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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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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 through activation of caspase-9 primarily by hydroxyl radical-mediated mitochondrial damage.

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 stress-induced intrinsic pathways [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

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; iNOS, inducible nitric oxide synthase; 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

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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 and characterized for insulin expression, content, and secretion as described before [22,23]. The expression of catalase was analyzed by immunoblotting analysis or enzyme activity measurement [23].

2.2. Rat islet isolation and single cell preparation

Pancreatic islets were isolated from 250 to 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. This cornea-derived ECM not only improves islet cell attachment and formation of a cellular mono-layer, but also maintains glucose-induced insulin secretion to a higher extent than conventionally coated plastic surfaces [24]. 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 1×10^6 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) or 5 mM Nω-nitro-L-arginine (Sigma-Aldrich, Munich, Germany). After 24 h incubation the cells were trypsinized and collected by centrifugation at 700×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. Quantitative RT-PCR (qRT-PCR)

Isolation of RNA, reverse transcription and the qRT-PCR reaction were performed as described in detail earlier [7]. The primer sequences for CHOP were as follows: forward, CCAGCAGAGGTCACAAGCAC, and reverse, CGCACTGACCACTCTGTTTC. Data are expressed as relative gene expression after normalization to the beta actin housekeeping gene using the Qgene96 and LineRegPCR software.

2.6. 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 the 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-Aldrich, Munich, Germany) [25]. The addition of the caspase-12 inhibitor Z-ATAD-FMK to the culture medium had no influence on cell viability under control conditions (absolute optical density $(OD)_{550}$ absorbance rates of the MTT assay were 0.99 ± 0.06 (RINm5F cells without Z-ATAD-FMK, $n=4$) and 0.95 ± 0.06 (RINm5F cells with Z-ATAD-FMK, $n=4$)).

2.7. 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 [26]. Control and MitoCatalase overexpressing RINm5F cells were seeded at a density of 7×10^5 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×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).

2.8. Analysis of H_2O_2 generation using the H_2O_2 sensitive HyPer protein

RINm5F cells overexpressing the H_2O_2 sensitive fluorescence protein HyPer in peroxisomes (HyPer-Peroxi) [27] were seeded at a density of 25×10^4 cells per well of a black 96-well-plate and allowed

to attach for 24 h. Thereafter the cells were exposed to the indicated cytokines or palmitic acid (100 μM) for 24 h. The fluorescence ratio (504/520 nm to 427/520 nm), which is an indicator of H_2O_2 production, was measured spectrofluorometrically as recently published [27,28].

2.9. 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 5×10^5 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×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).

2.10. 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 [29,30]. Control and MitoCatalase overexpressing RINm5F cells were seeded at a density of 7×10^5 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×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).

2.11. 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 5×10^4 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-Aldrich, Munich, Germany). 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).

2.12. 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).

