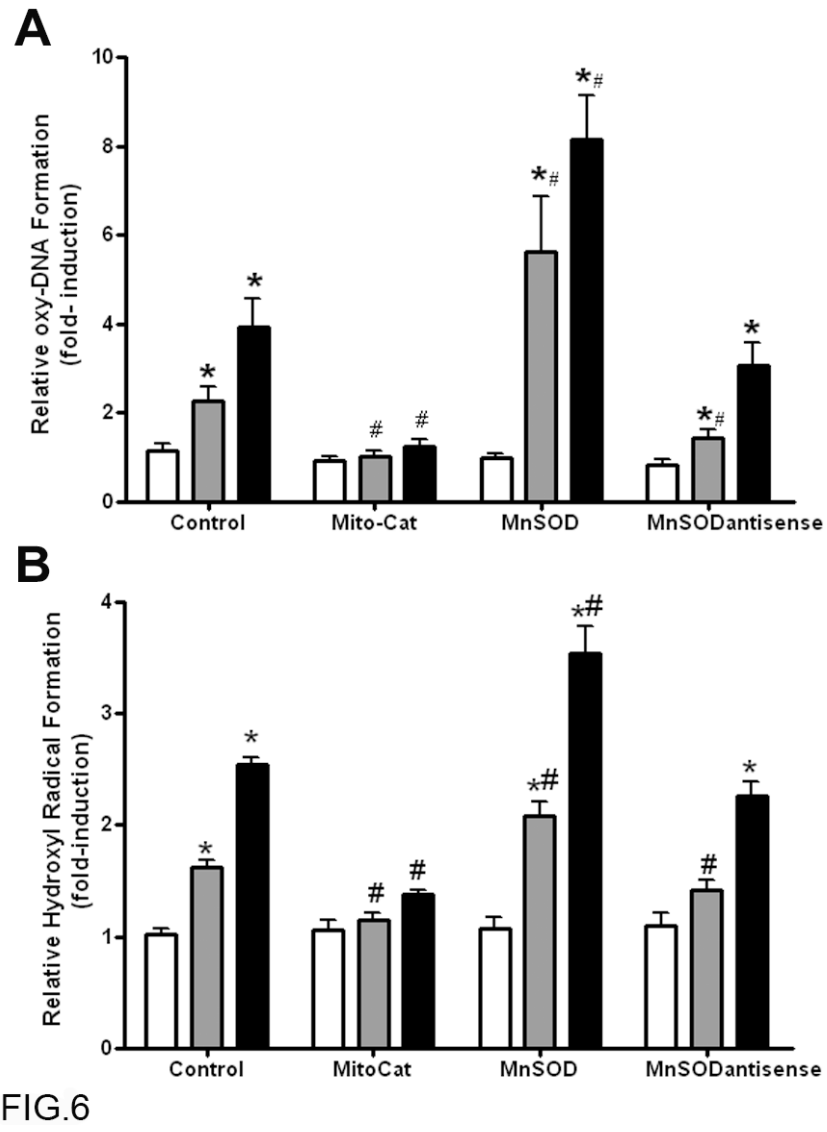
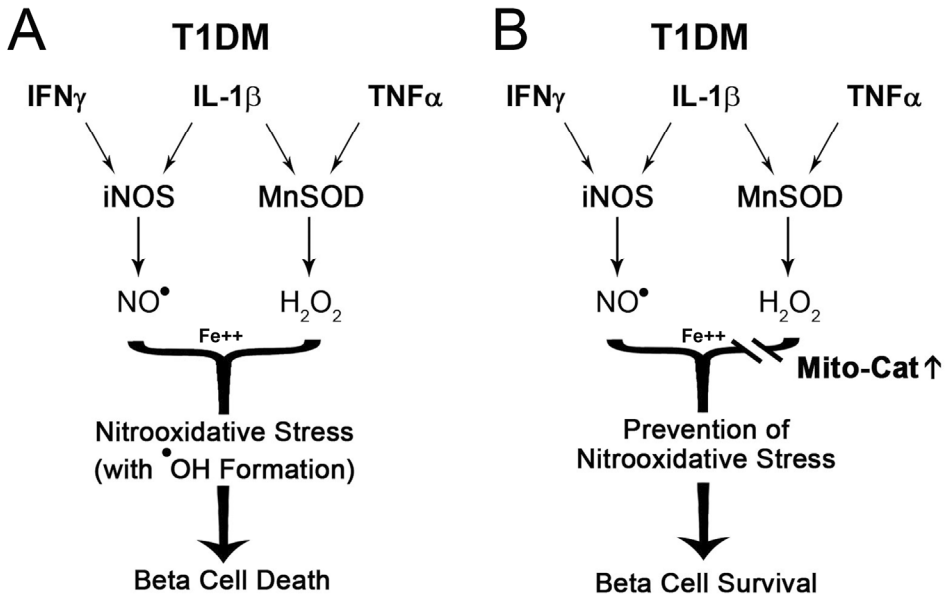


FIG.6

Fig. 6. Oxy-DNA formation and hydroxyl radical formation in control, Mito-Cat, and MnSOD insulin-producing cell clones after incubation with cytokines for 72 h. Insulin-producing RINm5F cells were incubated for 72 h with 600 U/ml IL-1β or a cytokine mixture (60 U/ml IL-1β, 185 U/ml TNFα, 14 U/ml IFNγ) before oxy-DNA damage was quantified or hydroxyl radical formation was estimated. *Open bars*: untreated, *grey bars*: IL-1β, *black bars:* cytokine mixture. Data are means (fold-induction) ± SEM of 4 independent experiments ± SEM. *$p < 0.05$ vs. untreated, #$p < 0.05$ vs. control cells. ANOVA followed by Bonferroni.



Scheme 1

Scheme 1. The concept of nitrooxidative stress-induced hydroxyl radical formation in proinflammatory cytokine-mediated (IL-1β, TNFα, and IFNγ) cell death in insulin-producing cells in type 1 diabetes (T1DM) and the protection by mitochondrial catalase overexpression. (**A**) nitrooxidative stress during T1DM, (**B**) prevention of nitrooxidative stress by catalase overexpression in mitochondria.

Abbreviations used: T1DM, type 1 diabetes mellitus; IFNγ, interferon γ; IL-1β, interleukin 1β; TNFα,. tumor necrosis factor α; iNOS, inducible NO synthase; MnSOD manganese superoxide dismutase; $NO^{\bullet}$, nitric oxide; H_2O_2, hydrogen peroxide; $^{\bullet}OH$, hydroxyl radical; Mito-Cat, catalase overexpressed in mitochondria.

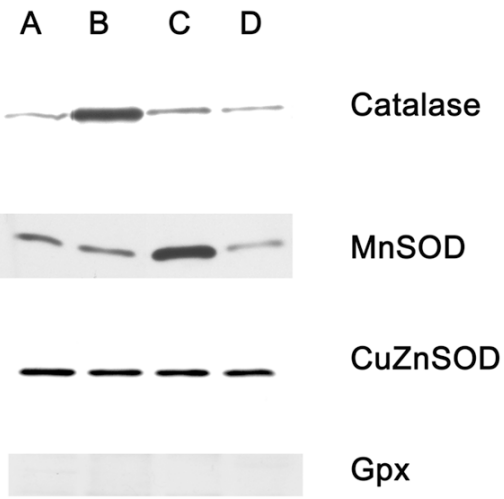


FIG.S1

Fig. S1. Antioxidant enzyme protein expression in insulin-producing cell clones. Insulin-producing RINm5F cells were stably transfected. Total cellular proteins were isolated, and Western blotting was used to analyze expression of the catalase, MnSOD, CuZnSOD and GPX protein in (A) control, (B) Mito-Cat, (C) MnSOD and (D) MnSODantisense cells.

Induction of the intrinsic apoptosis pathway in insulin-secreting cells is dependent on oxidative damage of mitochondria but independent of caspase-12 activation

Running title: cytokine-mediated mitochondrial degradation

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Keywords: mitochondrial ROS; oxidative damage; mitochondrial apoptosis pathway; beta cell death; caspase-9; ER-specific caspase-12: cytokines

Abstract

Pro-inflammatory cytokine-mediated beta cell apoptosis is activated through multiple signaling pathways involving mitochondria and endoplasmic reticulum. Activation of organelle-specific caspases has been implicated in the progression and execution of cell death. This study was therefore performed to elucidate the effects of pro-inflammatory cytokines on a possible cross-talk between the compartment-specific caspases 9 and 12 and their differential contribution to beta cell apoptosis. Moreover, the occurrence of ROS-mediated mitochondrial damage in response to beta cell toxic cytokines has been quantified.

ER-specific caspase-12 was strongly activated in response to pro-inflammatory cytokines; however, its inhibition did not abolish cytokine-induced mitochondrial caspase-9 activation and loss of cell viability. In addition, there was a significant induction of oxidative mitochondrial DNA damage and elevated cardiolipin peroxidation in insulin-producing RINm5F cells and rat islet cells. Overexpression of the H_2O_2 detoxifying enzyme catalase effectively reduced the observed cytokine-induced oxidative damage of mitochondrial structures. Taken together, the results strongly indicate that mitochondrial caspase-9 is not a downstream substrate of ER-specific caspase-12 and that pro-inflammatory cytokines cause apoptotic beta cell death primarily through hydroxyl radical-mediated mitochondrial damage.

Research Highlights:

- Caspase-9 is not a target of the ER-specific caspase-12 in insulin-producing cells.
- Activation of caspase-12 is not involved in cytokine-induced beta cell apoptosis.
- Cytokines induce beta cell death by ROS-mediated mitochondrial damage.
- Mitochondrially located catalase prevents ROS-induced damage of mitochondrial structures.

Abbreviations: 8-oxoG, 8-oxoguanine; CHOP, CAAT/enhancer binding protein homologous transcription factor; H_2O_2, hydrogen peroxide; IAP, inhibitor of apoptosis protein; IFN-γ, interferon-γ; IL-1β, interleukin-1β; JNK, c-Jun N-terminal kinases; MAPK, mitogen-activated protein kinase; NFκB, nuclear factor kappa B; NAO, 10-n-nonyl-acridine orange; NO, nitric oxide; ROS, reactive oxygen species; Smac/DIABLO, second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI; STAT-1, signal transducer and activator of transcription 1; T1DM, Type 1 Diabetes mellitus; TNF-α, tumor necrosis factor-γ; TRAF-2, TNF-receptor-associated factor 2.

1. Introduction

Type 1 diabetes mellitus (T1DM) is an immune mediated disease characterized by selective and progressive destruction of insulin-producing beta cells in the islets of Langerhans. Pro- inflammatory cytokines, such as interleukin 1β (IL-1β), tumor necrosis factor α (TNF-α) and interferon γ (IFN-γ) secreted by islet-infiltrating macrophages and T cells have been identified as the key soluble mediators of beta cell death in autoimmune diabetes [1-4]. IL-1β is the prototype pro-inflammatory cytokine, which significantly contributes to beta cell dysfunction. In synergism with TNF-α and IFN-γ, IL-1β induces the formation of high levels of nitric oxide (NO) and reactive oxygen species (ROS) finally leading to pancreatic beta cell death [5]. Beta cell apoptosis appears to be the result of a complex network of signaling events induced by pro-inflammatory cytokines and their key signaling pathways such as NF-κB, MAPK/STAT-1, and MAPK/JNK [1]. This ultimately culminates in endoplasmic reticulum (ER) stress [6], oxidative stress [5], and activation of the mitochondrial apoptosis machinery [7]. However, a possible interaction between ER stress- and mitochondria-mediated cell death pathways and their differential contribution to cytokine-induced beta cell apoptosis has not been conclusively demonstrated so far.

Available evidence suggests that the activation of cysteinyl aspartate-specific proteases (caspases) is crucial for the signal transduction and execution of apoptosis. This cysteine protease family can be activated through two main pathways: the extrinsic and the stress-induced intrinsic pathway [8-11]. The activation of the intrinsic pathway involves loss of mitochondrial homeostasis and release of pro-apoptotic factors from the intermembrane space to the cytosol, including SMAC/DIABLO and cytochrome c. On release from mitochondria, cytochrome c together with Apaf-1 activates caspase-9, which in turn activates downstream effector caspases [7, 12]. Recently we have shown that the mitochondrial generation of reactive oxygen species in response to pro-inflammatory cytokines specifically triggers the mitochondrial apoptosis pathway by increasing the Bax/Bcl-2 ratio, subsequently activating downstream caspases, which could be prevented by overexpressing the H_2O_2 detoxifying catalase in mitochondria [7].

Apart from cytochrome c-dependent caspase-9 activation, it has previously been suggested that caspase-9 can be directly activated by caspase-12 in the murine myoblast cell line C2C12 [13]. Caspase-12, predominantly localized on the cytoplasmic side of the ER, is considered to be an initiator caspase in ER stress-mediated apoptosis [14-16]. Several ER-stress stimuli such as tunicamycin, thapsigargin [16], and also pro-inflammatory cytokines

have been shown to promote caspase-12 activation in different cell lines including beta cell lines [17-19]. However, a putative crosstalk between compartment-specific caspases and their contribution to cytokine-induced beta cell death has not been studied so far.

The aim of this study was to investigate the interdependence of cytokine-induced ER stress-specific caspase-12 and the mitochondrial caspase cascade. In addition mitochondrial ROS-mediated key events involved in the induction of the intrinsic apoptosis pathway in response to pro-inflammatory cytokines were characterized in rat islet cells and insulin-producing cells overexpressing H_2O_2 detoxifying catalase in mitochondria.

2. Materials and methods

2.1. Tissue culture of RINm5F cells

Insulin-producing RINm5F tissue culture cells were cultured as described earlier in RPMI-1640 medium supplemented with 10 mM glucose, 10 % (v/v) fetal calf serum (FCS), penicillin, and streptomycin in a humidified atmosphere at 37 °C and 5 % CO_2 [20, 21]. RINm5F cells stably overexpressing catalase in the mitochondria (MitoCatalase) have been generated as described before [22]. The expression of catalase was analyzed by immunoblotting analysis or enzyme activity measurement [22]

2.2. Rat islet isolation and single cell preparation

Pancreatic islets were isolated from 250-300 g adult male Lewis rats by collagenase digestion and handpicked under a stereo microscope. Thereafter 70-100 uniformly sized isolated islets were cultured on extracellular matrix (ECM)-coated plates (35 mm) (Novamed, Jerusalem, Israel) in RPMI-1640 medium containing 5 mM glucose, 10 % FCS, penicillin, and 2 % streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 [23]. The islets were cultured for 7-10 days on the ECM plates to adhere and spread before they were incubated with pro-inflammatory cytokines.

2.3. Exposure to cytokines and the intramitochondrial ROS generator menadione

RINm5F control (mock-transfected) and MitoCatalase overexpressing RINm5F insulin-producing cells were seeded at different concentrations depending on further experimentation and allowed to attach for a period of 24 h. Thereafter the cells were exposed to 600 U/ml human IL-1β or a combination of cytokines (cytokine mixture) consisting of 60 U/ml IL-1β, 185 U/ml human TNF-α, and 14 U/ml IFN-γ (PromoCell, Heidelberg, Germany) for 24-72 h. As ROS generator, menadione was freshly dissolved and cells were exposed at a final concentration of 10 μM in RPMI 1640 medium for 2 h. After removal of the menadione containing medium the cells were cultured overnight in fresh RPMI 1640 medium.

2.4. Flow cytometric quantification of caspase-9 and -12 activation

Caspase-9 and -12 activation was determined with the CaspGLOW fluorescein active caspase-9 and -12 staining kits (Promocell, Heidelberg, Germany). Control and MitoCatalase overexpressing RINm5F cells were seeded at a density of 1 x 10^6 cells per well of a six-well-plate and allowed to attach for 24 h before incubation with the indicated cytokines in the presence or absence of caspase-12 inhibitor (Z-ATAD-FMK; 15 μM, Promocell, Heidelberg, Germany). After 24 h incubation the cells were trypsinized and collected by centrifugation at 700 x g for 5 min. Cell pellets were resuspended in 1 ml culture medium and 300 μl of each

sample were transferred to Eppendorf tubes. According to the manufacturer's protocol, FITC-LEHD-FMK (caspase-9) or FITC-ATAD-FMK (caspase-12) was added to these cells and incubated for 45 min at 37°C followed by washing twice with wash buffer. The cells were resuspended in 1 ml of washing buffer and 20 000 cells of each sample were analyzed by flow cytometry (CyFlow ML, Partec, Münster, Germany) using the FL-1 channel (488 nm excitation/527 nm emission). Data were analyzed by FlowJo software (Tree Star, Ashland, OR).

2.5. Assessment of cell viability

RINm5F control cells were seeded at 10 000 cells per well in 100 µl culture medium in 96-well-plates and allowed to attach for 24 h before they were incubated for 72 h with the indicated cytokines in presence or absence of the caspase-12 specific inhibitor Z-ATAD-FMK. Cell viability was then determined by microplate-based MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma, St. Louis, MO,USA) [24].

