Inhibitory Effect of Unsaturated Fatty Acids on Saturated Fatty Acid-Induced Apoptosis in Human Pancreatic β -Cells: Activation of Caspases and ER Stress Induction

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Key Words

Fatty acids • Apoptosis • β -cell • Caspases • Endoplasmic reticulum stress

Abstract

Aims: In this study we have tested the effect of unsaturated fatty acids on the proapoptotic effects of saturated fatty acids in the human pancreatic β -cells NES2Y. **Results:** We found that unsaturated palmitoleic and oleic acid at a concentration of 0.2 mM and higher are able to completely inhibit the proapoptotic effect of their counterpart saturated palmitic and stearic acid at a concentration of 1 mM. Apoptosis induced by stearic acid was associated with significant activation of caspase-6, -7, -9, -2 and -8, but not with significant activation of caspase-3. The activation of caspases was blocked by incubation with oleic acid. Stearic acid treatment was not associated with a significant change in mitochondrial membrane potential, reactive oxygen species level and with cytochrome c release from mitochondria. Furthermore, stearic acid treatment was not associated with changes in p21^{WAF1/CIP1}, PIDD, Fas receptor and Fas ligand expression. However, we detected endoplasmic reticulum (ER) stress markers, i. e. a significant upregulation of BiP and CHOP expression as

well as XBP1 mRNA splicing. These changes were inhibited by incubation with oleic acid. **Conclusion:** Presented data indicate that oleic acid inhibits apoptosis induction by stearic acid in NES2Y cells upstream of caspase activation and ER stress induction. It does not involve an interference with the mitochondrial pathway of apoptosis induction, with p53 activation and PIDD expression as well as with Fas receptor and Fas ligand expression.

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Introduction

Type 2 diabetes is a metabolic disorder characterized by peripheral insulin resistance and insufficient insulin production in respect to the demands of the body. Loss of β -cells by apoptosis contributes to insulin deficiency and has been demonstrated in Langerhans islets of patients with type 2 diabetes as well as with type 1 diabetes [1, 2]. Strong evidence indicates that the rise in the incidence of type 2 diabetes is correlated with increasing level of obesity and that the main factors responsible for triggering β -cell death are hyperglycemia and the increased level of circulating non-esterified fatty acids [3, 4]. The toxicity of fatty acids particularly

depends on the degree of their saturation [5-8].

Saturated fatty acid (e.g. palmitic and stearic acid) induced β -cell death that has been shown to be a caspase-dependent process involving the activation of caspase-3 [7, 9-14], caspase-6 [12] and caspase-8 [15]. A role of the mitochondrial pathway of apoptosis induction seems likely since the release of cytochrome c and AIF from mitochondria [5, 7, 13, 16] together with the decrease in Bcl-2 protein level [7] and the increase in Bax protein level [16] was reported after the treatment of β -cells with saturated fatty acids. A role for p53 activation in the course of apoptosis induction by fatty acids has also been suggested [17, 18].

Recently, we have shown newly that caspase-2 is also activated during saturated fatty acid-induced β -cell apoptosis [8]. Caspase-2 can be activated after its recruitment into a so-called PIDDosome complex, consisting of PIDD (p53-induced protein with death domain), adaptor protein RAIDD and caspase-2 [19]. However, the mechanism of caspase-2 activation and the involvement of PIDDosome in fatty acid-induced apoptosis have not yet been studied.

In contrast to the effect of saturated fatty acids on pancreatic β -cells, unsaturated fatty acids (e.g. palmitoleic acid, oleic acid, linoleic acid) do not induce cell death [5-8, 20, 21] and even may have a stimulatory effect on proliferation of pancreatic β -cells [5, 7, 8]. Unsaturated fatty acids were also shown to counteract the proapoptotic effects of saturated fatty acids [5-7, 22-24]. Several lines of evidence indicate that saturated and unsaturated fatty acids differ in their capacity to induce endoplasmic reticulum stress [21, 25-28]. However, most of these findings were obtained with β -cells of animal pancreatic islets or animal cell lines and there are few studies concerning the protective effect of unsaturated fatty acids on human pancreatic β -cells [6, 7]. Despite intensive research, molecular mechanisms of the protective effect of unsaturated fatty acids are unclear.