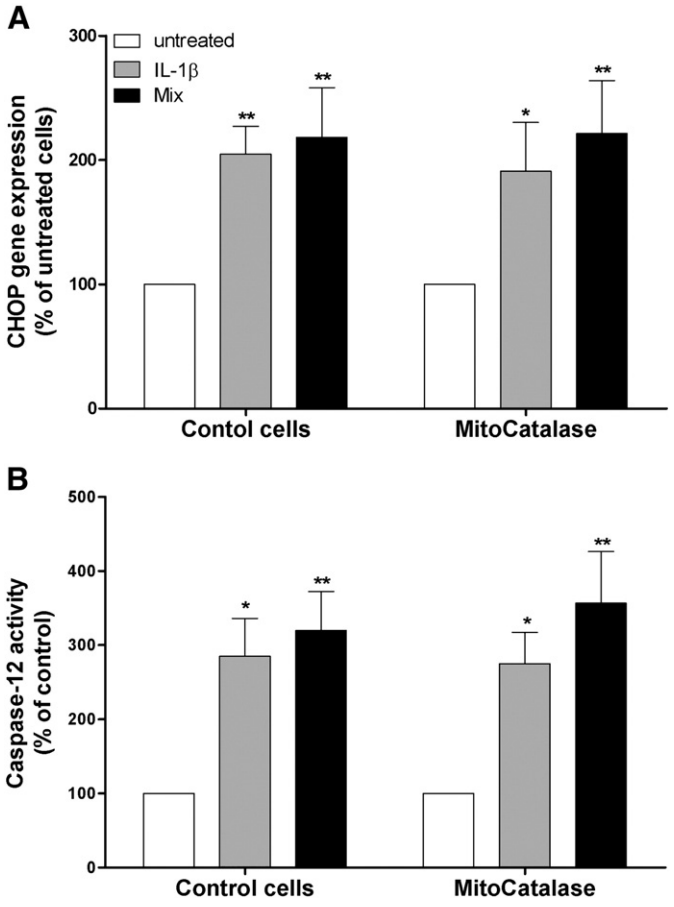

Fig. 1. Effects of pro-inflammatory cytokines on CHOP gene expression and caspase-12 activation in insulin-producing RINm5F control cells and cells overexpressing MitoCatalase. RINm5F control cells (mock transfected) and cells overexpressing catalase in mitochondria (MitoCatalase) were incubated under control conditions (open bars), with 600 U/ml IL-1β (gray bars) or with a cytokine mixture consisting of 60 U/ml IL-1β, 185 U/ml TNF-α, 14 U/ml IFN-γ (black bars) for 24 h. (A) Total RNA was isolated, qRT-PCR was performed with a CHOP specific primer set, and the relative expression levels were normalized to the housekeeping gene β-actin. The CHOP gene expression in each cell clone under control conditions was set as 100%. (B) 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).

3. Results

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

In order to investigate the induction of ER-stress in response to pro-inflammatory cytokines the expression of CHOP, a key regulator of the ER stress response, was analyzed by qRT-PCR in insulin-producing RINm5F control cells and cells overexpressing the H_2O_2 detoxifying enzyme catalase in mitochondria (MitoCatalase). A 24 h exposure to IL-1β alone or the cytokine mixture consisting of IL-1β, TNF-α, and IFN-γ resulted in a significant increase of CHOP gene expression in RINm5F control and MitoCatalase overexpressing cells (control cells: IL-1β 204%, $p<0.01$ and cytokine mixture 218%, $p<0.01$ compared with untreated cells; MitoCatalase cells: IL-1β 191%, $p<0.05$ and cytokine mixture 221%, $p<0.01$ compared with untreated cells) (Fig. 1A). Subsequently, the activation of the ER-specific caspase-12 was evaluated after the same cytokine treatments. Exposure to IL-1β or the cytokine mixture caused 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. 1B). Inhibition of cytokine-induced NO production by using the iNOS inhibitor Nω-nitro-L-arginine resulted in a significant reduction of caspase-12 activation (IL-1β −56%, $p<0.05$ and cytokine mixture −59%, $p<0.05$ compared with cells incubated without iNOS inhibitor), indicating that ER-stress and caspase-12 activation is strongly related to NO formation (Suppl. Fig. 1). Thus, the activation of ER-specific caspase-12 is caused by cytokine-mediated ER stress and it is not influenced by the overexpression of the H_2O_2 detoxifying enzyme catalase in mitochondria.

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 and only partially in presence of the iNOS inhibitor (Suppl. Fig. 1), suggesting that pro-caspase-9 is not a downstream target of caspase-12 and that caspase-9 activity is only partially modulated by NO (IL-1β −9% and cytokine mixture −39% compared with cells incubated without iNOS inhibitor) 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. Importantly, the findings from the RINm5F cell lines could be confirmed in primary rat islet cells (Fig. 4).