2.6. Assessment of intramitochondrial superoxide radical generation

Superoxide radical generation within the mitochondria was assessed using MitoSOX Red (Invitrogen, Karlsruhe, Germany), a highly specific fluorescent probe for superoxide detection in mitochondria of living cells [25]. Control and MitoCatalase overexpressing RINm5F cells were seeded at a density of 7 x 10^5 cells per well of a six-well-plate and allowed to attach for 24 h, while 70-100 uniformly sized rat islets were cultured for 7-10 days on the ECM plates to adhere and spread before they were incubated with IL-1β alone or the cytokine mixture for 24 h. Thereafter the cells were trypsinized and collected by centrifugation at 700 x g for 5 min. Cell pellets were resuspended in 0.5 ml culture medium containing MitoSOX Red at a final concentration of 5 µM and incubated for 30 min at 37 °C. Then the cells were washed twice with PBS and analyzed by flow cytometry (CyFlow ML, Partec, Münster, Germany) using the FL-10 channel (405 nm excitation/580 nm emission). Data were analyzed by FlowJo software (Tree Star, Ashland, OR).

2.7. Assessment of intracellular 8-oxoguanine content

The generation of 8-oxoguanine as sensitive and specific indicator of hydroxyl radical-induced DNA damage, was assessed with the OxyDNA Assay Kit (Argutus Medical, Dublin, Ireland). Control and MitoCatalase overexpressing RINm5F cells were seeded at a density of 5 x 10^5 cells per well of a six-well-plate and allowed to attach for 24 h, while 70-100 uniformly sized rat islets were cultured for 7-10 days on the ECM plates to adhere and spread before incubation with the indicated cytokines. After 72 h exposure to IL-1β alone or the

cytokine mixture, the cells were trypsinized and collected by centrifugation at 700 x g for 5 min. After fixation with 1 % paraformaldehyde for 15 min on ice, the cells were permeabilized with 70 % ethanol for 30 min at -20 °C. According to the manufacturer's protocol, FITC- labeled conjugate was added to these cells and incubated for 1 h at room temperature in the dark, followed by washing twice with wash buffer. The cells were resuspended in 1 ml of washing buffer and 20 000 RINm5F and 8000 rat islet cells of each sample were analyzed by flow cytometry (CyFlow ML, Partec, Münster, Germany) using the FL-1 channel (488 nm excitation/527 nm emission). Data were analyzed by FlowJo software (Tree Star, Ashland, OR).

2.8. Assessment of cardiolipin peroxidation

Cardiolipin peroxidation was assessed by using 10-N-nonyl acridine orange (NAO) (Invitogen, Karlsruhe, Germany), a highly specific fluorescent probe for cardiolipin. After the peroxidation of cardiolipin NAO loses its affinity for peroxidized cardiolipin, resulting in a decreased fluorescence signal [26, 27]. Control and MitoCatalase overexpressing RINm5F cells were seeded at a density of 7 x 10^5 cells per well of a six-well-plate and allowed to attach for 24 h, while 70-100 uniformly sized rat islets were cultured for 7-10 days on the ECM plates to adhere and spread before they were incubated at 37°C with IL-1β alone or the cytokine mixture for 24 h. Thereafter the cells were trypsinized and collected by centrifugation at 700 x g for 5 min. Cell pellets were resuspended in 0.5 ml culture medium containing NAO at a final concentration of 100 nM. After incubation for 30 min at 37 °C the cells were then washed twice with PBS and analyzed by flow cytometry (CyFlow ML, Partec, Münster, Germany) using the FL-1 channel (488 nm excitation/527 nm emission). Data were analyzed by FlowJo software (Tree Star, Ashland, OR).

2.9. Immunocytochemical staining of 8-oxoguanine and cardiolipin

For immunocytochemical staining of control and MitoCatalase overexpressing RINm5F cells, the cells were seeded at a density of 5 x 10^4 cells per well on LabTek chamber slides (Nunc, Roskilde, Denmark) 24 h before incubation. To visualize 8-oxoguanine, 72 h after incubation with the indicated cytokines the cells were stained with MitoTracker Deep Red (Invitogen, Karlsruhe, Germany) according the manufacturer's instruction, washed twice with PBS and subsequently fixed with 4 % paraformaldehyde at room temperature for 60 min. After washing, the cells were permeabilized and blocked with PBS containing 0.2 % Triton X-100 and 1 % BSA. The cells were incubated with primary antibody (anti-8-oxoguanine, clone 483.15, 1:200, Millipore, Schwalbach, Germany) diluted in PBS containing 0.1 % Triton X-100 and 0.1 % BSA at room temperature for 60 min. Then, the cells were washed

with PBS and incubated with secondary antibody (anti-mouse-FITC, 1:500, Dianova, Hamburg, Germany) for 60 min in the dark. To visualize cardiolipin, 24 h after incubation with the indicated cytokines the cells were co-incubated with 100 nM 10-n-nonyl-acridine orange (NAO) and MitoTracker Deep Red (Invitogen, Karlsruhe, Germany) for 30 min at 37 °C. For nuclear counterstaining, 300 nM DAPI was applied for 5 min at room temperature. Finally, the cells were washed and mounted with Mowiol/DABCO anti-photobleaching mounting media (Sigma, St. Louis, MO, USA). Stained cells were examined with an Olympus IX81 inverted microscope (Olympus, Hamburg, Germany) and microscopic images were post-processed using AutoDeblur and AutoVisualize (Autoquant Imaging, New York, USA).

2.10. Data analysis

The data are expressed as means ± SEM. Statistical analyses were performed by ANOVA followed by Dunnett's test for multiple comparisons or by t-test (unpaired, two-tailed) using the Prism analysis program (Graphpad, San Diego, CA, USA).

3. Results

3.1. Effects of pro-inflammatory cytokines on caspase-12 activation in insulin-producing RINm5F control cells and cells overexpressing MitoCatalase

In order to evaluate the role of ER-specific caspase-12 activation in cytokine-induced beta cell death, insulin-producing RINm5F control cells and cells overexpressing the H_2O_2 detoxifying enzyme catalase in mitochondria (MitoCatalase) were incubated with IL-1β alone or with a pro-inflammatory cytokine mixture consisting of IL-1β, TNF-α, and IFN-γ. A 24 h exposure of RINm5F cells to IL-1β or the cytokine mixture resulted in a significant activation of caspase-12 in RINm5F control cells as well as in cells overexpressing MitoCatalase (control cells: IL-1β 285 %, $p < 0.05$ and cytokine mixture 320 %, $p < 0.001$ compared with untreated cells; MitoCatalase cells: IL-1β 275 %, $p < 0.05$ and cytokine mixture 357 %, $p < 0.001$ compared with untreated cells) (Fig. 1). The overexpression of the H_2O_2 detoxifying enzyme catalase in mitochondria thus had no influence on the cytokine-induced ER-specific caspase-12 activation.

3.2. Effects of the caspase-12 specific inhibitor Z-ATAD-FMK on cytokine-induced caspase-9 activation and cell viability in insulin-producing RINm5F cells

To elucidate the contribution of activated caspase-12 in cytokine-induced beta cell death and a possible interaction with caspase-9, the effects of Z-ATAD-FMK, a caspase-12 specific inhibitor, were investigated in insulin-producing RINm5F cells treated with beta cell toxic cytokines (Fig. 2). Treatment of cells with the caspase-12 specific inhibitor significantly attenuated the cytokine-induced caspase-12 activation by IL-1β or the cytokine mixture ($p < 0.05$, Z-ATAD-FMK treated cells compared with Z-ATAD-FMK untreated cells) (Fig. 2). However, the inhibition of caspase-12 had no influence on cytokine-induced caspase-9 activation (Fig. 2). The cytokine-mediated caspase-9 activation was not abrogated in the presence of the caspase-12 inhibitor, suggesting that pro-caspase-9 is not a downstream target of caspase-12 in insulin-producing RINm5F cells. Moreover, cell viability after cytokine stimulation was significantly decreased to the same extent, both in the presence or absence of the caspase-12 inhibitor, indicating that caspase-12 activation is not essential for the initiation of the mitochondrial apoptosis pathway in beta cells and for cytokine-induced beta cell damage (Fig. 3).

3.3. Effects of pro-inflammatory cytokines on intramitochondrial superoxide radical production in insulin-producing RINm5F control or MitoCatalase cells and rat islet cells

Having demonstrated that the inhibition of ER-specific caspase-12 is not sufficient to prevent cytokine-induced activation of caspase-9 and beta cell death, we next examined other potential mediators responsible for the cytokine-induced initiation of mitochondrial destruction pathways. Therefore the cytokine effects on superoxide radical generation within the mitochondria were analyzed. For selective monitoring of superoxide anions within the mitochondria, mitochondria-targeted hydroethidine (MitoSOX Red) has been used at excitation/emission wavelengths of 404/580 nm. Exposure of insulin-producing RINm5F control and MitoCatalase overexpressing cells to IL-1β alone or a cytokine mixture did not result in a statistically significant increase of mitochondrial superoxide anion generation. A comparable result was observed in rat islet cells (Fig. 4).

3.4. Effects of pro-inflammatory cytokines on 8-oxoguanine formation in insulin-producing RINm5F control or MitoCatalase overexpressing cells and rat islet cells

We next determined 8-oxoguanine accumulation as a specific biomarker for hydroxyl radical generation in response to pro-inflammatory cytokines. As shown in Fig. 5A the exposure of RINm5F control cells to IL-1β alone or a cytokine mixture exhibited a significant induction of oxidative DNA damage (IL-1β 235 %, $p < 0.05$ and cytokine mixture 310 %, $p < 0.001$ compared with untreated cells). A comparable cytokine-mediated 8-oxoguanine accumulation was observed in rat islet cells (IL-1β 192 %, $p < 0.001$ and cytokine mixture 177 %, $p < 0.01$ compared with untreated cells). The overexpression of mitochondrially located catalase (MitoCatalase) effectively diminished the cytokine-induced formation of 8-oxoguanine, indicating that pro-inflammatory cytokines strongly induced oxidative damage of DNA (Fig. 5A).

3.5. Subcellular localization of 8-oxoguanine (8-oxoG) in insulin-producing RINm5F control cells after incubation with pro-inflammatory cytokines and menadione

In order to evaluate the subcellular localization of the generated 8-oxoguanine, immunofluorescent staining of the insulin-producing cells was performed. Co-staining approaches with MitoTracker Deep Red, a florescent probe that selectively accumulates in mitochondria and DAPI, a nuclear probe, illustrated that the observed 8-oxoguanine formation was mainly localized in the mitochondria (Fig. 5B). Again, corresponding to the quantitative data shown in Fig. 5A, following treatment with IL-1β alone or a cytokine mixture, a dramatic increase in immunofluorescence of 8-oxoguanine could be observed, reflecting an enhanced accumulation of 8-oxoguanine. Contrary, in MitoCatalase overexpressing cells no increase of 8-oxoguanine immunofluorescence was seen in response

to IL-1β alone or the cytokine mixture, respectively (data not shown). This observation strongly suggests that pro-inflammatory cytokines directly trigger ROS formation in mitochondria and that these organelles are the primary target for the deleterious effects of hydroxyl radicals.

To further verify the specificity of 8-oxoguanine formation and its localization in mitochondria, RINm5F control and MitoCatalase overexpressing cells were treated with menadione, a chemical intramitochondrial ROS generator. After a 2 h exposure to menadione a massive accumulation of 8-oxoguanine could be detected in control cells (Fig. 6A), whereas in MitoCatalase overexpressing cells no striking accumulation of 8-oxoguanine was observed (Fig. 6B). Furthermore, 8-oxoguanine immunoreactivity showed a clear co-localization with the mitochondria specific probe MitoTracker Deep Red, indicating that 8-oxoguanine was formed selectively in mitochondria.

3.6. Effects of pro-inflammatory cytokines on cardiolipin peroxidation in insulin-producing RINm5F control or MitoCatalase overexpressing cells and rat islet cells

In order to obtain more information about the deleterious effects of cytokine-mediated ROS in pancreatic beta cells, we next examined the effects of pro-inflammatory cytokines on cardiolipin, an inner membrane phospholipid of mitochondria. A 24 h exposure of insulin-producing RINm5F control cells to IL-1β alone or a cytokine mixture caused a significant increase of cardiolipin peroxidation (IL-1β 219 %, $p < 0.001$ and cytokine mixture 222 %, $p < 0.01$ compared with untreated cells) (Fig. 7A). Comparable effects were seen in rat islet cells under the same treatment conditions (IL-1β 220 %, $p < 0.001$ and cytokine mixture 207 %, $p < 0.01$ compared with untreated cells) (Fig. 7A). Again the overexpression of MitoCatalase greatly diminished the cytokine-induced cardiolipin peroxidation (Fig. 7A).

To further confirm the specificity of cytokine-induced cardiolipin peroxidation, immunofluorescent staining of insulin-producing RINm5F cells before and after cytokine treatment was performed. Untreated RINm5F cells showed an intense staining for cardiolipin and a clear co-localization with mitochondria (Fig. 7B, upper panel). After cytokine treatment the cardiolipin content of the treated cells was drastically reduced compared with untreated cells (Fig. 7B, middle and lower panel).

4. Discussion

Pro-inflammatory cytokines are the main mediators of beta cell apoptosis in type 1 diabetes [1, 3]. These trigger multiple intracellular signaling pathways, which converge into ER stress [6], oxidative stress, and mitochondrial dysfunction [5, 7]. Although organelle specific signaling pathways, emanating from the ER or mitochondria, can induce apoptosis independently, it has previously been suggested that ER stress specifically activates caspase-12, which promotes caspase-9 activation independent of cytochrome c release [13]. In the present study, we showed that exposure of insulin-producing RINm5F control cells but also of cells overexpressing mitochondrially located catalase (MitoCatalase) to beta cell toxic cytokines strongly induced the caspase-12 activation, in contrast to the cytokine-induced caspase-9 activation, which was effectively suppressed along with protection against cytokine-mediated cell death by overexpression of MitoCatalase in these cells [7]. However, inhibition of ER-specific caspase-12 had no influence on cytokine-induced caspase-9 activation and decrease of cell viability. In addition, we demonstrated that the pro-inflammatory cytokines predominately induced mitochondrial oxidative DNA damage and cardiolipin peroxidation, both in RINm5F and rat islet cells. The appearance of these oxidative injuries could be successfully prevented by overexpression of the H_2O_2 detoxifying enzyme catalase in mitochondria. Taken together, these data indicate that caspase-9 is not a downstream target of ER-specific caspase-12 and that activation of caspase-12 is no prerequisite for cytokine-induced beta cell death. Moreover, cytokine-induced mitochondrial dysfunction mediated by ROS is a central event in beta cell death.

Several reports have linked prolonged ER stress to apoptosis induction, involving various pathways such as transcriptional activation of CHOP, activation of the JNK by IRE1-dependent recruitment of TNF-receptor-associated factor 2 (TRAF-2), and activation of caspase-12, which might be implicated in the execution of ER stress-triggered apoptosis [28-30]. Caspase-12 is phylogenetically related to the interleukin-1β converting subfamily of caspases and is specifically activated in cells subjected to ER-stress stimuli including thapsigargin, tunicamycin, and also pro-inflammatory cytokines [16-19]. In rodent non insulin-secreting cells several studies have proposed caspase-12 as a specific mediator of ER stress-induced apoptosis, since caspase-12-deficient cells appear to be resistant to ER stressors [16, 31, 32]. Moreover, Morishima et al. recently suggested that processed caspase-12 activates caspase-9 independent of cytochrome c release, indicating that pro-caspase-9 is a downstream substrate in the murine myoblast cell line C2C12 [13]. In the present study, we demonstrated that caspase-12 was activated in response to pro-inflammatory cytokines in

insulin-producing cells. However, its inhibition had no effect on caspase-9 activation and cell viability, indicating that caspase-9 activation by cytokines is independent from ER-specific caspase-12 activation and that this caspase is not required for cytokine-mediated beta cell apoptosis. These results are in agreement with earlier reports showing that activated caspase-12 did not aggravate the rate of apoptosis significantly [18, 19]. Furthermore, this observation was also confirmed in MitoCatalase overexpressing cells in which the activation of caspase-12 was similar to control cells, but caspase-9 activation and consequently beta cell death was significantly reduced [7, 22]. Nevertheless, the exact function of caspase-12 during the ER-stress is still unclear, since cellular substrates of this caspase are currently unknown.

