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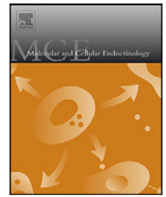

Modulation of Bcl-2-related protein expression in pancreatic beta cells by pro-inflammatory cytokines and its dependence on the antioxidative defense status

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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.

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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,

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