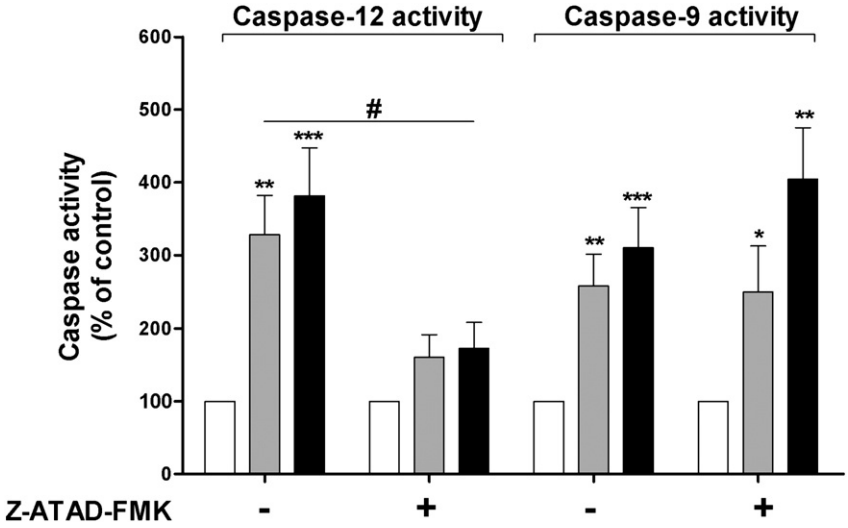

Fig. 2. Effects of the caspase-12 specific inhibitor Z-ATAD-FMK on cytokine-induced caspase-9 activation in insulin-producing RINm5F cells. RINm5F cells were incubated under control conditions (open bars), with 600 U/ml IL-1β (gray bars) or with a cytokine mixture consisting of 60 U/ml IL-1β, 185 U/ml TNF-α, 14 U/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 FITC-LEDH-FMK and the intracellular fluorescence intensity was measured by flow cytometry. Data are expressed as means ± SEM of four independent experiments; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ 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).

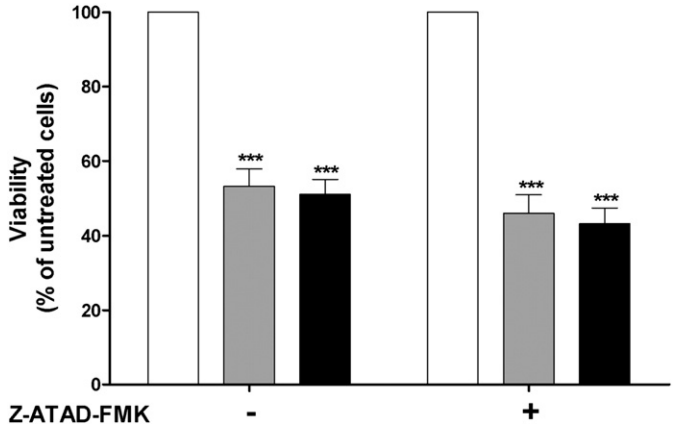

Fig. 3. Effects of the caspase-12 specific inhibitor Z-ATAD-FMK on cytokine toxicity in insulin-producing RINm5F cells. RINm5F cells were incubated under control conditions (open bars), with 600 U/ml IL-1β (gray bars) or with a cytokine mixture consisting of 60 U/ml IL-1β, 185 U/ml TNF-α, 14 U/ml IFN-γ (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).

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).

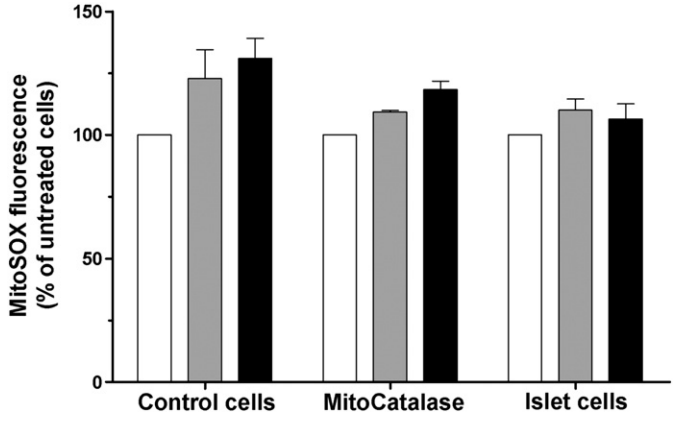

Fig. 4. Effects of pro-inflammatory cytokines on intramitochondrial superoxide radical ($O_2\cdot^-$) production in insulin-producing RINm5F control or MitoCatalase overexpressing cells and rat islet cells. RINm5F control cells or cells overexpressing catalase in mitochondria (MitoCatalase) and rat islet cells were incubated under control conditions (open bars), with 600 U/ml IL-1β (gray bars) or with a cytokine mixture consisting of 60 U/ml IL-1β, 185 U/ml TNF-α, 14 U/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.