Excluding caspase-9 activation by caspase-12, the reason for the intrinsic apoptotic pathway activation resides within the mitochondria. This organelle has been generally considered as a main source of ROS generation and also as a possible primary target for a ROS attack. Oxidative DNA modifications and peroxidation of the inner mitochondrial membrane phospholipid cardiolipin have been identified as susceptible structures for ROS-mediated mitochondrial dysfunction [33]. Such structural alterations can lead to extensive impairment of mitochondrial metabolism and facilitate the detachment of cytochrome c from cardiolipin, followed by translocation into the cytosol [34]. The extent of ROS-mediated oxidative damage depends on the antioxidative defense system. Importantly, one major characteristic of beta cells is the extraordinarily low antioxidative enzyme status and the resultant particular vulnerability [5, 35]. While the superoxide-radical-inactivating enzyme capacity is adequate, the expression level of H_2O_2-inactivating enzymes is extremely low [21]. This apparent imbalance is further accentuated by the cytokine-increased MnSOD expression, rendering beta cells vulnerable to accumulated H_2O_2 [36]

Indeed, neither in insulin-producing RINm5F cells nor in rat islet cells a significantly increased mitochondrial $O_2^{\bullet-}$ generation in response to beta cell toxic cytokines could be observed, apparently due to the efficient superoxide radical dismutation in these cells. The result of this rapid dismutation are increased H_2O_2 concentrations, providing the basis for the rapid generation of the highly toxic hydroxyl radicals via the Haber-Weiss or Fenton reaction [37]. The massive accumulation of 8-oxoguanine in mtDNA and the observed cardiolipin peroxidation in insulin-producing RINm5F control cells and rat islet cells after cytokine treatment are direct evidences for this hydroxyl radical formation. Furthermore, H_2O_2 dissipation through overexpression of catalase in mitochondria prevented the direct damaging effect of mitochondrially formed ROS and suppressed the release of apoptotic factors from the mitochondrial intermembrane space into the cytosol as recently published [7].

In conclusion, the present results show that cytokine-mediated activation of caspase-9 is independent of ER-specific caspase-12 activation. Instead the data suggest that mitochondrial ROS, in particular hydroxyl radicals, preferentially cause oxidative damage to mtDNA and profound loss of cardiolipin, resulting in mitochondrial disintegration and caspase-9 activation. Conversely, detoxification of mitochondrial H_2O_2 by mitochondrially expressed catalase preserved mitochondrial integrity. Hence oxidative damage induced by ROS, specifically hydroxyl radicals, is the major source of mitochondrial instability leading to the release of pro-apoptotic factors and finally to caspase-9 dependent beta cell death.

Disclosures

The authors have no conflicts of interest to declare.

Acknowledgments

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Figures

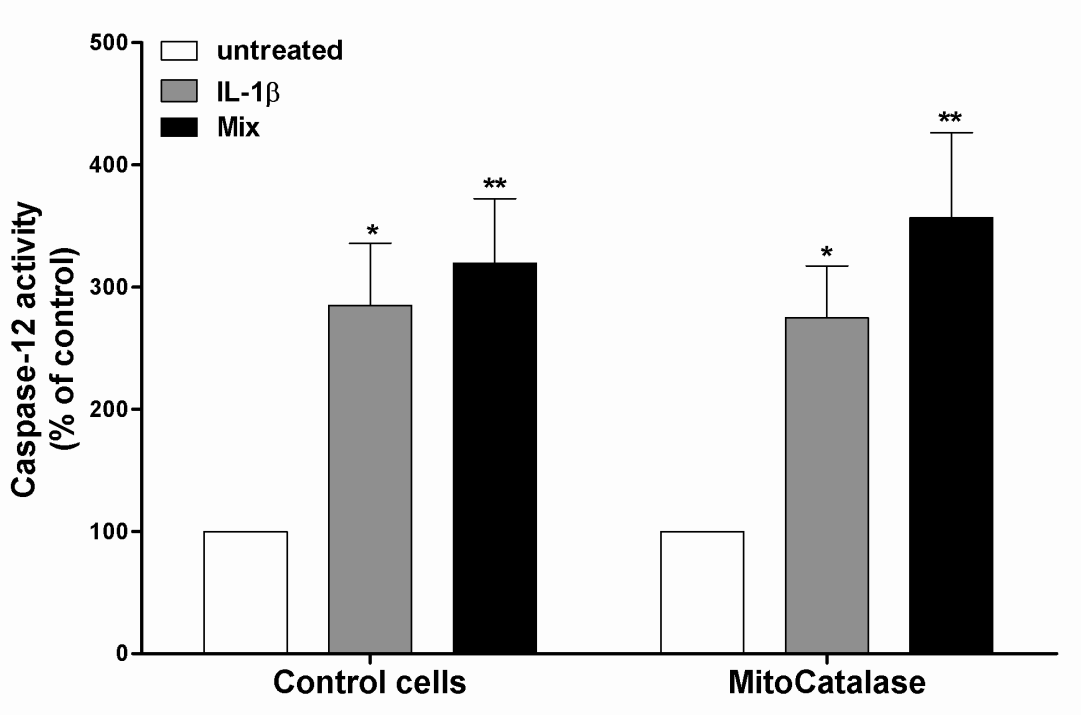

Fig. 1. Effects of pro-inflammatory cytokines on caspase-12 activation in insulin-producing RINm5F control cells and cells overexpressing MitoCatalase. RINm5F control cells (mock transfected) and cells overexpressing catalase in mitochondria (MitoCatalase) were incubated under control conditions (open bars), with 600 units/ml IL-1β (gray bars) or with a cytokine mixture consisting of 60 units/ml IL-1β, 185 units/ml TNF-α, 14 units/ml IFN-γ (black bars) for 24 h. The cells were trypsinized, incubated for 45 min with the fluorescence caspase-12 substrate FITC-ATAD-FMK, and the intracellular fluorescence intensity was measured by flow cytometry. Data are expressed as means ± SEM of four independent experiments; *, $p < 0.05$; **, $p < 0.01$ compared with cells incubated under control conditions (ANOVA/Dunnett's-test).

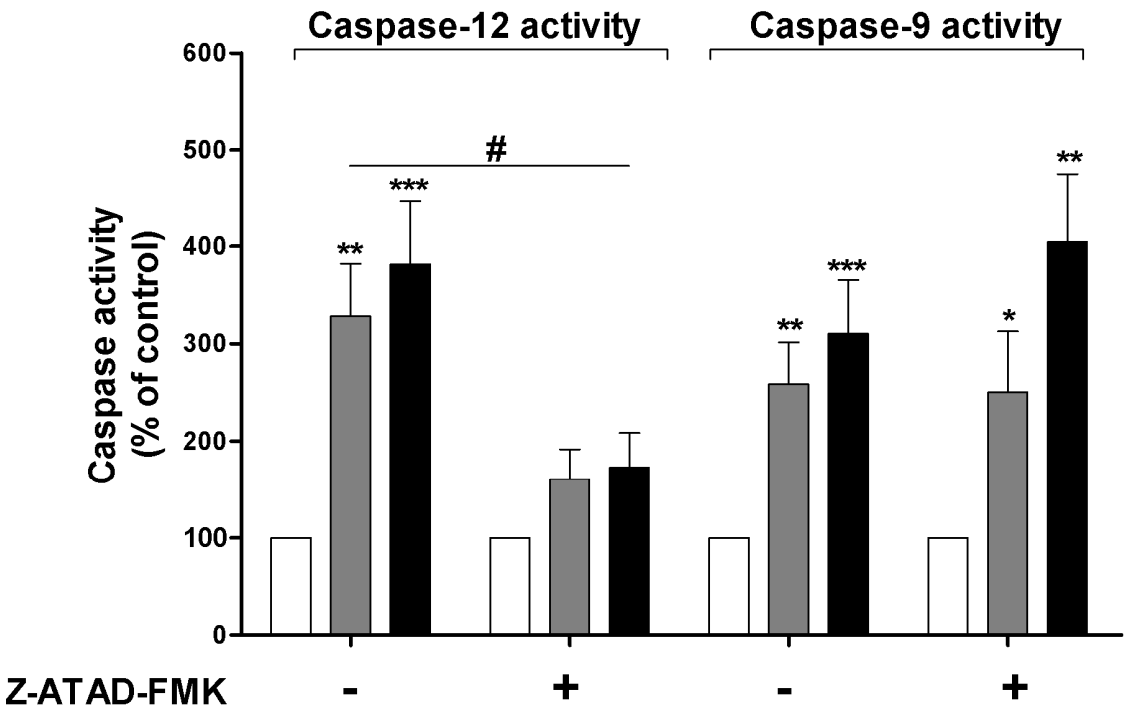

Fig. 2. Effects of the caspase-12 specific inhibitor Z-ATAD-FMK on cytokine-induced caspase-9 activation in insulin-producing RINm5F cells. RINm5F cells were incubated under control conditions (open bars), with 600 units/ml IL-1β (gray bars) or with a cytokine mixture consisting of 60 units/ml IL-1β, 185 units/ml TNF-α, 14 units/ml IFN-γ (black bars) for 24 h in the presence or absence of the caspase-12 specific inhibitor Z-ATAD-FMK (15 μM). The cells were trypsinized, incubated for 45 min with the fluorescence caspase-12 substrate FITC-ATAD-FMK or with the caspase-9 substrate FAM-LEDH-FMK and the intracellular fluorescence intensity was measured by flow cytometry. Data are expressed as means ± SEM of four independent experiments; *, $p < 0.05$; **, $p < 0.01$ compared with cells incubated under control conditions (ANOVA/Dunnett's-test), #, $p < 0.05$ compared with cells incubated under the same conditions without caspase-12 inhibitor (t-test, unpaired, two-tailed).

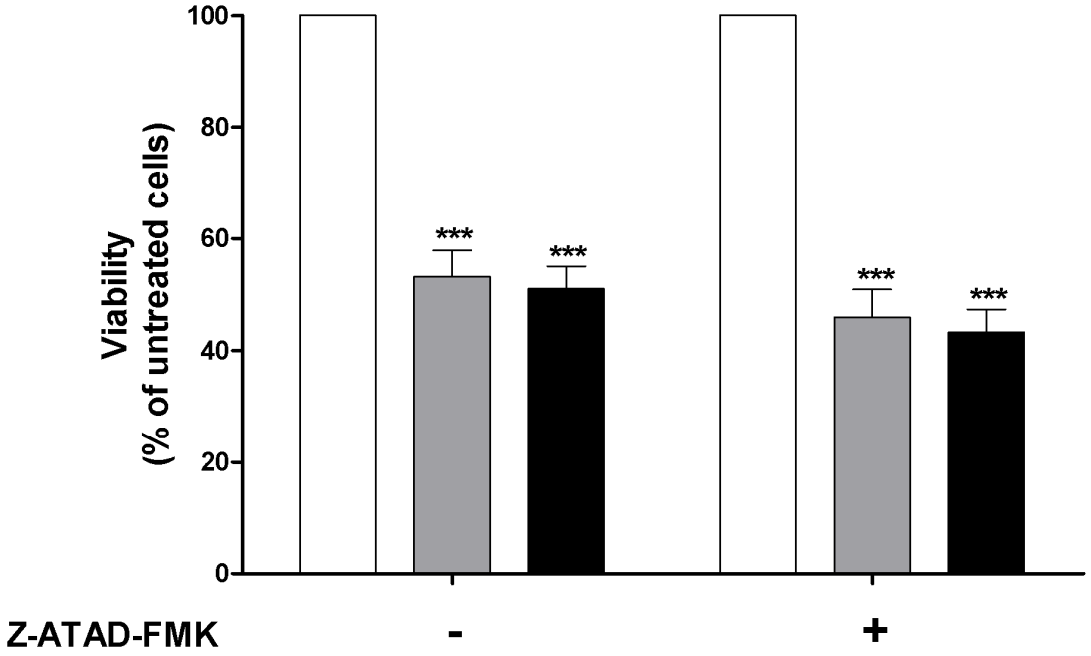

Fig. 3. Effects of the caspase-12 specific inhibitor Z-ATAD-FMK on cytokine toxicity in insulin-producing RINm5F cells. RINm5F cells were incubated under control conditions (open bars), with 600 units/ml IL-1β (gray bars) or with a cytokine mixture consisting of 60 units/ml IL-1β, 185 units/ml TNF-α, 14 units/ml IFN-γ (black bars) for 72 h in the presence or absence of the caspase-12 specific inhibitor Z-ATAD-FMK (15 μM). Thereafter, viability of the cells was determined by the MTT assay and expressed as a percentage of untreated cells. Data are expressed as means ± SEM of four independent experiments. ***, $p < 0.001$ compared with cells incubated under control conditions (ANOVA/ Dunnett's-test).

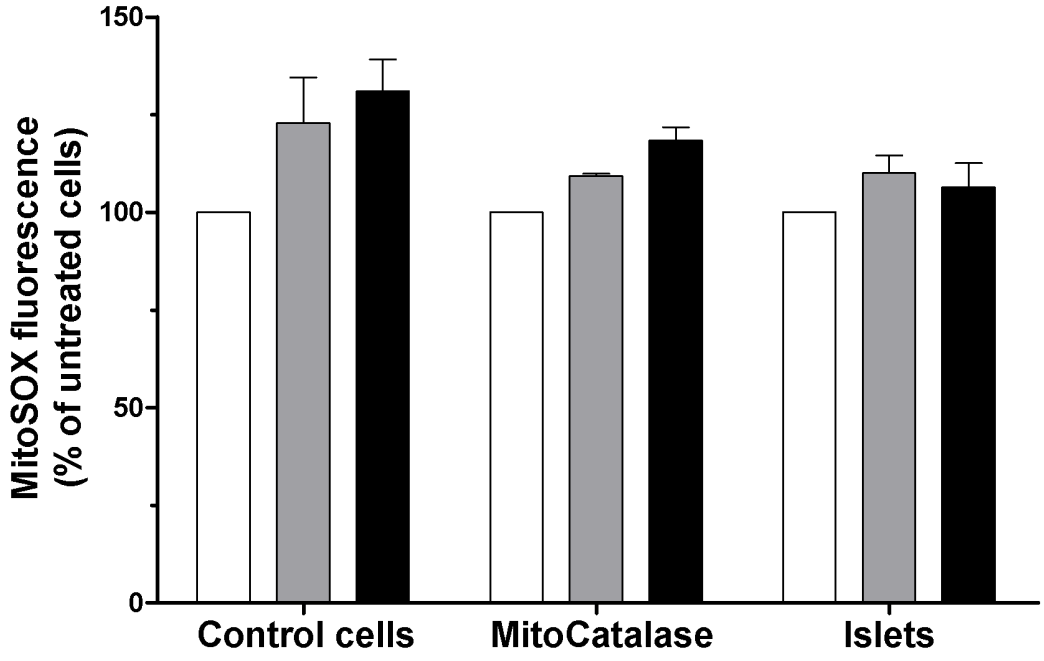

Fig. 4. Effects of pro-inflammatory cytokines on intramitochondrial superoxide radical ($O_2^{\bullet-}$) production in insulin-producing RINm5F control or MitoCatalase overexpressing cells and rat islet cells. RINm5F control cells or cells overexpressing catalase in mitochondria (MitoCatalase) and rat islet cells were incubated under control conditions (open bars), with 600 units/ml IL-1β (gray bars) or with a cytokine mixture consisting of 60 units/ml IL-1β, 185 units/ml TNF-α, 14 units/ml IFN-γ (black bars) for 24 h. The cells were then trypsinized, incubated for 30 min with the MitoSOX Red probe at a final concentration of 5 μM and the intracellular fluorescence intensity was measured by flow cytometry. Data are expressed as means ± SEM of four independent experiments.