In our previous study, we showed that saturated fatty acids at a concentration of 1 mM and higher induced death of human pancreatic β -cells NES2Y while unsaturated fatty acids did not induce any cell death even at a concentration of 3 mM [8]. In the present study, we tested the effect of unsaturated fatty acids on death induction by saturated fatty acids in these cells. We found that unsaturated palmitoleic acid and oleic acid are able to completely block the death-inducing effect of saturated palmitic acid and stearic acid. The cell death induced by stearic acid was accompanied by a significant activation of caspase-6, -7, -9, -2 and -8, but not by significant

activation of caspase-3. The activation of caspases was inhibited by coincubation with oleic acid. We also found that stearic acid application did not lead to significant changes in mitochondrial membrane potential ($\Delta\Psi_m$) and in the level of reactive oxygen species (ROS) and it did not also cause cytochrome c release from mitochondria. In addition, the expression of p21^{WAF1/CIP1} and PIDD (p53-induced protein with death domain) as well as the expression of Fas receptor and Fas ligand were not affected by stearic acid treatment. However, after stearic acid treatment we did show a significantly increased expression of the ER chaperone BiP and ER stress marker CHOP as well as XBP1 mRNA splicing. These changes were blocked by coincubation with oleic acid.

Materials and Methods

Materials

All chemicals were from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise indicated. For western blot analysis, the following primary antibodies were used: mouse monoclonal antibody against human p21 (F-5), mouse monoclonal antibody against human FasR (B-10) and goat polyclonal antibody against human PIDD from Santa Cruz Biotechnology (Santa Cruz, CA, USA), mouse monoclonal antibody against human FasL (NOK-1) from BD Pharmingen (San Diego, CA, USA), rabbit monoclonal antibody against BiP (C50B12), mouse monoclonal antibody against CHOP, mouse monoclonal antibody against caspase-2 and caspase-8, rabbit polyclonal antibody against cleaved caspase-3, cleaved caspase-6, cleaved caspase-7 and cleaved caspase-9 from Cell Signaling Technology (Danvers, MA, USA) and mouse monoclonal antibody against human actin (clone AC-40) from Sigma-Aldrich (St. Louis, MO, USA). Staurosporine was from Enzo Life Sciences (Farmingdale, NY, USA).

Preparation of stock media with fatty acids

Solutions of stearic and oleic acids (0.3 M) were prepared in ethanol. Stearic acid in ethanol was warmed to 45°C to completely dissolve. The solutions were then mixed with the serum-free medium (see below) containing 10% fatty acid-free bovine serum albumin (BSA, pH 7.4) and were shaken intensively for 6 h at 37°C in order to prepare 10 mM fatty acid/10% BSA solutions. After sterilization by filtration, actual concentrations of individual fatty acids were determined using the Nefa C kit (Wako, Neuss, Germany). These stock media were stored at -80°C.

Cells and culture conditions

The human pancreatic β -cell line NES2Y [8, 29] was used. NES2Y are proliferating insulin-secreting cells with insulin promotor unresponsive to glucose. The cells (5×10^5 cells/0.5 ml of serum-free medium) produced insulin concentration of 28.0 ± 5.4 pmol/l within 24-h incubation, as quantified using the

IMMULITE 2000 Insulin assay (Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA). The insulin secretion by NES2Y cells was not affected by fatty acid treatment. Insulin secretion in response to common secretory stimuli was assessed earlier [29, 30]. The response of different human and animal pancreatic β -cell lines or primary β -cells and the physiological response of β -cells *in vivo* may differ. However, despite this obvious disadvantage, β -cell lines still represent very useful model for studies concerning molecular mechanisms of various cell processes because they represent easily accessible and “inexhaustible” source of homogenic β -cells.