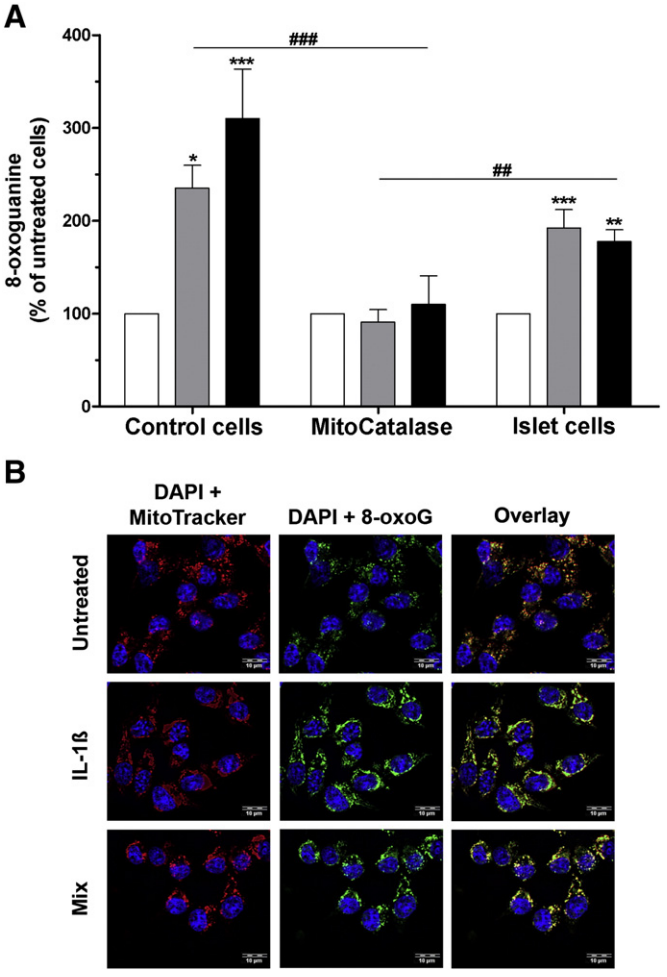

Fig. 5. Effects of pro-inflammatory cytokines on 8-oxoguanine formation in insulin-producing RINm5F control or MitoCatalase overexpressing cells and rat islet cells. (A) RINm5F control cells or cells overexpressing catalase in mitochondria (MitoCatalase) and rat islet cells were incubated under control conditions (open bars), with 600 U/ml IL-1β (gray bars) or with a cytokine mixture consisting of 60 U/ml IL-1β, 185 U/ml TNF-α, 14 U/ml IFN-γ (black bars) for 72 h. After fixation and permeabilization, 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; ##, $p<0.01$; ###, $p<0.001$ 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 panels) compared to the untreated cells (upper panel).

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.