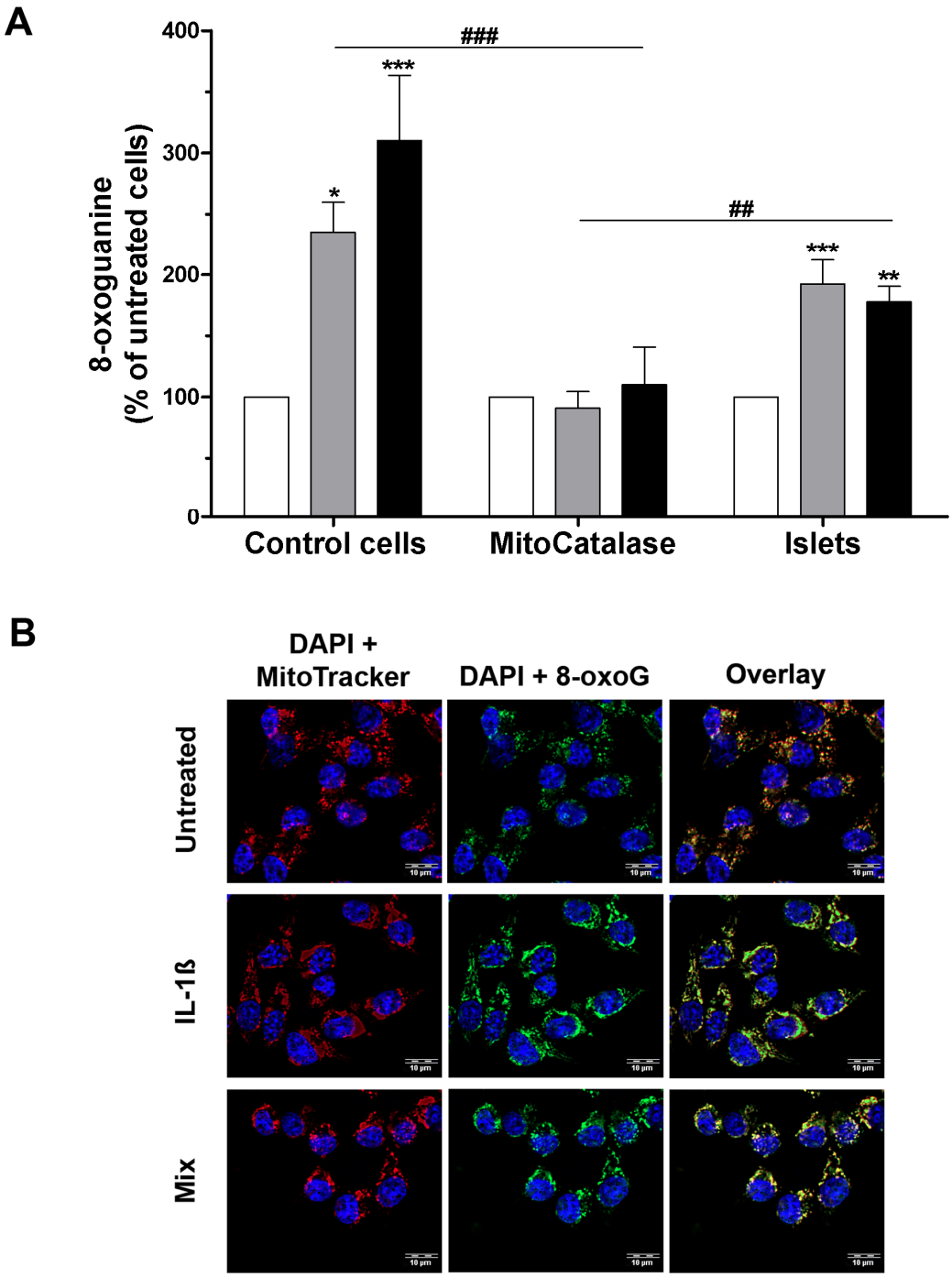

Fig. 5. Effects of pro-inflammatory cytokines on 8-oxoguanine formation in insulin-producing RINm5F control or MitoCatalase overexpressing cells and rat islet cells. (A) RINm5F control cells or cells overexpressing catalase in mitochondria (MitoCatalase) and rat islet cells were incubated under control conditions (open bars), with 600 units/ml IL-1β (gray bars) or with a cytokine mixture consisting of 60 units/ml IL-1β, 185 units/ml TNF-α, 14 units/ml IFN-γ (black bars) for 72 h. After fixation and permeablisation, the cells were stained with the FITC-labeled 8-oxoguanine probe for 1 h at room temperature and the fluorescence

intensity was measured by flow cytometry. Data are expressed as means ± SEM of six (rat islets) and eight (RINm5F control and MitoCatalase cells) independent experiments; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with cells incubated under control conditions; #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$ compared with MitoCatalase cells incubated under the same conditions (ANOVA/Dunnett's-test). (B) Subcellular localization of 8-oxoguanine (8-oxoG) in insulin-producing RINm5F control cells after incubation with pro-inflammatory cytokines. RINm5F control cells were incubated as in (A). After fixation with 4 % paraformaldehyde, the cells were stained for mitochondria (red) and for 8-oxoguanine (green) followed by nuclear counterstaining with DAPI (blue). These images revealed the localization of 8-oxoG in mitochondria and confirmed its accumulation after cytokine treatment (middle and lower panel) compared to the untreated cells (upper panel).

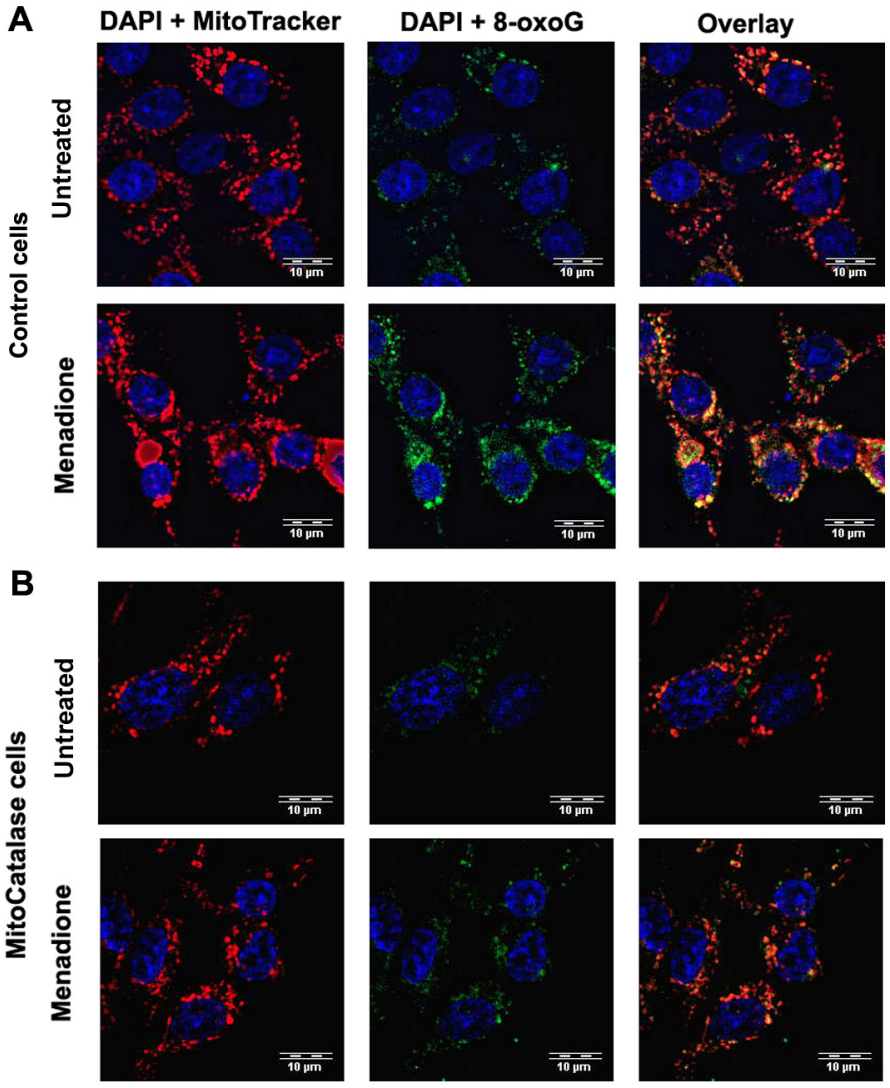

Fig. 6. Subcellular localization of 8-oxoguanine (8-oxoG) in insulin-producing RINm5F control and MitoCatalase overexpressing cells after incubation with menadione. RINm5F control cells (A) and MitoCatalase overexpressing cells (B) were incubated for 2 h under control conditions or with the intramitochondrial ROS generator menadione (10 µM). Thereafter the menadione containing culture medium was removed and the cells were then incubated overnight in fresh medium. After fixation with 4 % paraformaldehyde, the cells were stained for mitochondria (red) and for 8-oxogunaine (green) followed by nuclear counterstaining with DAPI (blue). These images revealed the menadione-induced accumulation of 8-oxoG in mitochondria of control cells (A), comparable to the cytokine-induced 8-oxoG formation. By contrast, MitoCatalase overexpressing cells showed significantly lower 8-oxoG accumulation under the same conditions (B).

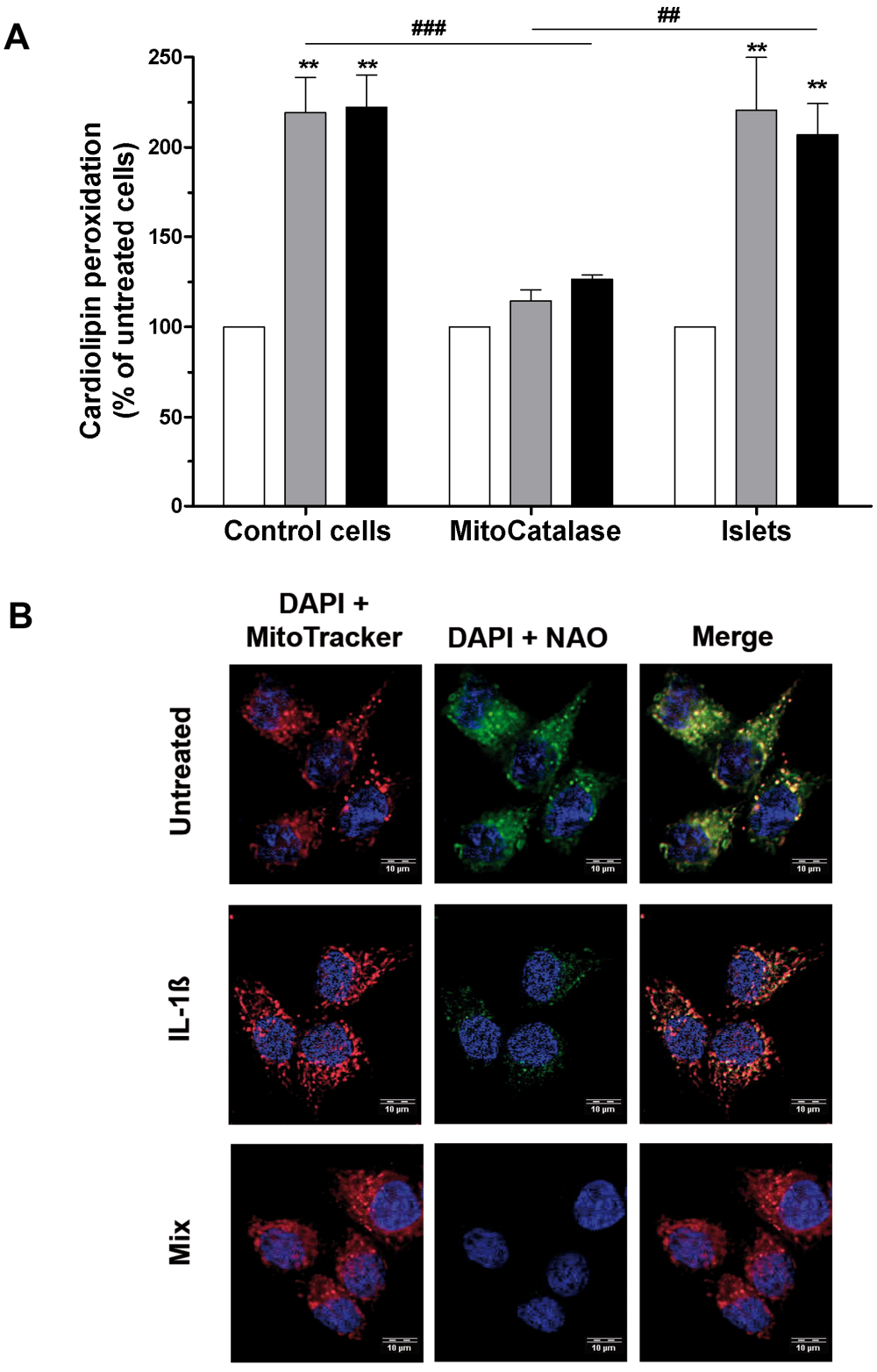

Fig. 7. Effects of pro-inflammatory cytokines on cardiolipin peroxidation in insulin-producing RINm5F control or MitoCatalase overexpressing cells and rat islet cells. (A) RINm5F control cells or cells overexpressing catalase in mitochondria (MitoCatalase) and rat islet cells were incubated under control conditions (open bars), with 600 units/ml IL-1β (gray

bars) or with a cytokine mixture consisting of 60 units/ml IL-1β, 185 units/ml TNF-α, 14 units/ml IFN-γ (black bars) for 24 h. The cells were trypsinized and incubated for 30 min at 37 °C with 10-n-nonyl-acridine orange (NAO) at a final concentration of 100 nM. Thereafter the intracellular fluorescence intensity was measured by flow cytometry. Data are expressed as means ± SEM of four (rat islets) and eight (control and MitoCatalase cells) independent experiments; **, $p < 0.01$ compared with cells incubated under control conditions; ##, $p < 0.01$; ###, $p < 0.001$ compared with MitoCatalase cells under the same conditions (ANOVA/Dunnett's-test). (B) Subcellular localization of 10-n-nonyl-acridine orange (NAO) in insulin-producing RINm5F control cells after incubation with pro-inflammatory cytokines. RINm5F control cells were incubated as in (A). After fixation with 4 % paraformaldehyde, the cells were stained for mitochondria (red) and for NAO (green) followed by nuclear counterstaining with DAPI (blue). The observed decrease of NAO immunofluorescence after exposure to IL-1β alone (middle panel) or cytokine mixture (lower panel) represents the cytokine-induced cardiolipin peroxidation.

3 Diskussion

Der Typ 1 Diabetes mellitus ist eine Autoimmunerkrankung, die von einer progredienten Zerstörung der insulinproduzierenden β-Zellen des Pankreas hervorgerufen wird und durch einen absoluten Insulinmangel gekennzeichnet ist. Die Zerstörung der pankreatischen β-Zellen ist ein komplexer Prozess, der durch zelluläre Elemente des Immunsystems vermittelt wird. Inselinfiltrierende Makrophagen und T-Lymphozyten sezernieren proinflammatorische Zytokine, welche intrazelluläre Signalkaskaden, bestehend aus Caspasen und anderen proapoptotischen Proteinen initiieren, an deren Ende der apoptotische β-Zelltod steht (Eizirik & Mandrup-Poulsen 2001; Kim & Lee 2009). Freie Sauerstoffradikale (ROS) und Stickstoffmonoxid (NO), die von den aktivierten mononukleären Immunzellen freigesetzt sowie infolge der Zytokinwirkung gebildet werden, spielen für die β-Zellschädigung eine entscheidende Rolle. Der durch Zytokine induzierte β-Zelltod ist dabei die Folge eines Ungleichgewichts zwischen antioxidativen Schutzsystemen und der während des Autoimmunprozesses intrazellulär gebildeten Menge ROS (Tiedge *et al.* 1997; Lenzen 2008). Eine Steigerung des antioxidativen Abwehrstatus, insbesondere die Überexpression der H_2O_2-detoxifizierenden Katalase in Mitochondrien, bewirkte in insulinproduzierenden RINm5F Gewebekulturzellen einen signifikanten Schutz gegenüber chemischen Sauerstoff-radikalbildnern und zytokinvermittelter β-Zelltoxizität (Gurgul *et al.* 2004). Dies weist einerseits auf den Ursprungsort von intrazellulären reaktiven Sauerstoffspezies und anderseits auf die Beteiligung mitochondrial generierter ROS an dem Untergang der insulinproduzierenden β-Zellen hin.

Mitochondrien, als Ort der oxidativen Phosphorylierung, sind nicht nur die Hauptquelle der intrazellulären reaktiven Sauerstoffspezies, sondern auch der Ausgangspunkt des intrinsischen Apoptosesignalwegs. Die Permeabilisierung der äußeren Mitochondrienmembran und die damit verbundene Freisetzung proapoptotischer Faktoren definieren den Punkt der unumkehrbaren Apoptoseinduktion (Chipuk *et al.* 2006; Kroemer *et al.* 2007). Die proapoptotischen Bcl-2 Familienmitglieder Bax und dessen Homolog Bak spielen dabei eine entscheidende Rolle. Der genaue Einfluss dieser Proteine auf die Permeabilisierung der Mitochondrien und den damit bedingten Ausstrom proapoptotischer Faktoren aus diesen Organellen ist nach wie vor Gegenstand der wissenschaftlichen Diskussion. Antiapoptotische Bcl-2 Familienmitglieder wie Bcl-2 und Bcl-X_L wirken der Permeablisierung und der Freisetzung mitochondrialer apoptotischer Faktoren entgegen, indem sie aktiviertes Bax und/oder Bak binden und so ihre proapototische Wirkung aufheben. Die Balance zwischen anti- und proapoptotisch wirkenden Bcl-2 Familienmitgliedern scheint demnach über das

Schicksal der Zelle zu bestimmen (Adams & Cory 1998; Wong & Puthalakath 2008; Brenner & Mak 2009).

Zahlreiche Studien an nicht endokrinen Zellarten konnten zeigen, dass ROS, insbesondere H_2O_2, die Expression von Bcl-2 vermindert und die des proapoptotischen Proteins Bax erhöht. Das daraus resultierende Missverhältnis zwischen Bax und Bcl-2 spielt dabei eine zentrale Rolle für die hohe Sensitivität der Zellen gegenüber H_2O_2-induzierten Schäden (Gomez Sarosi *et al.* 2003; Li *et al.* 2004; Juknat *et al.* 2005; Siu *et al.* 2009).