Cells were routinely maintained in an RPMI 1640 based culture medium [31] supplemented with 10% heat-inactivated fetal bovine serum (Biochrom AG, Berlin, Germany) at 37°C in a humidified atmosphere of 5% CO₂ in air. RPMI 1640 medium contains 11 mM glucose. In experiments, a defined serum-free medium [32] supplemented with fatty acids bound to 2% fatty-acid free bovine serum albumin (BSA) was used as described previously [8]. The serum-free medium containing 2% BSA alone and without bound fatty acids was used as a control medium. Stock solutions containing individual fatty acids bound to the 10% BSA in the serum-free medium were prepared as described above and were diluted to the required concentration of fatty acid and BSA prior to experiments. Fatty acid/BSA molar ratios used in experiments were lower than the ratios known to exceed the binding capacity of BSA [20].

Cell growth and survival experiments

Cells maintained in culture medium were harvested and then seeded at 5x10³ cells/100 μ l of the culture medium into wells of a 96-well plastic plate. Cells were allowed to attach during a 24-h incubation period (approximately 15% confluency) and afterwards the culture medium was replaced by the serum-free medium containing stearic and/or oleic acid at tested concentrations or by control medium. Cell growth and survival were evaluated after 96 h of incubation. This time period was shown as optimal to detect most of effects on cell growth and survival previously [8]. The number of living cells was determined by hemocytometer counting after staining with trypan blue.

Measurement of caspase-3, caspase-9, caspase-2 and caspase-8 activation

Commercial CaspGLOW™ Active Caspase Staining Kits (Biovision, Mountain View, CA, USA) for detection of active forms of caspase-3, caspase-9, caspase-2 and caspase-8 were used. The kits utilize specific caspase inhibitors conjugated to fluorescent markers which are cell permeable and nontoxic. These inhibitors irreversibly bind to activated caspases allowing their detection in living cells. Cells (approximately 3x10⁵ cells per sample) were seeded and after a 24-h preincubation period allowing cells to attach, the culture medium was replaced by the serum-free medium containing fatty acids at the required concentration or by control medium. After 12 h, 24 h and 36 h of incubation the cells were harvested by low-speed centrifugation and staining was performed according to the manufacturer's instructions. The fluorescence was measured using a FACS Calibur cytometer (Becton Dickinson, San Jose, CA, USA).

Flow cytometric analysis of the mitochondrial membrane potential ($\Delta\psi_m$)

Cells (approximately 5x10⁵ cells per sample) were seeded and fatty acids were applied after 24-h preincubation as described above (see “Measurement of caspase-3, caspase-9, caspase-2 and caspase-8 activation”). After 24-h incubation, the cells were harvested by low-speed centrifugation and resuspended in PBS. The $\Delta\psi_m$ was measured as described previously [33]. Briefly, cells were kept on ice and 20 nM 3,3'-dihexyloxacarbocyanine iodide [DiOC₆(3)] from Molecular Probes (Eugene, OR, USA) was added. After 20 min of incubation at 37°C, cells were again kept on ice. As a negative control, aliquots of cells were incubated in the presence of 100 μ M carbonyl cyanide m-chlorophenylhydrazone (CCCP), a protonophore causing a complete disruption of the $\Delta\psi_m$. The fluorescence was measured using a FACS Calibur cytometer (Becton Dickinson, San Jose, CA, USA).

Flow cytometric analysis of reactive oxygen species (ROS) level

The ROS level was measured by dihydroethidine probe according to protocol described by Castedo et al. [34] with minor modifications. Briefly, cells (approximately 5x10⁵ cells per sample) were seeded and fatty acids were applied after 24-h preincubation as described above (see “Measurement of caspase-3, caspase-9, caspase-2 and caspase-8 activation”). After 24-h incubation, the cells were harvested by low-speed centrifugation and resuspended in the serum-free medium. Cells were kept on ice and 5 μ M dihydroethidium (Sigma-Aldrich, St. Louis, MO, USA) was added. After 30 min of incubation at 37°C in dark, cells were again kept on ice. The fluorescence was measured using a FACS Calibur cytometer (Becton Dickinson, San Jose, CA, USA).