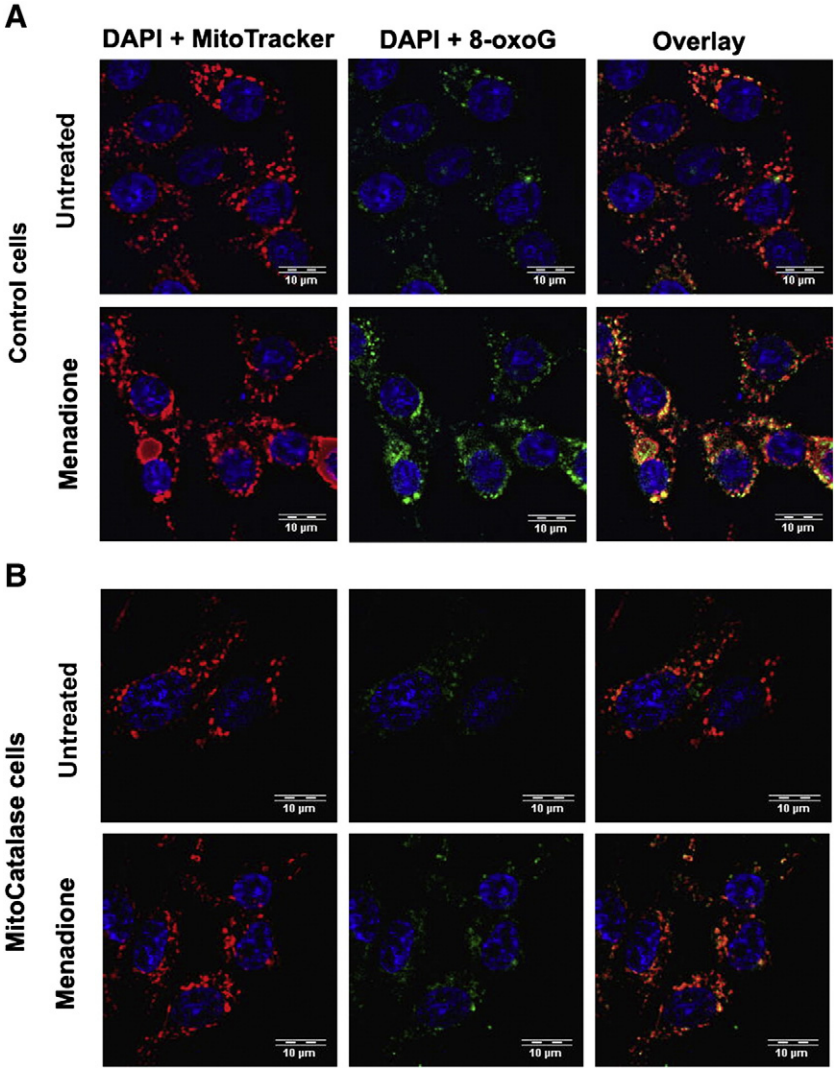

Fig. 6. Subcellular localization of 8-oxoguanine (8-oxoG) in insulin-producing RINm5F control and MitoCatalase overexpressing cells after incubation with menadione. RINm5F control cells (A) and MitoCatalase overexpressing cells (B) were incubated for 2 h under control conditions or with the intramitochondrial 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).

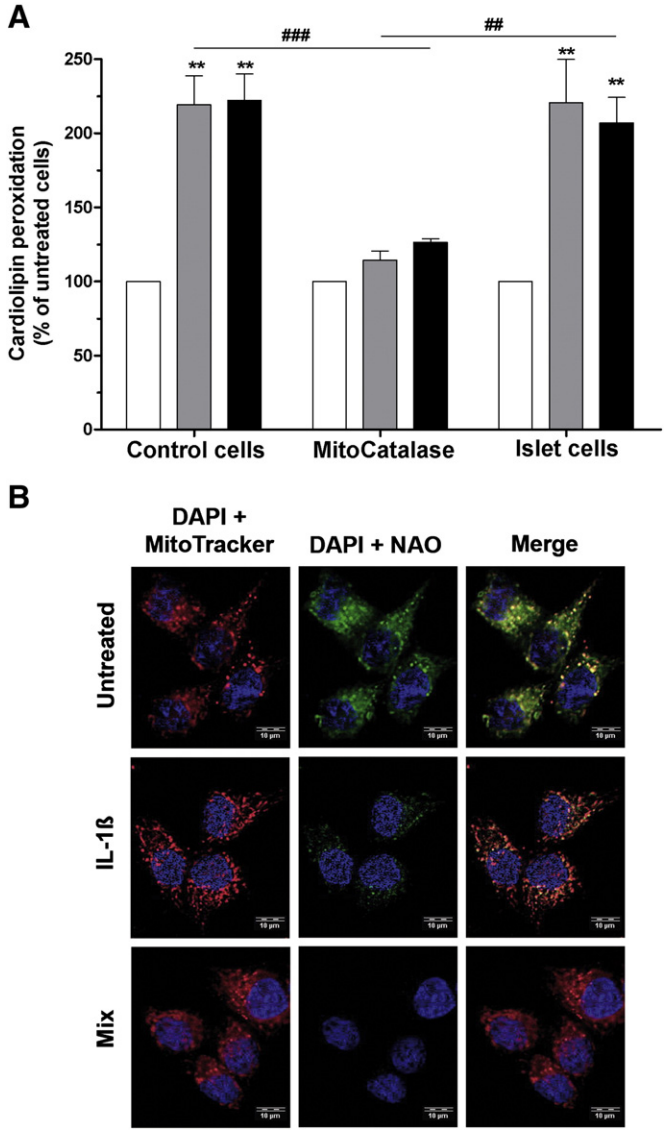

Fig. 7. Effects of pro-inflammatory cytokines on cardiolipin peroxidation in insulin-producing RINm5F control or MitoCatalase overexpressing cells and rat islet cells. (A) RINm5F control cells or cells overexpressing catalase in mitochondria (MitoCatalase) and rat islet cells were incubated under control conditions (open bars), with 600 U/ml IL-1β (gray bars) or with a cytokine mixture consisting of 60 U/ml IL-1β, 185 U/ml TNF-α, 14 U/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.