β-zelltoxische Zytokine entfalten ihre zytotoxische Wirkung auf insulinproduzierende β-Zellen durch eine Interaktion von intrazellulär generierten reaktiven Sauerstoff- und Stickstoffspezies. Daher sollte im Rahmen der vorliegenden Arbeit die Auswirkung des veränderten antioxidativen Abwehrstaus auf die zytokinvermittelte Modulation von anti- (Bcl-2 und Bcl-X_L) und proapoptotischen (Bax, Bad, Bim und Bid) Bcl-2 Familienmitgliedern aufgezeigt werden. Dabei galt es insbesondere, die durch den zytokinvermittelten oxidativen Stress induzierten Signalkaskaden in primären Inselzellen sowie in insulinproduzierenden RINm5F-Zellen zu charakterisieren. Darüber hinaus sollte in Expositionsversuchen mit Zytokinen die zellkompartimentspezifische Bildung reaktiver Sauerstoffspezies quantifiziert und deren schädigende Wirkung an zellulären Strukturen sowie die Bedeutung der klassischen bzw. alternativen NF-κB Signaltranstuktion untersucht werden.

3.1 Die Bedeutung von anti- und proapoptotischen Bcl-2 Proteinen für die β-Zellzerstörung beim Typ 1 Diabetes mellitus

Eine Vielzahl experimenteller Daten zeigt, dass die Apoptose die vorherrschende Form des β-Zelltodes in der Entstehung des Typ 1 Diabetes mellitus ist. Die β-Zellapoptose kann durch unterschiedliche Signale und Signalwege induziert werden. So können extrazelluläre Todesliganden (wie z.B. FasL oder TNF), die an entsprechenden Rezeptoren auf der β-Zelloberfläche binden, den extrinsischen Signalweg auslösen. Der intrinsische Signalweg wird hingegen über intrazellulär gebildete Signale eingeleitet, z.B. nach der Schädigung intrazellulärer Strukturen, der Akkumulation ungefalteter sowie missgefalteter Proteine im ER-Lumen (ER-Stress) bzw. massiver Bildung reaktiver Sauerstoffspezies (oxidativer Stress) (Eizirik & Mandrup-Poulsen 2001; Pirot *et al.* 2008; Thomas *et al.* 2009). Hierbei spielen die Mitochondrien neben ihrer physiologischen Funktion der ATP-Synthese und der damit verbundenen Generierung des Signals für die glukoseinduzierte Insulinsekretion eine ausschlaggebende Rolle. Sie enthalten eine Vielzahl proapoptotischer Faktoren, die nach dem

Verlust der mitochondrialen Membranintegrität freigesetzt werden und so die Exekution der Apoptose einleiten. Dieser Prozess wird maßgeblich durch die Mitglieder der Bcl-2 Familie reguliert. Die antiapoptotisch wirkenden Bcl-2 Proteine, insbesondere Bcl-2, sind in der Lage, die mitochondriale Integrität zu stabilisieren und aufrechtzuerhalten, während die proapoptotischen Proteine, insbesondere die Aktivierung der Bax-homologen Multidomänenproteinen, für die Destabilisierung der mitochondrialen Integrität und Freisetzung proapoptotischer Faktoren entscheidend ist (Kluck *et al.* 1997; Yang *et al.* 1997; Danial 2007; Kroemer *et al.* 2007). Da die Bcl-2 Familienmitglieder miteinander interagieren und sich dadurch gegenseitig in ihrer Funktion beeinflussen, wird derzeit postuliert, dass das Verhältnis von pro- und antiapoptotisch wirkenden Bcl-2 Familienmitgliedern über die Empfindlichkeit oder Resistenz der Zelle gegenüber apoptotischen Stimuli entscheidet (Youle & Strasser 2008).

Ein Indiz dafür, dass das relative Verhältnis zwischen pro- und antiapoptotisch wirkenden Bcl-2 Proteinen auch für die β-Zellzerstörung während der Manifestation des Typ 1 Diabetes mellitus verantwortlich ist, ist der Schutz humaner und muriner β-Zellen vor Apoptose und Nekrose durch Überexpression des antiapoptotisch wirkenden Bcl-2 Proteins (Rabinovitch *et al.* 1999; Saldeen 2000; Barbu *et al.* 2002; Tran *et al.* 2003). Auch die Sequestrierung des proapoptotischen Proteins Bax durch das Bax-Suppressor-Pepid V5, auch bekannt als Ku70, führte zu einer erhöhten Resistenz gegenüber der zytokinvermittelten Toxizität in humanen pankreatischen β-Zellen (Grunnet *et al.* 2009).

In den vorliegenden Untersuchungen konnte gezeigt werden, dass das proapoptotische Protein Bax unter Kontrollbedingungen sowohl in primären Ratteninseln als auch in insulinproduzierenden RINm5F Zellen am stärksten von den untersuchten Bcl-2 Familienmitgliedern exprimiert wurde. Im Vergleich zu seinem Antagonist Bcl-2 wies Bax in primären Ratteninseln eine um den Faktor 5 und in insulinproduzierenden RINm5F Zellen eine um den Faktor 8,5 höhere basale Expression auf. Diese Ergebnisse stimmen mit den Expressionsdaten einer in humanen Inseln durchgeführten Studie überein (Thomas *et al.* 2002). Als Ursache für das erhöhte Bax/Bcl-2 Verhältnis wurde die stressige Prozedur der Inselisolation angenommen. Aus den erhobenen Daten an primären Ratteninseln und insulinproduziereden RINm5F Zellen geht jedoch hervor, dass das Bax/Bcl-2 Ungleichgewicht ein Charakteristikum der insulinproduzierenden β-Zellen ist, welches durch die β-zelltoxischen Zytokine noch zusätzlich verstärkt wird.

3.1.1 Einfluss von proinflammatorischen Zytokinen auf das Expressionsprofil von anti- und proapoptotischen Bcl-2 Proteinen

Proinflammatorische Zytokine wie IL-1β, TNF-α und das IFN-γ sind als lösliche Mediatoren an der funktionellen Inhibierung und Zerstörung sowohl humaner als auch muriner pankreatischer β-Zellen beteiligt. Sie werden von infiltrierten Makrophagen und aktivierten T-Zellen sezerniert, die ihre Wirkung auf zellulärer Ebene durch Oberflächenrezeptoren vermitteln (Mandrup-Poulsen 1996; Eizirik & Mandrup-Poulsen 2001; Lenzen 2008). IL-1β, das als zentraler Mediator bei der Zerstörung der pankreatischen β-Zellen gilt, vermittelt seine β-zelltoxische Wirkung im Wesentlichen über die Aktivierung von NF-κB sowie über die MAP (*mitogen-activated protein*) Kinasekaskade und die Proteinkinase C Aktivierung. Die zellulären Effekte von TNF-α werden vorwiegend durch die Bindung an TNF-Rezepor 1 (TNF-R1) mediiert. Dabei können zwei unterschiedliche Signalwege induziert werden: Zum einen kann direkt durch die Rekrutierung des Adapterproteins FADD die Initiatorcaspase-8 aktiviert werden, zum anderen ist über TRAF-2 (*TNF receptor-associated factor*-2) die Induktion der NF-κB und des MAPK Signaltransduktionswegs möglich. IFN-γ vermittelt hingegen die intrazelluläre Signaltransduktion über die Aktivierung rezeptorassoziierter Januskinasen (Jak 1/2) und nachfolgender Phosphorylierung von STAT-1 (*signal transducer and activator of transcription*). Zudem können weitere Signalkaskaden durch IFN-γ induziert werden, wie die MAPK und die ERK (*extracellular-siganl-regulated kinase*) Kaskade. Während die von IL-1β und TNF-α induzierten Signaltransduktionswege in der Aktivierung von NF-κB und MAPK-Kaskade konvergieren, interagiert der von IFN-γ initiierte Signalweg nur über die MAPK-Kaskade sowie distal auf der transkriptionellen Ebene bei der Induktion der induzierbaren NO-Synthase (Eizirik & Mandrup-Poulsen 2001; Pirot *et al.* 2008). Dieses synergistische und additive Zusammenspiel der drei proinflammatorischen Zytokine macht die Toxizität auf die pankreatischen β-Zellen aus (Abb. 1.6).

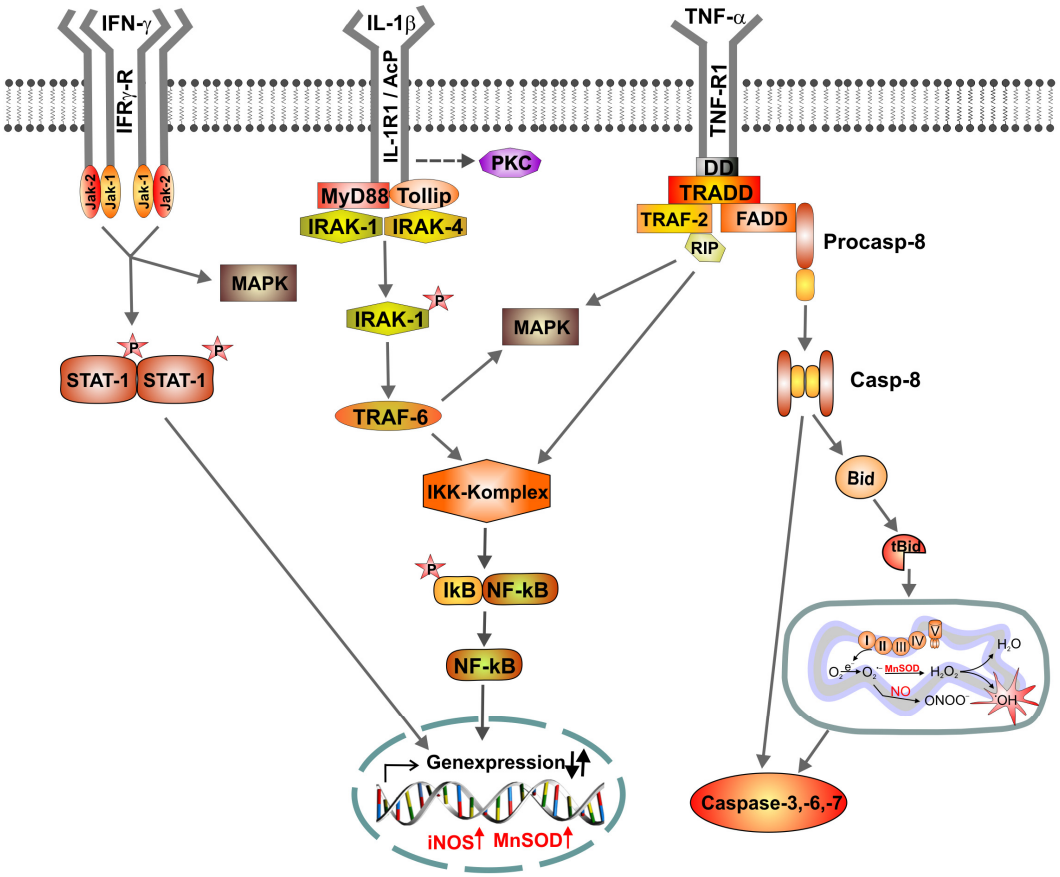

Abb. 1.6. Schematische Darstellung der IFN-γ, IL-1β und TNF-α induzierten Signaltransduktionswege in insulinproduzierenden Zellen (modifiziert nach Pirot *et al.* (2008))

IFN-γ führt nach Bindung an den entsprechenden Rezeptor zur Oligomerisierung und nachfolgend zur Aktivierung der rezeptorassoziierten Janus-Kinasen (Jak-1 und Jak-2). Die aktivierten Jaks rekrutieren und phosphorylieren den zytosolischen Transkriptionsfaktor STAT-1, der nach seiner Oligomerisierung in den Zellkern transloziert. Dort wird durch Bindung an spezifische DNA-Sequenzen u.a. die Expression der iNOS induziert. Zusätzlich zum STAT-Signalweg können die aktivierten Jaks den MAPK Signalweg initiieren. Nach Bindung von IL-1β an den Rezeptor (IL-1R1) kommt es zu einer Heterodimerisierung mit IL-1AcP (IL-1R *accessory protein*) und Bildung eines Rezeptorkomplexes bestehend aus MyD88 (*Myeloid differentiation primary response gene* 88), Tollip (*Toll interacting protein*), IRAK-1 und -4 (IL-1R *associated kinase*). IRAK-4 phosphoryliert IRAK-1, welche daraufhin von dem Rezeptorkomplex dissoziert und TRAF-6 (TNF-*receptor-associated factor*-6) aktiviert. Aktiviertes TRAF-6 initiiert anschließend den NF-κB und/oder den MAPK Signalweg. TNF-α induziert nach Bindung an den Rezeptor TNF-R1 über DD (*death domain*) die Rekrutierung des Adapterproteins TRADD. TRADD rekrutiert wiederum TRAF-2 und die Serin/Threoninkinase RIP, die ähnlich wie TRAF-6 den NF-κB und/oder den MAPK Signalweg auslösen. Zudem kann durch TRADD auch das Adapterprotein FADD rekrutiert werden, welches mit Procaspase-8 interagiert und diese nach dem Prinzip der induzierten Proximität aktiviert. Die Caspase-8 kann direkt Effektorcaspasen prozessieren und/oder über die Spaltung von Bid zu tBid eine Verbindung zum intrinsischen Signalweg herstellen.

Untersuchungen an isolierten primären β-Zellen von Menschen und Nagern ergaben, dass weder TNF-α noch IFN-γ allein β-zellschädigend sind, und IL-1β allein lediglich eine Funktionsstörung mit Hemmung der Insulinbiosynthese und der glucoseinduzierten Insulinsekretion in murinen β-Zellen zur Folge hat. Jedoch führt IL-1β in Kombination mit

TNF-α und IFN-γ nach 2-7 Tagen zum apoptotischen β-Zelltod (Mandrup-Poulsen 2001; Cnop *et al.* 2005). Dieses spricht dafür, dass für die zytokinvermittelte β-Zellapoptose eine komplexe Veränderung der Gen- und Proteinexpression erforderlich ist. Anhand von Microarray- und Proteomanalysen konnte gezeigt werden, dass IL-1β, TNF-α und IFN-γ die Expression zahlreicher Gene, u.a. die der Bcl-2 Familie modulieren, die möglicherweise zur β-Zelldysfunktion und letztlich zur β-Zellapoptose führen können (Kutlu *et al.* 2003; Ortis *et al.* 2006; D'Hertog *et al.* 2007; Ortis *et al.* 2010). Aus diesem Grund wurde in der vorliegenden Arbeit der Einfluss proinflammatorischer Zytokine auf das Expressionsprofil von wichtigen Mitgliedern der Bcl-2 Familie untersucht. Es konnte gezeigt werden, dass durch die verwendeten Zytokine (IL-1β allein und der Zytokinmix bestehend aus IL-1β, TNF-α und IFN-γ) sowohl in primären Ratteninseln als auch in insulinproduzierenden RINm5F Zellen eine Expressionsveränderung der untersuchten Bcl-2 Proteine hervorgerufen wurde. Die synergistische Verstärkung der durch IL-1β bewirkten β-zelltoxischen Effekte durch TNF-α und IFN-γ spiegelte sich auch in ihrer verstärkten Wirkung auf die Expression von Bcl-2 und den untersuchten BH3-only Proteinen wieder. Eine 24stündige Exposition von primären Ratteninseln und insulinproduzierenden RINm5F Zellen mit IL-1β allein oder mit dem Zytokinmix verursachte eine signifikante Abnahme der Expression des antiapoptotischen Proteins Bcl-2 und eine Expressionsinduktion proapoptotischer BH3-only Proteine. Dabei zeigten die dem Zytokinmix exponierten Zellen einen deutlich stärkeren Effekt, als die mit IL-1β allein behandelten Zellen. Der potenzierte Effekt von IL-1β in der Kombination mit TNF-α und IFN-γ stimmte mit denen in vorangegangen Vitalitätsuntersuchungen an RINm5F Zellen überein. So wurde nach dreitägiger Inkubation mit IL-1β eine Restvitalität von 75 % beobachtet, während unter dem Einfluss des Zytokinmix die Zellvitalität bis auf 35 % abnahm (Lortz *et al.* 2000).

Die erfasste zytokinvermittelte Reduktion der Bcl-2 Expression steht im Einklang mit bereits veröffentlichten Studien. So konnte in murinen und humanen pankreatischen Inseln ebenfalls eine Abnahme der Bcl-2 Expression beobachtet werden (Piro *et al.* 2001; Trincavelli *et al.* 2002; Van de Casteele *et al.* 2002), während eine Überexpression die pankreatischen β-Zellen vor zytokinvermittelter Toxizität schützte (Rabinovitch *et al.* 1999; Saldeen 2000; Barbu *et al.* 2002; Tran *et al.* 2003). Durch die zytokinmediierte Abnahme des antiapoptotisch wirkenden Proteins Bcl-2 bei gleichbleibender Expression des proapoptotisch wirkenden Proteins Bax vergrößerte sich zusätzlich die Bax/Bcl-2 Dysbalance zugunsten des Proteins Bax. Dieses Missverhältnis könnte ein Grund für die Vulnerabilität der β-Zellen gegenüber proinflammatorischer Zytokinen sein.