Flow cytometric analysis of cytochrome c release

Commercial InnoCyte™ Flow Cytometric Cytochrome c Release Kit (Merck, Darmstadt, Germany) was used for the assessment of cytochrome c release from mitochondria. Briefly, cells (approximately 2x10⁶ cells per sample) were seeded and fatty acids were applied after 24-h preincubation as described above (see “Measurement of caspase-3, caspase-9, caspase-2 and caspase-8 activation”). After 24-h incubation, the cells were harvested by low-speed centrifugation and resuspended in PBS. The cells were permeabilized with Permeabilization Buffer, then fixed with 8 % paraformaldehyde in PBS and repeatedly washed. Blocking Buffer was added and the cells were incubated for 1 h at room temperature. Then the cells were incubated with the primary antibody against cytochrome c for 1 h at room temperature. After washing, the cells were incubated with the secondary anti-IgG FITC antibody for 1 h at room temperature. After subsequent washing, the cells were resuspended and the fluorescence was measured using a FACS Calibur cytometer (Becton Dickinson, San Jose, CA, USA).

Confocal microscopy analysis of cytochrome c release

Cells were seeded onto coverslips (approximately 2 x 10⁵ cells per coverslip) and fatty acids were applied after 24-h preincubation as described above (see “Measurement of

caspase-3, caspase-9, caspase-2 and caspase-8 activation”). After 24-h induction with fatty acids, coverslips were incubated with 1 μ M MitoTracker Red from Molecular Probes (Eugene, OR, USA) at 37°C for 45 min in order to stain mitochondria. Then, the staining solution was replaced by fresh serum-free medium and the dye was allowed to accumulate in mitochondria for 45 min at 37°C. Afterwards, cells were fixed with 4% paraformaldehyde for 20 min at 37°C. Fixed cells were washed several times with PBS and permeabilized with cold (-20°C) acetone for 10 min. After washing with PBS, cells were incubated with 30 μ l of the primary antibody against cytochrome c (clone 6H2 B4, Pharmingen, San Diego, CA, USA) diluted 1:50 in PBS for 2 h at room temperature. Then, cells were washed with PBS and incubated with 30 μ l of the secondary FITC-conjugated antibody from Sigma-Aldrich (St. Louis, MO, USA) diluted 1:100 in PBS for 2 hour in the dark at room temperature. Finally, cells were washed again with PBS. Stained cells on coverslips were transferred onto a droplet of Vectashield® Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA) and sealed. Samples were analyzed employing a confocal microscope Leica TCS SP5 (Bannockburn, IL, USA) with 63x oil objective at relevant excitation and emission wavelengths. As a positive control for cytochrome c release, cells were incubated with 1 μ M staurosporine for 24 hours.

Western blot analysis

Cells (approximately 20x10⁶ cells per sample) were seeded and fatty acids were applied after 24-h preincubation as described above (see “Measurement of caspase-3, caspase-9, caspase-2 and caspase-8 activation”). After 24-h incubation, the cells were harvested by low-speed centrifugation, washed twice with PBS and lysed in 150 μ l RIPA lysis buffer (Sigma-Aldrich, St. Louis, MO, USA) containing 1.5 μ l Protease Inhibitor Cocktail (Sigma-Aldrich, St. Louis, MO, USA). Cell lysates were then centrifuged (18,000 g, 20 min, 4°C). Supernatants were collected into new Eppendorf tubes and frozen at -80°C until further analysis. Total protein content was determined by the bicinchoninic acid assay [35]. Samples (10 μ l) containing 40 μ g of proteins were mixed with 10 μ l of sample loading buffer (0.125 mM Tris-HCl, pH 6.8, 10% glycerol, 4% SDS, 250 mM DTT, 0.004% bromophenol blue), heated for 10 min at 95°C and then quickly cooled on ice. SDS-PAGE was performed as described previously [36, 37] with minor modifications. Briefly, proteins were separated on 12% polyacrylamide gel (4% polyacrylamide stacking gel) at 30 mA and then blotted onto 0.2 μ m nitrocellulose transfer membrane (Protran BA83, Schleicher-Schuell, Dassel, Germany) for 2 h at 0.25 A using a Mini-Protein 3 apparatus (Bio-Rad, Hercules, CA). The membrane was blocked with 5% non-fat milk in TBS (100 mM Tris-HCl, 150 mM NaCl, pH 7.5) for 20 min and then washed with 0.1% Tween-20/TBS three times. The washed membrane was incubated with the primary antibody in 0.1% Tween-20/TBS containing 1% non-fat milk overnight at 4°C. Following dilutions of primary antibodies (see “Materials”) were used: 1:800 for anti-actin antibody, 1:100 for anti-PIDD, anti-FasR and anti-p21 antibodies and 1:1,000 for anti-BiP, anti-CHOP, anti-caspase-2, anti-caspase-3, anti-caspase-6, anti-caspase-7, anti-caspase-8 and anti-caspase-9 antibody. After the