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 panels).

4. Discussion

In the complex autoimmune processes of the pathogenesis of type 1 diabetes, pro-inflammatory cytokines are important humoral mediators for beta cell apoptosis [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 not only insulin-producing RINm5F control cells but also of cells overexpressing mitochondrially located catalase (MitoCatalase) to beta cell toxic cytokines strongly induced ER-stress as shown by increased CHOP gene expression and 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 [31–33]. 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 [16,34,35]. 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 pro-inflammatory cytokines significantly induced CHOP gene expression and the activation of caspase-12 in insulin-producing cells. Inhibition of iNOS activity by an iNOS inhibitor significantly attenuated the cytokine-mediated activation of caspase-12 and also the induction of CHOP as recently shown [36]. This observation indicates that ER stress induced by beta cell toxic cytokines is mainly mediated by iNOS induction and NO formation as described in earlier studies [36–38]. However, inhibition of caspase-12 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. However, caspase-9 activation and consequently beta cell death was significantly reduced in this cell clone, suggesting that cytokine-mediated activation of caspase-9 is crucial for the activation of the intrinsic apoptosis

pathway in insulin-producing cells [7,23,28]. By contrast, 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 [39]. 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 [40]. 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 [5,41]. 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 [42].

Indeed, neither in insulin-producing RINm5F cells nor in rat islet cells a significantly increased mitochondrial $O_2\cdot^-$ 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 is an increased mitochondrial H_2O_2 concentration. As recently shown, cytokine treatment of RINm5F cells overexpressing the hydrogen peroxide sensitive fluorescence protein HyPer in different cellular compartments resulted in an elevated H_2O_2 production exclusively within the mitochondria [28]. Importantly, in contrast to long-chain non-esterified fatty acids [27] cytokines did not induce H_2O_2 generation in peroxisomes (Suppl. Fig. 2). This mitochondrially accumulated H_2O_2 provides the basis for the rapid generation of highly toxic hydroxyl radicals via the Haber-Weiss or Fenton reaction [43] in mitochondria in response to cytokines. 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, independent of ER-specific caspase-12 activation, is sufficient to induce beta cell apoptosis. 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.

Supplementary materials related to this article can be found online at doi:10.1016/j.bbamcr.2011.06.022.

Disclosures

The authors have no conflicts of interest to declare.