Möglicherweise tragen aber auch die BH3-only Proteine, die als Sensoren und Mediatoren apoptotischer Stimuli stromaufwärts von Bcl-2 Multidomänproteinen agieren, zu dieser Dysbalance bei. Es wird angenommen, dass die im Rahmen dieser Arbeit untersuchten BH3-only Proteine Bid, Bad und Bim die Wirkung antiapoptotischer Bcl-2 Proteine neutralisieren und die des proapoptotischen Proteins Bax fördern (Kuwana *et al.* 2005; Kim *et al.* 2006; Wong & Puthalakath 2008).

Bid liegt unter physiologischen Bedingungen zytosolisch und inaktiv vor und wird u.a. durch die Caspase-8 proteolytisch zum *truncated* Bid (tBid) aktiviert, das anschließend über eine Interaktion mit Multidomänproteinen der Bcl-2 Familie zur Induktion der Apoptose beitragen kann (Gross *et al.* 1999; Billen *et al.* 2008). Bid ist somit das einzige Bcl-2 Protein, das in der Lage ist, den extrinsischen mit dem intrinsischen Apoptosesignalweg zu verknüpfen.

Da die Expression dieses Proteins sowohl in primären Ratteninseln als auch in insulinproduzierenden RINm5F Zellen durch die β-zelltoxischen Zytokine signifikant induziert wurde, ist anzunehmen, dass eine Amplifizierung der Caspasekaskade über den mitochondiralen Apoptoseweg für eine zytokinvermittelte Induktion der β-Zellapoptose notwendig ist. Dieser Befund steht in Übereinstimmung mit einer kürzlich an primären Mausinseln durchgeführten Studie. McKenzie *et al*. konnten zeigen, dass Bid für die FasL- und TNF-α-induzierten DNA-Strangbrüche von entscheidender Bedeutung ist (McKenzie *et al.* 2008). Ein weiterer Hinweis für die Aktivierung und Beteiligung von Bid am zytokinvermittelten β-Zelltod ist die bereits gezeigte Aktivierung der Caspase-8 durch proinflammatorische Zytokine (Cottet *et al.* 2002; Papaccio *et al.* 2005).

Bad liegt in vitalen Zellen im phosphorylierten Zustand (Ser^{112} und Ser^{136}) assoziiert mit Proteinen der 14-3-3 Familie zytosolisch vor. Nach seiner Dephosphorylierung durch die Ca^{2+}-abhängige Phosphatase Calcineurin (Wang *et al.* 1999a) transloziert es in apoptotischen Zellen zur äußeren Mitochondrienmembran, wo es die antiapoptotische Wirkung der Bcl-2 Familienmitglieder antagonisiert (Zha *et al.* 1996; Chiang *et al.* 2001). Die Dissoziation und damit die Aktivierung von Bad kann zudem durch die JNK (*c-Jun NH2-terminal kinase*)-vermittelte Phosphorylierung des 14-3-3 Proteins induziert werden (Sunayama *et al.* 2005). So zeigten Studien, dass die β-zelltoxischen Zytokine zur Aktivierung von JNK (Mokhtari *et al.* 2008) führen und eine Erhöhung der intrazellulären Ca^{2+}-Konzentration in pankreatischen β-Zellen hervorrufen können (Wang *et al.* 1999b). Daher wurde der Phosphorylierungsstatus von Bad unter dem Einfluss von proinflammatorischen Zytokinen in der vorliegenden Arbeit untersucht. Die Exposition der primären Ratteninseln und insulinproduzierenden RINm5F

Zellen mit IL-1β oder einem Zytokinmix verursachte eine Dephosphorylierung des Proteins Bad, so dass in diesen Zellen möglicherweise ebenfalls Bad zur zytokininduzierten Apoptose beiträgt.

Auch die Expression des proapoptotisch wirkenden Proteins Bim wurde unter den gleichen Expositionsbedingungen stark induziert. Seine proapoptotische Aktivität wird transkriptionell sowie posttranslational durch eine große Zahl extra- und intrazellulär generierender Signale reguliert. Wie aus den vorherigen Studien an nicht endokrinen Zellarten hervorgeht, wird Bim bei anhaltendem ER-Stress durch den Transkriptionsfaktor CHOP stark induziert, während auf der posttranslationalen Ebene durch die Phosphoprotein Phosphatase 2A-mediierte Dephosphorylierung das Protein stabilisiert und seine proteosomale Degradierung verhindert wird (Puthalakath *et al.* 2007). Es konnte bereits in pankreatischen β-Zellen nachgewiesen werden, dass die proinflammatorischen Zytokine auch zum ER-Stress und infolgedessen zur Aktivierung von CHOP führen (Eizirik *et al.* 2008). Die beobachtete zytokinvermittelte Induktion von Bim könnte daher als Bindeglied zwischen dem ER-Stress und dem mitochondrialen Apoptoseweg fungieren.

Bei zusammenfassender Betrachtung der im Rahmen dieser Arbeit ermittelten Expressionsdaten der anti- und proapoptotischen Bcl-2 Familienmitglieder wird deutlich, dass die verschiedenen zytokinvermittelten Signalkaskaden durch ein komplexes Zusammenspiel dieser Proteinfamilie im mitochondrialen Apoptoseweg münden.

3.1.2 Einfluss von reaktiven Sauerstoffspezies auf das Expressionsprofil von anti- und proapoptotischen Bcl-2 Proteinen

Freie reaktive Sauerstoffspezies, die extrazellulär durch aktivierte mononukleäre Zellen freigesetzt sowie während des Autoimmunprozesses durch die β-zelltoxischen Zytokine intrazellulär generiert werden, spielen eine zentrale Rolle bei die Entstehung des Typ 1 Diabetes mellitus (Lenzen 2008). Die Bildungsmenge und die zytotoxische Wirkung von freien ROS hängen maßgeblich von den effizienten antioxidativen Schutzmechanismen einer Zelle ab (Halliwell & Gutteride 2007).

β-Zellen sowie insulinproduzierende Gewebekulturzellen zeichnen sich durch einen geringen antioxidativen Abwehrstatus und eine außerordentliche Vulnerabilität gegenüber freien Sauerstoffspezies aus. Während das Expressionsniveau superoxidinaktivierender Enzyme CuZnSOD und MnSOD in den β-Zellen moderat ist, ist das der H_2O_2-inaktivierenden Katalase und Glutathionperoxidase besonders niedrig und macht nur 1–3 % des Expressionsniveaus der Leber aus (Lenzen *et al.* 1996; Tiedge *et al.* 1997). Dieses Ungleichgewicht

zwischen Superoxid- und H_2O_2-inaktivierenden Enzymsystemen wird noch zusätzlich durch die zytokinvermittelte Induktion von MnSOD verstärkt (Lortz *et al.* 2005), was eine Akkumulation von H_2O_2 und infolgedessen die Bildung von außerordentlich aggressiven Hydroxylradikalen in der metallkatalysierten Fenton Reaktion zur Folge hat. Das toxische Potential freier Sauerstoffradikale gegenüber Zellen beruht nicht nur auf der direkten Reaktion mit zellulären Komponenten wie z.B. der Schädigung von Proteinen, Nukleinsäuren und Lipiden, sondern sie können auch als Signalmoleküle wirken und zelluläre Vorgänge wie die Apoptose modulieren (Halliwell & Gutteride 2007).

Eine Vielzahl experimenteller Daten an nicht endokrinen Zellen lässt vermuten, dass H_2O_2 die Expression von Bcl-2 vermindert und die des proapoptotischen Proteins Bax erhöht (Gomez Sarosi *et al.* 2003; Li *et al.* 2004; Juknat *et al.* 2005; Siu *et al.* 2009), während die Detoxifizierung von H_2O_2 durch Antioxidantien und H_2O_2-inaktivierende Enzyme die Verminderung der Bcl-2 Expression verhindert (Hildeman *et al.* 2003; Pugazhenthi *et al.* 2003; Faucher *et al.* 2005; Wang *et al.* 2008). Um die Beteiligung von H_2O_2 in der Regulation von Bcl-2 Familienmitgliedern in insulinproduzierenden β-Zellen zu untersuchen, wurden RINm5F Zellen genutzt, die das H_2O_2-inaktivierende Enzym Katalase im Zytosol (ZytoKatalase) bzw. in Mitochondrien (MitoKatalase) stabil überexprimieren. Experimente mit diesen Zellen zeigten, dass nur die mitochondrial lokalisierte Katalase in der Lage war, die basale Expression von Bcl-2 signifikant zu erhöhen und die des proapoptotisch wirkenden Proteins Bax zu erniedrigen. Auch die zytokinvermittelte Reduktion des antiapoptotisch wirkenden Proteins Bcl-2 konnte durch die Überexpression der Katalase in Mitochondrien vollständig verhindert werden, was mit dem effektiven Schutz gegenüber β-zelltoxischen Zytokinen in diesen Zellen korreliert (Gurgul *et al.* 2004). Im Gegensatz dazu hatte die zytosolisch lokalisierte Katalase keinen Einfluss auf die untersuchten Bcl-2 Familienmitglieder. Demnach könnten mitochondrial generierte ROS, insbesondere H_2O_2, als modulierender Faktor der Bcl-2 bzw. Bax Expression angesehen werden. Eine ähnliche Beobachtung wurde in Glutathionperoxidase (GPx) überexprimierenden Endothelgewebe-kulturzellen (ECV304) sowie in Lungenepithelzellen (H460) gemacht, die mit dem synthetischen mitochondrial lokalisierten Antioxidanz MnTBAP behandelt wurden (Faucher *et al.* 2005; Wang *et al.* 2008). Es konnte gezeigt werden, dass die Überexpression von GPx bzw. die Behandlung der Zellen mit MnTBAP die Expression von Bcl-2 erhöhen und die von Bax erniedrigen. Ein möglicher Mechanismus, wie H_2O_2 die Expression des Bcl-2 Proteins moduliert, könnte die H_2O_2-vermittelte Abnahme des phosphorylierten Proteins CREB (*cAMP-responsive element binding protein*) sein (See & Loeffler 2001). Als

Transkriptionsfaktor führt CREB nach seiner Phosphorylierung an Ser^{133} zur Induktion einer Reihe von Genen, deren Promotor das sog. CRE- (cAMP *responsive element*) Bindungsmotiv enthält (Gonzalez & Montminy 1989). Auch in der Bcl-2 Promotorregion konnte das CRE-Bindungsmotiv und eine CREB-vermittelte Induktion an neuronalen Zellen nachgewiesen werden (Pugazhenthi *et al.* 1999). Ferner konnte gezeigt werden, dass ROS zu einer Abnahme des CREB Proteins führen, während seine Überexpression in einem signifikanten Schutz gegenüber ROS-induzierter Apoptose aufgrund gesteigerter Bcl-2 Expression resultiert (Pugazhenthi *et al.* 2003).

Die Untersuchungen zur Promotoraktivität des Bcl-2 Proteins in insulinproduzierenden RINm5F Zellen ergaben, dass die proinflammatorischen Zytokine eine signifikante Abnahme der Bcl-2 Promotoraktivität verursachten, wohingegen die Überexpression der MitoKatalase die zytokinvermittelte Reduktion verhinderte. Dieses deutet darauf hin, dass mitochondrial generierte ROS hauptsächlich auf der transkriptionellen Ebene über das CREB Protein die Expression von Bcl-2 beeinflussen.

Die Überexpression H_2O_2-inaktivierender Enzyme mit zytosolischer oder mitochondrialer Lokalisation schien dagegen keinen Einfluss auf das Expressionsverhalten der untersuchten BH3-only Proteine zu haben, da das basale Expressionsniveau und die Expressionsmuster nach Zytokinexposition sich nicht von den untransfizierten RINm5F Zellen unterschieden. Dies lässt den Schluss zu, dass die Expression der BH3-only Proteine im Vergleich zu den Multidomänproteinen nicht von ROS beeinflusst wird.

Zusammenfassend ist festzustellen, dass ROS mitochondrialen Ursprungs offenbar Hauptmediatoren für die zytokinvermittelte β-Zellzerstörung sind. Dabei kann H_2O_2 als membranpermeable reaktive Sauerstoffspezies von ihrem Entstehungsort heraus diffundieren und u.a. die Expression antiapoptotisch wirkender Proteine negativ beeinflussen. Des Weiteren konnte durch die Überexpression der mitochondrial lokalisierten Katalase das Bax/Bcl-2 Ungleichgewicht kompensiert werden, was mit der Hemmung der mitochondrialen Freisetzung apoptotischer Signalmoleküle nach Zytokinexposition einherging.

3.2 Die Bedeutung der anti- und proapoptotischen NF-κB Signaltransduktion für die β-Zellzerstörung beim Typ 1 Diabetes mellitus

Die zytokinvermittelte Modulation vieler anti- und proapoptotischer Proteine wird primär auf transkriptioneller Ebene durch Aktivierung spezifischer Transkriptionsfaktoren reguliert (Eizirik & Mandrup-Poulsen 2001). Eine Vielzahl von Experimenten deutet darauf hin, dass

die Aktivierung des Transkriptionsfaktors NF-κB ein zentraler Schritt in der zytokinvermittelten β-Zelldestruktion ist (Giannoukakis *et al.* 2000; Naamane *et al.* 2007; Ortis *et al.* 2008). So konnte durch die Überexpression eines IκB Superrepressors, der die Translokation von NF-κB in den Zellkern inhibiert, die zytokinvermittelte Induktion von iNOS und MnSOD und infolgedessen die β-Zelldestruktion verhindert werden (Heimberg *et al.* 2001). Andererseits wurde berichtet, dass eine Inhibierung von NF-κB die Diabetesentwicklung eher beschleunigt (Norlin *et al.* 2005; Liuwantara *et al.* 2006; Kim *et al.* 2007). Während NF-κB in anderen Zellsystemen hauptsächlich antiapoptotische Effekte vermittelt (Karin & Lin 2002; Karin 2006), scheint seine genaue Funktion in β-Zellen noch unklar zu sein.

Die NF-κB Proteinfamilie besteht aus fünf Untereinheiten, p50, p52, p65 (RelA), c-Rel und RelB, die untereinander Homo- und Heterodimere bilden können. Alle Untereinheiten haben eine N-terminale Domäne (RHD, *Rel Homology Domain*) gemeinsam, welche die Bindung mit der DNA, die Homo- und Heterodimerisierung sowie die Interaktion mit IκB (*inhibitor of kappa-B*) Proteinen ermöglicht. Im Ruhezustand liegt die NF-κB Dimäre assoziiert mit einem der inhibitorischen IκB Proteine zytosolisch vor. Zu diesen inhibitorischen Proteinen gehören IκBα, IκBβ, IκBγ, IκBε, Bcl-3, p100 und p105. Die Aktivierung von NF-κB kann über zwei Signalwege, den klassischen und den alternativen Signalweg, erfolgen. Beim klassischen Signalweg werden die IκBs durch den aktivierten IκB Kinase Komplex-β (IKK-β) an Ser^{32} und Ser^{36} phosphoryliert, welche anschließend ubiquitinyliert und durch das 26S Proteasom degradiert werden. Dadurch wird sequestriertes NF-κB bzw. p65/p50 freigesetzt und kann in den Zellkern translozieren. Der alternative Signalweg dagegen führt über die spezifische Aktivierung des IKKα-Komplexes zur Prozessierung von p100 zu p52 und nachfolgender Dimerisierung mit der RelB Untereinheit (Perkins 2007; Hayden & Ghosh 2008).

Die genaue funktionelle Rolle der NF-κB Untereinheiten ist trotz intensiver Forschung in humanen pankreatischen Inseln noch weitestgehend ungeklärt und wird kontrovers diskutiert. Deshalb wurde der Einfluss β-zelltoxischer Zytokine auf eine mögliche Induktion des klassischen bzw. alternativen NF-κB Aktivierungssignalweges an humanen Inselzellen untersucht. In Übereinstimmung mit Studien an nicht endokrinen Zellarten (Xiao & Ghosh 2005) konnte eine zytokinvermittelte Translokation von p65 Untereinheit in den Zellkern und damit die Aktivierung des klassischen Signalwegs an humanen Inseln beobachtet werden. Im Gegensatz dazu konnte weder die Translokation von p52 noch von RelB unter den gleichen Inkubationsbedingungen detektiert werden, was darauf hindeutet, dass der alternative

Signalweg in humanen Inseln durch Zytokine nicht aktiviert wird. Darüber hinaus wurde eine nukleäre Translokation des ribosomalen Proteins S3 (rpS3) nach Exposition mit IL-1β festgestellt. rpS3 ist ein Bestandteil der kleinen ribosomalen Untereinheit 40S und ist für Proteinbiosynthese von entscheidender Bedeutung (Wan *et al.* 2007). Zudem besitzt rpS3 offenbar noch extraribosomale Funktionen. So konnte gezeigt werden, dass rpS3 die spezifische Bindung von p65 an der DNA unterstützt und wurde somit als non-Rel Untereinheit des p65 Homo- oder p56/p50 Heterodoimer identifiziert (Wan *et al.* 2007).

Um die Aktivierung des klassischen NF-κB Signalweges nachzuahmen, wurden dispergierte humane Inselzellen mit einem adenoviralen Vektorkonstrukt transduziert, wodurch die NF-κB Untereinheit c-Rel unter der Kontrolle des CMV Promotors exprimiert wurde. c-Rel wurde ausgewählt, da keine Expression in humanen Inseln detektierbar war und es in anderen Zellarten eine p65 ähnliche Funktion ausübt (Kucharczak *et al.* 2003). Hierdurch konnte eine mögliche Beeinflussung der endogen exprimierten NF-κB Untereinheiten durch die gezielte Überexpression ausgeschlossen werden. Die Überexpression von c-Rel resultierte im Vergleich zu den untransfizierten humanen Inselzellen in einem signifikanten Schutz gegenüber β-zelltoxischen Zytokinen, H_2O_2 und Streptozotocin. Dieses spricht dafür, dass die Aktivierung des klassischen NF-κB Signalweges über eine verstärkte und selektive c-Rel Translokation zu einem protektiven Effekt in humanen Inseln führt. In nicht endokrinen Zellarten scheint NF-κB seinen antiapoptotischen Effekt durch die Induktion einer Vielzahl antiapoptotisch wirkender Gene auszuüben. Dazu gehören beispielsweise c-FLIP (Caspase-8 Inhibitor), c-IAP1 und c-IAP2 (Caspase-3, 7 und 9 Inibitoren) und das antiapoptotisch wirkende Bcl-2 Familienmitglied Bcl-X_L (Micheau *et al.* 2001; Karin & Lin 2002; Karin 2006; Varfolomeev & Vucic 2008). Auch in c-Rel überexprimierenden humanen Inselzellen konnte eine gesteigerte Expression des Bcl-X_L Proteins sowie von c-IAP2 nachgewiesen werden. In Anbetracht dieser Ergebnisse könnte mit einer Aktivierung des antiapoptotischen und Reduktion des inflammatorischen NF-κB Signalweges in pankreatischen β-Zellen auch eine erhöhte Resistenz gegenüber proinflammatorischen Zytokinen erzielt werden.

3.3 Die Bedeutung von reaktiven Sauerstoffspezies für die β-Zell-zerstörung beim Typ 1 Diabetes mellitus

Der oxidative Stress wird als zentraler pathophysiologischer Prozess für die Entstehung zahlreicher Krankheiten, zu denen Diabetes, Arteriosklerose, rheumatoide Arthritis aber auch neurodegenerative Erkrankungen gehören, angesehen. Dieser Prozess ist durch eine vermehrte Bildung von ROS und eine niedrige endogene Kapazität an antioxidativen

Abwehrmechanismen gekennzeichnet (Droge 2002; Halliwell & Gutteride 2007). ROS werden fortwährend als Nebenprodukte des oxidativen Stoffwechsels gebildet. Eine der wichtigsten intrazellulären Quellen für ROS stellen die Mitochondrien dar. Schätzungsweise werden etwa 1-2 % des konsumierten molekularen O_2 während der oxidativen Phosphorylierung zum Superoxidradikalanion ($O_2^{\bullet-}$) umgewandelt. $O_2^{\bullet-}$ dient als Vorläufer der meisten ROS und wird spontan oder durch Superoxiddismutasen zu H_2O_2 dismutiert, welches wiederum in Anwesenheit von Fe^{2+} rasch zur Bildung des reaktiven Hydroxylradikal ($HO^{\bullet}$) führt (Turrens 2003; Orrenius *et al.* 2007).

Obwohl die Toxizität von ROS als Ursache für den Untergang der β-Zellen beim Typ 1 Diabetes mellitus angesehen wird, ist eine differenzierte Bestimmung einzelner ROS und ihrer Entstehungssorte sowie eine abschließende Charakterisierung der ROS-mediierten Zellschäden bisher nicht erfolgt. Für die Beteiligung von ROS an der toxischen Zytokinwirkung spricht der ausgeprägte Schutz, der durch die Überexpression zytoprotektiver Enzyme, insbesondere von H_2O_2-detoxifizierender Katalase und Glutathionperoxidase, erreicht wurde (Tiedge *et al.* 1997; Tiedge *et al.* 1998; Lortz *et al.* 2000). Dieser Schutz konnte durch die zellkompartiment gerichtete Überexpression der Katalase in Mitochondrien signifikant verbessert werden (Gurgul *et al.* 2004), was auf eine zentrale Rolle von mitochondrial generierten ROS nach einer Zytokinexposition hindeutet.

Tatsächlich konnte im Rahmen dieser Arbeit ein Anstieg der ROS Konzentration unter dem Einfluss β-zelltoxischer Zytokine mittels des Fluorochroms Dichlorofluorescein (DCF) festgestellt werden, die jedoch in MitoKatalase überexprimierenden Zellen deutlich niedriger war. Die DCF Fluoreszenzmessung lässt allerdings keinen Schluss auf die Art und den Ursprung der gebildeten ROS zu, da dieses Fluorochrom unspezifisch durch verschiedene ROS oxidiert wird, sowie keine kompartimentspezifische Akkumulation des DCF möglich ist (Tarpey *et al.* 2004; Wardman 2007). Um intramitochondrial generierte ROS nach einer Zytokininkubation in primären Ratteninseln und in insulinproduzierenden RINm5F Zellen selektiv zu quantifizieren, wurden organellspezifische Nachweismethoden angewandt. Zur Bestimmung der intrazellulären $O_2^{\bullet-}$-Konzentration wurde der mitochondrial akkumulierende Superoxidindikator MitoSOX verwendet, der ausschließlich durch $O_2^{\bullet-}$ zu einem rot fluoreszierenden Farbstoff oxidiert wird (Robinson *et al.* 2006).

Nach einer 24stündigen Inkubation mit proinflammatorischen Zytokinen konnte weder in primären Ratteninselzellen noch in Kontroll- und MitoKatalase überexpremierenden RINm5F Zellen ein signifikanter Anstieg intramitochondrialer $O_2^{\bullet-}$ beobachtet werden. Eine Erklärung hierfür könnte die effiziente MnSOD katalysierte Dismutation von $O_2^{\bullet-}$ zu H_2O_2 und O_2 sein.

Die relativ hohe basale Expression des $O_2^{\bullet-}$-inaktivierenden Enzyms MnSOD in insulin-produzierenden Zellen wird zusätzlich durch die benutzten Zytokine gesteigert (Borg *et al.* 1992; Bigdeli *et al.* 1994; Tiedge *et al.* 1997). Diese hohe MnSOD Expression in Kombination mit einer extrem niedrigen Expression an H_2O_2-detoxifizierenden Enzymen (Lenzen *et al.* 1996; Tiedge *et al.* 1997) könnte zu einer H_2O_2 Akkumulation in Mitochondrien führen. Um dieses zu überprüfen, wurde das H_2O_2-spezifische Sensorprotein HyPer im Zytosol (HyPerZyto) oder durch vorhergehende Fusion mit der entsprechenden Zielsequenz in Mitochondrien (HyPerMito) überexprimiert. Dieser Fluoreszenzsensor weist zwei Anregungsmaxima bei 420 und 500 nm und ein Emissionsmaximum bei 516 nm auf. Die Absorption bei 420 nm nimmt bei einer H_2O_2 vermittelten Oxidation proportional zur H_2O_2-Konzentration ab, im Gegenzug nimmt die Absorption bei 500 nm zu. Durch diese Veränderung der Fluoreszenzratio können mikromolare H_2O_2-Konzentrationen spezifisch detektiert werden (Belousov *et al.* 2006). Die in der vorliegenden Arbeit ermittelte Fluoreszenzratio ergab, dass die H_2O_2-Konzentration selbst unter Kontrollbedingungen in den Mitochondrien deutlich höher ist als im Zytosol. Nach einer Zytokinexposition konnte lediglich in den Mitochondrien eine signifikant gesteigerte H_2O_2-Konzentration beobachtet werden, die durch die Überexpression der H_2O_2-detoxifizierenden Katalase in diesen Organellen vollständig reduziert wurde. Ferner konnte festgestellt werden, dass die basale H_2O_2-Konzentration in MitoKatalase überexprimierenden Zellen niedriger ist als in Kontroll- und ZytoKatalase Zellen. Auch in primären Ratteninselzellen konnte mit Hilfe von HyPerMito ein Anstieg der mitochondrialen H_2O_2-Konzentration nach Inkubation mit Zytokinen detektiert werden. Demnach entfalten die proinflammatorischen Zytokine ihre toxische Wirkung auf β-Zellen durch Bildung und Akkumulation von ROS in den Mitochondrien. Vorangegangene Studien an verschiedenen zellulären Systemen haben gezeigt, dass proinflammatorische Zytokine, insbesondere TNF-α zur Dysfunktion der Elektronentransportkette und somit zur massiven Bildung intramitochondrialer ROS (insbesondere $O_2^{\bullet-}$) führen (Yang *et al.* 2007; Mariappan *et al.* 2009). Zudem können sie über die Induktion der iNOS und nachfolgender Bildung von NO (Lenzen 2008) die ROS Bildung in den Mitochondrien anregen. NO ist ein membranpermeables Radikal und kann nach seiner Bildung in die Mitochondrien diffundieren und dort selektiv aber reversibel den Atmungskettenkomplex IV inhibieren. Zum andern kann NO mit $O_2^{\bullet-}$ zum Peroxinitrit ($ONOO^-$) reagieren, das dann durch Modifikation von Fe-S-Zentren und S-Nitrosylierung unselektiv die Atmungskettenkomplexe I, III und IV irreversibel inhibiert, was anschließend zur verstärkten ROS Bildung führt (Brown & Borutaite 2002; Brown & Borutaite 2004).

Auch in RINm5F Zellen konnte nach einer Zytokinbehandlung eine vermehrte Bildung von NO und die daraus resultierende Entstehung des $ONOO^-$ detektiert werden. Allerdings führte die Inhibierung der iNOS mittels L-Nitroarginin, ein iNOS spezifischer Inhibitor, zu einer Verminderung von reaktiven NO Verbindungen, jedoch wurde die zytokinvermittelte Vitalitätsabnahme durch die Inhibierung der iNOS nicht vollständig verhindert. Dies deutet darauf hin, dass das NO nicht allein für den β-Zelltod verantwortlich ist.

Mitochondrien sind nicht nur die Hauptquellen intrazellulärer reaktiver Sauerstoffspezies, sondern auch gleichzeitig das Hauptziel der schädigenden Effekte dieser Moleküle (Orrenius 2007; Maechler *et al.* 2010). Wird das in den Mitochondrien akkumulierte H_2O_2 enzymatisch nicht detoxifiziert, kann es unter Umständen durch die metallkatalysierte Disproportionierung in der Fenton Reaktion zum hoch reaktiven $HO^\bullet$ reagieren. $HO^\bullet$ hat eine extrem kurze Halbwertzeit (10^{-9} Sekunden) und verursacht oxidative Schäden an nahezu allen intrazellulären Strukturen in direkter Umgebung ihrer Entstehung (Halliwell & Gutteride 2007). Zielmoleküle sind beispielsweise die DNA, Lipide und Proteine. Das mitochondriale Genom ist besonders vulnerabel gegenüber oxidativem Stress, da es in unmittelbarer Nähe der ROS Generierung lokalisiert ist und im Gegensatz zur nukleären DNA keine schützenden Histone enthält. Es wird angenommen, dass die oxidative Schädigung der mitochondrialen DNA (mtDNA) 10-20fach höher ist als die der nuklearen DNA (Orrenius *et al.* 2007). Von den DNA Basen ist Guanin aufgrund seines niedrigen Redoxpotentials die reaktionsfreudigste Base und damit als Hauptangriffspunkt für $HO^\bullet$ prädisponiert. In Gegenwart von $HO^\bullet$ wird es bevorzugt am Kohlenstoffatom in der Position 8 hydroxyliert. Das entstehende Produkt ist 8-Hydroxyguanin bzw. die Ketoform 8-Oxoguanin (8-oxoG) und steht in direktem Zusammenhang mit einer erhöhten $HO^\bullet$ Bildung in der Zelle (Kasai 1997; Marczynski & Wilhelm 2001; Halliwell & Gutteride 2007).

Untersuchungen an primären Ratteninselzellen und insulinproduzierenden RINm5F Gewebekulturzellen ergaben, dass sowohl IL-1β allein als auch der Zytokinmix massive oxidative DNA Schäden in Form von 8-oxoG hervorrufen. Darüber hinaus konnte mittels Immunfluoreszenzfärbung gezeigt werden, dass die zytokininduzierte 8-oxoG Bildung hauptsächlich in den Mitochondrien erfolgt. Die Spezifizität und die subzelluläre Lokalisation von 8-oxoG wurden zusätzlich durch Versuche mit Menadion, einer spezifisch in Mitochondrien Sauerstoffradikale generierenden Verbindung (Frei *et al.* 1986), bestätigt. Durch die mtDNA werden neben zwei ribosomalen RNAs und 22 tRNAs 13 Untereinheiten der Atmungskettenkomplexe I, III, IV sowie die ATP Synthase kodiert (Anderson *et al.* 1981; DiMauro & Schon 2003). Das mitochondriale Genom ist daher für die Synthese der

Atmungskettenkomplexe von entscheidender Bedeutung. Eine oxidative Schädigung der mtDNA, wie im Rahmen dieser Arbeit gezeigt wurde, ist somit mit einer unzureichenden *de novo* Synthese und Dysfunktion der Atmungskettenkomplexe assoziiert, was eine verringerte ATP- und vermehrte ROS-Produktion zu Folge haben kann.

Neben der mtDNA können auch mehrfach ungesättigte Phospholipide durch ROS geschädigt werden. Cardiolipin ist ein mitochondriales Phospholipid und stellt den Hauptbestandteil der inneren Mitochondrienmembran dar. Es verleiht ihr Fluidität und Stabilität und sorgt durch die Assemblierung der Atmungskettenkomplexe zum sog. Superkomplex für eine optimale Funktion der oxidativen Phosphorylierung (Ow *et al.* 2008; Paradies *et al.* 2009). Degradation des Cardiolipins und Änderungen seiner Zusammensetzung werden in Zusammenhang mit einer mitochondrialen Dysfunktion und in der Folge mit der Entstehung zahlreicher Krankheiten gebracht (Chicco & Sparagna 2007). Bedingt durch einen hohen Anteil an ungesättigten Fettsäuren ist Cardiolipin besonders anfällig für Schädigungen durch ROS. Daher wurde der Einfluss proinflammatorischer Zytokine auch auf eine mögliche Cardiolipinperoxidation als zusätzlicher Indikator für die ROS Generierung in β-Zellen untersucht. So wiesen primäre Ratteninselzellen und RINm5F Zellen nach der Exposition mit β-zelltoxischen Zytokinen im Vergleich zu den unbehandelten Zellen eine signifikant erhöhte Cardiolipinperoxidation auf. In MitoKatalase überexprimierenden Zellen konnte dagegen weder eine gesteigerte oxidative DNA Schädigung noch eine erhöhte Peroxidation von Cardiolipin festgestellt werden, so dass eine effiziente Detoxifizierung von H_2O_2 in Mitochondrien die Entstehung hochtoxischer $HO^{\bullet}$ verhindert und infolgedessen die zellulären Strukturen vor oxidativen Schäden schützt.

3.4 Die Bedeutung des mitochondrialen Apoptosesignalweges für die β-Zellzerstörung beim Typ 1 Diabetes mellitus

Die intrinsische Signaltransduktion und die Exekution der Apoptose hängen von der Aktivierung der Caspasenkaskade ab. An diesem Prozess sind Mitochondrien ganz wesentlich beteiligt. Sie enthalten eine Vielzahl proapoptotischer Faktoren, die nach dem Verlust der mitochondrialen Integrität freigesetzt werden und über die Initiatorcaspase-9 die zelltodfördernden Effektorcaspasen wie Caspase-3 aktivieren (Riedl & Salvesen 2007; Li & Yuan 2008). Die Freisetzung von Cytochrom c, ein lösliches und hämhaltiges Protein der Atmungskette, das die Elektronenübertragung zwischen dem Atmungskettenkomplex III und IV vermittelt, ist ausschlaggebend für die Aktivierung der Caspase-9. Nach der Freisetzung aus dem Intermembranraum in das Zytosol oligomerisiert das Cytochrom c ATP-abhängig mit dem zytosolischen Kofaktor Apaf-1 zum Apoptosom, welches anschließend die

Aktivierung von Caspase-9 durch die induzierte Nähe ermöglicht (Riedl & Salvesen 2007; Ow *et al.* 2008). Obwohl die mitochondriale Freisetzung von Cytochrom c als Schlüsselereignis bei der Apoptoseinitiierung angesehen wird, sind die zugrundeliegenden Mechanismen, die zu seiner Freisetzung führen, derzeit nicht eindeutig geklärt. Es gibt jedoch experimentelle Hinweise dafür, dass die Freisetzung von Cytochrom c als Folge der zellulären Schädigung in einem Zweistufenprozess erfolgt: (1) die Dissoziation von der inneren und (2) die Translokation durch die äußere Mitochondrienmembran. Cytochrom c ist unter physiologischen Bedingungen durch elektrostatische und hydrophobe Wechselwirkungen mit Cardiolipin an der inneren Mitochondrienmembran verankert (Ow *et al.* 2008). Erst wenn Cardiolipin z.B. durch ROS peroxidiert wird, verliert es seine Affinität gegenüber Cytochrom c, das dann durch die von Bax permeablisierte äußere Mitochondrienmembran ins Zytosol gelangt (Ott *et al.* 2002). Weitere Hinweise auf den Zweistufenprozess als Mechanismus der Cytochrom c Freisetzung finden sich in Untersuchungen an neuronalen Mitochondrien. Hier konnte gezeigt werden, dass rekombinantes, oligomerisiertes Bax Protein allein lediglich 18 %, jedoch zusammen mit Komplex I Inhibitoren, welche die ROS Bildung in den Mitochondrien stimulieren, mehr als 65 % der Cytochrom c Freisetzung triggert (Ott *et al.* 2007). Dies lässt den Schluss zu, dass die Permeabilisierung der äußeren Mitochondrienmembran allein nicht ausreicht, um Cytochrom c in großen Mengen freizusetzen. Vielmehr muss zuerst die bestehende Interaktion zwischen Cytochrom c und dem Cardiolipin durch ROS-mediierte Schäden aufgehoben werden.