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References

[1] D.L. Eizirik, T. Mandrup-Poulsen, A choice of death—the signal-transduction of immune-mediated beta-cell apoptosis, Diabetologia 44 (2001) 2115–2133.
[2] K.A. Kim, M.S. Lee, Recent progress in research on beta-cell apoptosis by cytokines, Front. Biosci. 14 (2009) 657–664.
[3] T. Mandrup-Poulsen, Beta cell death and protection, Ann. N. Y. Acad. Sci. 1005 (2003) 32–42.
[4] P. Pirot, A.K. Cardozo, D.L. Eizirik, Mediators and mechanisms of pancreatic beta-cell death in type 1 diabetes, Arq. Bras. Endocrinol. Metabol. 52 (2008) 156–165.
[5] S. Lenzen, Oxidative stress: the vulnerable beta-cell, Biochem. Soc. Trans. 36 (2008) 343–347.
[6] D.L. Eizirik, A.K. Cardozo, M. Cnop, The role for endoplasmic reticulum stress in diabetes mellitus, Endocr. Rev. 29 (2008) 42–61.
[7] I. Mehmeti, S. Lenzen, S. Lortz, Modulation of Bcl-2-related protein expression in pancreatic beta cells by pro-inflammatory cytokines and its dependence on the antioxidative defense status, Mol. Cell. Endocrinol. 332 (2011) 88–96.
[8] H. Hui, F. Dotta, U. Di Mario, R. Perfetti, Role of caspases in the regulation of apoptotic pancreatic islet beta-cells death, J. Cell. Physiol. 200 (2004) 177–200.
[9] J. Li, J. Yuan, Caspases in apoptosis and beyond, Oncogene 27 (2008) 6194–6206.
[10] S.E. Logue, S.J. Martin, Caspase activation cascades in apoptosis, Biochem. Soc. Trans. 36 (2008) 1–9.
[11] S.J. Riedl, Y. Shi, Molecular mechanisms of caspase regulation during apoptosis, Nat. Rev. Mol. Cell Biol. 5 (2004) 897–907.
[12] G. Kroemer, L. Galluzzi, C. Brenner, Mitochondrial membrane permeabilization in cell death, Physiol. Rev. 87 (2007) 99–163.
[13] N. Morishima, K. Nakanishi, H. Takenouchi, T. Shibata, Y. Yasuhiko, An endoplasmic reticulum stress-specific caspase cascade in apoptosis. Cytochrome c-independent activation of caspase-9 by caspase-12, J. Biol. Chem. 277 (2002) 34287–34294.
[14] M. Lamkanfi, M. Kalai, P. Vandenabeele, Caspase-12: an overview, Cell Death Differ. 11 (2004) 365–368.
[15] T. Momoi, Caspases involved in ER stress-mediated cell death, J. Chem. Neuroanat. 28 (2004) 101–105.
[16] T. Nakagawa, H. Zhu, N. Morishima, E. Li, J. Xu, B.A. Yankner, J. Yuan, Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta, Nature 403 (2000) 98–103.
[17] J.L. Contreras, C.A. Smyth, G. Bilbao, C. Eckstein, C.J. Young, J.A. Thompson, D.T. Curiel, D.E. Eckhoff, Coupling endoplasmic reticulum stress to cell death program in isolated human pancreatic islets: effects of gene transfer of Bcl-2, Transpl. Int. 16 (2003) 537–542.
[18] E. Gurgul-Convey, S. Lenzen, Protection against cytokine toxicity through endoplasmic reticulum and mitochondrial stress prevention by prostacyclin synthase overexpression in insulin-producing cells, J. Biol. Chem. 285 (2010) 11121–11128.
[19] M. Kalai, M. Lamkanfi, G. Denecker, M. Boogmans, S. Lippens, A. Meeus, W. Declercq, P. Vandenabeele, Regulation of the expression and processing of caspase-12, J. Cell Biol. 162 (2003) 457–467.
[20] S. Lortz, M. Tiedge, T. Nachtwey, A.E. Karlsen, J. Nerup, S. Lenzen, Protection of insulin-producing RINm5F cells against cytokine-mediated toxicity through overexpression of antioxidant enzymes, Diabetes 49 (2000) 1123–1130.
[21] M. Tiedge, S. Lortz, J. Drinkgern, S. Lenzen, Relation between antioxidant enzyme gene expression and antioxidative defense status of insulin-producing cells, Diabetes 46 (1997) 1733–1742.
[22] W. Gehrmann, M. Elsner, S. Lenzen, Role of metabolically generated reactive oxygen species for lipotoxicity in pancreatic beta-cells, Diabetes Obes. Metab. 12 (Suppl 2) (2010) 149–158.
[23] E. Gurgul, S. Lortz, M. Tiedge, A. Jörns, S. Lenzen, Mitochondrial catalase overexpression protects insulin-producing cells against toxicity of reactive oxygen species and proinflammatory cytokines, Diabetes 53 (2004) 2271–2280.
[24] N. Kaiser, A.P. Corcos, I. Sarel, E. Cerasi, Monolayer culture of adult rat pancreatic islets on extracellular matrix: modulation of B-cell function by chronic exposure to high glucose, Endocrinology 129 (1991) 2067–2076.
[25] T. Mosmann, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, J. Immunol. Methods 65 (1983) 55–63.
[26] K.M. Robinson, M.S. Janes, M. Pehar, J.S. Monette, M.F. Ross, T.M. Hagen, M.P. Murphy, J.S. Beckman, Selective fluorescent imaging of superoxide in vivo using ethidium-based probes, Proc. Natl. Acad. Sci. U.S.A. 103 (2006) 15038–15043.
[27] M. Elsner, W. Gehrmann, S. Lenzen, Peroxisome-generated hydrogen peroxide as important mediator of lipotoxicity in insulin-producing cells, Diabetes 60 (2011) 200–208.
[28] E. Gurgul-Convey, I. Mehmeti, S. Lortz, S. Lenzen, Cytokine toxicity in insulin-producing cells is mediated by nitro-oxidative stress-induced hydroxyl radical formation in mitochondria, J. Mol. Med. 89 (2011) 785–798.
[29] M.E. Rodriguez, K. Azizuddin, P. Zhang, S.M. Chiu, M. Lam, M.E. Kenney, C. Burda, N.L. Oleinick, Targeting of mitochondria by 10-N-alkyl acridine orange analogues:

role of alkyl chain length in determining cellular uptake and localization, Mitochondrion 8 (2008) 237–246.
[30] V. Umansky, M. Rocha, R. Breitkreutz, S. Hehner, M. Bucur, N. Erbe, W. Droge, A. Ushmorov, Glutathione is a factor of resistance of Jurkat leukemia cells to nitric oxide-mediated apoptosis, J. Cell. Biochem. 78 (2000) 578–587.
[31] S.G. Fonseca, F. Urano, M. Burcin, J. Gromada, Stress hypERactivation in the beta-cell, Islets 2 (2010) 1–9.
[32] X. Lei, S. Zhang, B. Emani, S.E. Barbour, S. Ramanadham, A link between endoplasmic reticulum stress-induced beta-cell apoptosis and the group VIA $Ca2^{+}$-independent phospholipase A2 (iPLA2beta), Diabetes Obes. Metab. 12 (Suppl 2) (2010) 93–98.
[33] D. Ron, P. Walter, Signal integration in the endoplasmic reticulum unfolded protein response, Nat. Rev. Mol. Cell Biol. 8 (2007) 519–529.
[34] G. Bajaj, R.K. Sharma, TNF-alpha-mediated cardiomyocyte apoptosis involves caspase-12 and calpain, Biochem. Biophys. Res. Commun. 345 (2006) 1558–1564.
[35] E. Szegezdi, U. Fitzgerald, A. Samali, Caspase-12 and ER-stress-mediated apoptosis: the story so far, Ann. N. Y. Acad. Sci. 1010 (2003) 186–194.
[36] S. Kacheva, S. Lenzen, E. Gurgul-Convey, Differential effects of proinflammatory cytokines on cell death and ER stress in insulin-secreting INS1E cells and the involvement of nitric oxide, Cytokine 55 (2011) 195–201.
[37] K.T. Chambers, J.A. Unverferth, S.M. Weber, R.C. Wek, F. Urano, J.A. Corbett, The role of nitric oxide and the unfolded protein response in cytokine-induced beta-cell death, Diabetes 57 (2008) 124–132.
[38] S. Oyadomari, K. Takeda, M. Takiguchi, T. Gotoh, M. Matsumoto, I. Wada, S. Akira, E. Araki, M. Mori, Nitric oxide-induced apoptosis in pancreatic beta cells is mediated by the endoplasmic reticulum stress pathway, Proc. Natl. Acad. Sci. U.S. A. 98 (2001) 10845–10850.
[39] P. Maechler, N. Li, M. Casimir, L. Vetterli, F. Frigerio, T. Brun, Role of mitochondria in beta-cell function and dysfunction, Adv. Exp. Med. Biol. 654 (2010) 193–216.
[40] S. Orrenius, V. Gogvadze, B. Zhivotovsky, Mitochondrial oxidative stress: implications for cell death, Annu. Rev. Pharmacol. Toxicol. 47 (2007) 143–183.
[41] S. Lenzen, J. Drinkgern, M. Tiedge, Low antioxidant enzyme gene expression in pancreatic islets compared with various other mouse tissues, Free Radic. Biol. Med. 20 (1996) 463–466.
[42] S. Lortz, E. Gurgul-Convey, S. Lenzen, M. Tiedge, Importance of mitochondrial superoxide dismutase expression in insulin-producing cells for the toxicity of reactive oxygen species and proinflammatory cytokines, Diabetologia 48 (2005) 1541–1548.
[43] B. Halliwell, J.M.C. Gutteridge, Free Radicals in Biology and Medicine, Fourth Edition ed. Oxford University Press, 2007.