Aufgrund der Tatsache, dass die β-zelltoxischen Zytokine die Bax/Bcl-2 Ratio zugunsten des Bax Proteins verschieben und extreme oxidative Schäden an mitochondrialen Strukturen verursachen, wurde die subzellulare Distribution von caspaseaktivierenden Faktoren wie Cytochrom c und nachfolgender Aktivierung der Caspasenkaskade nach einer Zytokin-exposition untersucht. Eine 24stündige Inkubation der insulinproduzierenden RINm5F Zellen mit β-zelltoxischen Zytokinen führte im Vergleich zu den unbehandelten Kontrollzellen zu einer vermehrten Cytochrom c Freisetzung aus dem Intermembranraum der Mitochondrien in das Zytosol. Dabei waren die Wirkungen des Zytokinmix, bedingt durch synergistische Effekte, deutlich stärker. Diese Beobachtung steht im Einklang mit einer kürzlich an humanen Inselzellen durchgeführten Studie, in der die Inkubation der isolierten Inseln mit einem Zytokinmix auch mit einer gesteigerten Cytochrom c Freisetzung einherging (Grunnet *et al.* 2009). Darüber hinaus wurde der Ausstrom des mitochondrialen SMAC/DIABLO Proteins unter den gleichen Inkubationsbedingungen erfasst. Dieses Protein neutralisiert die hemmende Wirkung von IAP (*inhibitor of apoptosis protein*) auf Caspasen und wirkt somit

synergistisch mit dem freigesetzten Cytochrom c (Kroemer *et al.* 2007). Eine Inkubation von β-Zellen mit Zytokinen führte nicht nur zum Ausstrom von Cytochrom c, sondern auch zur Translokation des SMAC/DIABLO Proteins in das Zytosol. Die hieraus resultierende Konsequenz ist die unmittelbare Caspase-9 Aktivierung, die wiederum die nachgeschaltete Effektorcaspase-3 proteolytisch aktiviert und so letztlich den apoptotischen Zelltod einleitet. Tatsächlich konnte im Rahmen dieser Arbeit ein signifikanter Anstieg der Initiatorcaspase-9 und Effektorcaspase-3 Aktivität nach Zytokininkubation der RINm5F Zellen festgestellt werden. Im Gegensatz dazu verhinderte die Überexpression der Katalase in Mitochondrien den zytokinvermittelten Ausstrom von Cytochrom c und SMAC/DIABLO sowie die Aktivierung der Caspase-9 und -3. Diese Ergebnisse zeigen, dass Mitochondrien die Zielorganellen für die schädigenden Effekte β-zelltoxischer Zytokine sind und dass der mitochondriale Apoptosesignalweg für die zytokinvermittelte β-Zellapoptose entscheidend ist.

Neben der bereits beschriebenen Cytochrom c abhängigen Caspase-9 Aktivierung wurde kürzlich postuliert, dass Caspase-9 direkt durch Caspase-12 aktiviert werden kann (Morishima *et al.* 2002). Dabei soll die Caspase-9 Aktivierung unabhängig von der mitochondrialen Apoptosesignalkaskade erfolgen. Caspase-12 ist spezifisch mit dem ER assoziiert und wird als Initiatorcaspase bei dem ER-Stress vermittelten Apoptosesignalweg angesehen. ER-Stress induzierende Stimuli wie Tunicamycin, Thapsigargin (Nakagawa *et al.* 2000) und proinflammatorische Zytokine verursachen die Aktivierung der Caspase-12 in unterschiedlichen Zellsystemen, einschließlich β-Zellen (Contreras *et al.* 2003; Gurgul-Convey & Lenzen 2010). Um eine mögliche Interaktion zwischen der ER-spezifischen Caspase-12 und mitochondrialen Caspase-9 sowie ihre Bedeutung für den zytokinvermittelten β-Zelltod aufzuklären, wurde die Wirkung des spezifischen Caspase-12 Inhibitors Z-ATAD-FMK an RINm5F Zellen untersucht. Die Behandlung von β-Zellen mit diesem Caspase-12 spezifischen Inhibitor führte im Vergleich zu den ohne Inhibitor inkubierten Zellen zu einer signifikant erniedrigten Caspase-12 Aktivität. Dabei hatte die Caspase-12 Inhibierung keinen Einfluss auf die Caspase-9 Aktivierung oder die Zellvitalität nach einer Zytokininkubation. Somit erfolgt die zytokinvermittelte Aktivierung der Caspase-9 und infolgedessen die β-Zellzerstörung unabhängig von der ER-spezifischen Caspase-12. Hierfür spricht auch die Beobachtung, dass die MitoKatalase überexprimierenden Zellen eine mit Kontrollzellen vergleichbare Aktivierung der ER-spezifischen Caspase-12 nach Zytokininkubation aufwiesen, wohingegen die Caspase-9 Aktivierung und damit die β-Zell-zerstörung durch die MitoKatalase erfolgreich verhindert werden konnte. Die genaue zelluläre

Funktion der durch ER-Stress aktivierten Caspase-12 ist jedoch noch weitestgehend ungeklärt. Ihre Auswirkungen auf Apoptoseinduktion scheinen dabei stark zellspezifisch zu sein und sind noch Gegenstand der wissenschaftlichen Diskussion (Lamkanfi *et al.* 2004).

Diese Ergebnisse unterstreichen, dass die Generierung von ROS in den Mitochondrien der β-Zellen ein entscheidendes Element der zytokinvermittelten Toxizität beim Typ 1 Diabetes mellitus ist. Des Weiteren zeigte sich, dass ROS-mediierte intramitochondriale Schäden und die damit verbundene Initiierung des mitochondrialen Apoptosesignalweges für den β-Zelltod ausschlaggebend sind. Daher muss die Bildung von ROS und die daraus resultierende β-Zellschädigung in möglichen Präventionsstrategien des Typ 1 Diabetes mellitus verhindert werden. Dabei könnte die Detoxifizierung der intrazellulär gebildeten ROS durch H_2O_2-inaktivierende Enzyme, die Inhibierung spezifischer an der β-Zellzerstörung beteiligter Signaltransduktionswege oder die Antagonisierung sezernierter proinflammatorischer Zytokine einen möglichen Ansatzpunkt zur Verhinderung der β-Zellzerstörung darstellen. Eine umfassende Protektion ist allerdings nur durch die Verhinderung der Immunzelleninfiltration und der damit assoziierten Sekretion proinflammatorischer Zytokine denkbar. Diese Primärprävention könnte durch eine kombinierte Gabe immunsuppressiver und immunmodulierender Substanzen erreicht werden. (Boettler & von Herrath 2010; Jörns *et al.* 2010).

4 Zusammenfassung

Der Typ 1 Diabetes mellitus ist eine komplexe, multifaktorielle Autoimmunerkrankung, die durch selektive Zerstörung der insulinproduzierenden β-Zellen des Pankreas hervorgerufen wird. Die Folge ist ein absoluter Insulinmangel, was eine lebenslange Substitution des Hormons Insulin erforderlich macht. Die Zerstörung wird insbesondere durch sezernierte zytotoxische Zytokine mediiert, die vielfältige intrazelluläre Signalkaskaden initiieren, an deren Ende der apoptotische β-Zelltod steht. Dabei kommt den durch infiltrierende Immunzellen freigesetzten und infolge der Zytokinwirkung intrazellulär gebildeten reaktiven Sauerstoffspezies (ROS) eine besondere Bedeutung zu. β-Zellen weisen eine ungewöhnlich niedrige Kapazität an antioxidativen Schutzmechanismen zur Detoxifikation von ROS auf. Insbesondere die H_2O_2-inaktivierenden Enzyme Katalase und Glutathionperoxidase werden in den β-Zellen nur in geringem Maße exprimiert, was eine Akkumulation von H_2O_2 begünstigt. Der intrazelluläre Ursprung der zytokinvermittelten ROS Generierung und der direkte kausale Zusammenhang mit der β-Zellzerstörung ist noch weitestgehend unklar. Daher war das Ziel der vorliegenden Arbeit, die Beteiligung von ROS an der durch proinflammatorische Zytokine vermittelten β-Zellapoptose, an primären Ratteninselzellen und insulin-produzierenden RINm5F Gewebekulturzellen, die als Modell der pankreatischen β-Zellen fest etabliert sind, aufzuklären. Dabei wurde in Expositionsversuchen mit IL-1β allein oder mit einer Zytokinkombination bestehend aus IL-1β, TNF-α und IFN-γ die Auswirkung eines erhöhten antioxidativen Abwehrstatus auf zelluläre Ereignisse untersucht.

Im Rahmen dieser Arbeit konnte gezeigt werden, dass in β-Zellen ein Ungleichgewicht zwischen dem antiapoptotisch wirkenden Protein Bcl-2 und dem proapoptotisch wirkenden Protein Bax herrscht. Durch die zytokinvermittelte Reduktion der Bcl-2 Expression bei gleichbleibender Expression von Bax verstärkte sich zusätzlich die bestehende Bax/Bcl-2 Dysbalance zugunsten des proapoptotischen Proteins Bax, so dass die Permeabilisierung der äußeren Mitochondrienmembran weiter begünstigt wird. Die stabile Überexpression von Katalase in Mitochondrien führte hingegen zu einer erhöhten basalen Expression des Bcl-2 Proteins und zu einer erniedrigten Expression des Bax Proteins. Des Weiteren konnte in diesen Zellen die zytokinvermittelte Reduktion von Bcl-2 verhindert werden. Dies lässt den Schluss zu, dass initial in den Mitochondrien gebildetes H_2O_2 u.a. die Expression von Bcl-2 supprimiert. Um die infolge der Zytokinwirkung gebildeten ROS zu quantifizieren, wurde der H_2O_2-sensitive Fluoreszenzsensor HyPer spezifisch im zytosolischen und mitochondrialen Kompartiment überexprimiert. So konnte gezeigt werden, dass die verwendeten Zytokine

lediglich in Mitochondrien eine signifikant gesteigerte H_2O_2 Bildung bewirken und dass dessen Akkumulation durch die Überexpression der mitochondrial lokalisierten Katalase vollständig verhindert wird. Mitochondrien sind nicht nur die Hauptquelle intrazellulärer ROS, sondern stellen auch gleichzeitig das Hauptziel für eine ROS mediierte Schädigung dar. Das in Mitochondrien akkumulierte H_2O_2 kann in Anwesenheit von Metallionen (Fe^{2+} oder Cu^{+}) rasch zum außerordentlich aggressiven Hydroxylradikal reagieren, das direkte Oxidationsschäden an Makromolekülen verursachen kann. So konnte gezeigt werden, dass eine Inkubation der Zellen mit β-zelltoxischen Zytokinen zu massiven oxidativen DNA Schäden führt, die in Form von 8-Oxoguanin spezifisch in den Mitochondrien identifiziert werden konnten. Zudem wurde eine erhöhte Peroxidation von Cardiolipin festgestellt. Als Folge der Schädigung mitochondrialer Strukturen und der Verschiebung der Bax/Bcl-2 Ratio zugunsten des proapoptotisch wirkenden Proteins Bax konnte die Freisetzung von Cytochrom c und SMAC/DIABLO aus den Mitochondrien in das Zytosol nachgewiesen werden. Auch die Aktivierung von Caspase-9 und der nachgeschalteten Effektorcaspase-3, die endgültig die Exekution der Apoptose einleitet, konnte unter dem Einfluss β-zelltoxischer Zytokine beobachtet werden. Die Detoxifizierung von H_2O_2 durch Katalase in den Mitochondrien verhinderte die oxidative Schädigung von zellulären Strukturen und damit die Destabilisierung der mitochondrialen Integrität sowie die Aktivierung von Caspase-9 und -3. Eine erhöhte Vitalität und Überlebensrate von β-Zellen konnte zudem durch eine selektive Überexpression der NF-κB Untereinheit c-Rel erzielt werden. Im Gegensatz zu der sonst in β-Zellen beobachteten proapoptotischen Wirkung des NF-κB Signalweges führte c-Rel zu einer gesteigerten Expression der antiapoptotisch wirkenden Proteine Bcl-X_L und c-IAP2, was die proapoptotischen Effekte von NF-κB unterdrückte. Darüber hinaus konnte eine Beteiligung der ER-spezifischen Caspase-12 im zytokinvermittelten β-Zelltod sowie eine Interaktion mit der Caspase-9 ausgeschlossen werden.

Diese Ergebnisse belegen, dass die intramitochondriale Bildung von ROS, speziell die Bildung des Hydroxylradikals, für den zytokinvermittelten β-Zelltod entscheidend ist. Daher scheint die Initiierung des mitochondrialen Apoptosesignalweges für die Apoptoseexekution von zentraler Bedeutung zu sein. Diese Befunde eröffnen weitere Möglichkeiten zum Verständnis der zytokinvermittelten β-Zelltoxizität, die zur Autoimmunzerstörung im Typ 1 Diabetes mellitus führen.

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6 Publikationen

Teilergebnisse der vorliegenden Dissertation sind bereits veröffentlicht.

Kurzbeiträge:

Mehmeti I., Lortz S., Lenzen S. (2007) Der Einfluss antioxidativer Enzyme auf die Expression von Proteinen der Bcl-2 Familie und ihre Modulation durch proinflammatorische Zytokine in insulinproduzierenden Zellen. *Diabetologie und Stoffwechsel* **16,** Suppl. 1, S50, P131, 42. Jahrestagung der Deutschen Diabetes-Gesellschaft, Hamburg, 2007

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Veröffentlichungen:

Mokhtari D., Barbu A., Mehmeti I., Vercamer C., Welsh N. (2009) Overexpression of the Nuclear Factor-{kappa}B subunit c-Rel protects against human islet cell death in vitro. *Am J Physiol Endocrinol Metab*. 2009 Aug 25. E1067–E1077.

Mokhtari D., Kerblom B., Mehmeti I., Wang X., Funa NS., Olerud J., Lenzen S., Welsh N., Welsh M. (2009) Increased Hsp70 expression attenuates cytokine-induced cell death in islets of Langerhans from Shb knockout mice. *Biochem Biophys Res Commun*. 2009 Sep 25; 387 (3): 553-7.

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Gurgul-Convey E., Mehmeti I., Lortz S., Lenzen S. (2011) Cytokine toxicity in insulin-producing cells is mediated by nitrooxidative stress-induced hydroxyl radical formation in mitochondria. *J Mol Medicine* (Im Druck)

Mehmeti I., Gurgul-Convey E., Lenzen S., Lortz S. (2011) Induction of the intrinsic apoptosis pathway in insulin-secreting cells is dependent on oxidative damage of mitochondria but independent of caspase-12 activation. BBA - *Mol Cell Research* (eingereicht)

7 Curriculum Vitae

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Studium:

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Promotion:

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8 Erklärung

Hierdurch erkläre ich, dass die Dissertation

Die Bedeutung von freien Radikalen für die Toxizität proinflammatorischer Zytokine und die Apoptoseinduktion in insulinproduzierenden Zellen

selbstständig verfasst und alle benutzten Hilfsmittel sowie evtl. zur Hilfeleistung herangezogene Institutionen vollständig angegeben wurden.

Die Dissertation wurde nicht schon als Diplom- oder ähnliche Prüfungsarbeit verwendet.

Hannover, den 21.03.2011

Ilir Mehmeti

Die vorliegende Dissertation wurde im Juli 2006 unter Anleitung von Herrn Professor Dr. S. Lenzen am Institut für Klinische Biochemie der Medizinischen Hochschule Hannover begonnen und im März 2011 abgeschlossen

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