incubation, the washed membrane was incubated with the corresponding horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h. After washing, the horseradish peroxidase-conjugated secondary antibody was detected by enhanced chemiluminescence using the Supersignal reagent from Pierce (Rockford, IL, USA) and LAS-4000 CCD device (Fujifilm, Tokyo, Japan) or Gel Logic 1500 Imaging System (Kodak).

Assessment of XBP1 mRNA splicing

Cells (approximately 1x10⁶ cells per sample) were seeded and fatty acids were applied after 24-h preincubation as described above (see “Measurement of caspase-3, caspase-9, caspase-2 and caspase-8 activation”). After 24-h incubation, the cells were harvested by low-speed centrifugation. The splicing of XBP1 mRNA was assessed by RT-PCR as described previously [38]. Reverse transcription from total RNA was performed according to Balusikova et al. [39]. Expression of housekeeping gene GAPDH was determined using primer sequences described previously [39]. The temperature profile for PCR amplification of spliced and unspliced XBP1 was: denaturation at 94°C for 5 min, 38 cycles of amplification at 94°C for 30 s, 60°C for 45 s and 72°C for 1 min, final elongation at 72°C for 10 min. The temperature profile for PCR amplification of GAPDH was: denaturation at 94°C for 5 min, 35 cycles of amplification at 94°C for 30 s, 55°C for 30 s and 72°C for 1 min, final elongation at 72°C for 10 min. Amplified PCR products of GAPDH and XBP1 (456 bp product of unspliced XBP1 and 430 bp product of spliced XBP1) were separated on 2% agarose gel containing ethidium bromide and signal was recorded by gel documentation system (Syngene, Frederick, MD, USA).

Statistical analysis

Statistical significance of differences was determined by Student's *t*-test. *P* < 0.05 was considered statistically significant.

Results

Effect of unsaturated fatty acids on cell death induced by saturated fatty acids

The cells were incubated with 1 mM palmitic acid or stearic acid applied together with palmitoleic acid or oleic acid at increasing concentrations (0.02-1 mM), respectively. These concentrations are within the range of concentrations physiologically available *in vivo* [40]. After 96 h of incubation, both unsaturated fatty acids significantly inhibited the cell death-inducing effect of saturated fatty acids at a concentration as low as 0.05 mM. Moreover, unsaturated fatty acids at a concentration of 0.2 mM and higher concentrations were able to completely block the cell death induced by saturated fatty acids. Unsaturated fatty acids even allowed the cells to grow in the presence of saturated fatty acids. However,

Fig. 1. Concentration-dependent effect of (A) palmitoleic acid (POA) and (B) oleic acid on the cell death-inducing effect of (A) palmitic acid (PA) and (B) stearic acid (SA) in NES2Y cells. The cells were seeded at 5×10^3 cells/100 μ l of culture medium in the well. The number of cells of the inoculum is shown as a dotted line. After 24-h incubation period allowing the cells to attach, the culture medium was replaced by media containing (A) palmitic acid and/or palmitoleic acid or (B) stearic acid and/or oleic acid at indicated concentrations bound to 2% fatty acid-free bovine serum albumin (BSA) or by control medium. Control cells were incubated without fatty acids but with 2% fatty acid-free BSA. The number of living cells was determined after following 96 h of incubation (see “Materials and methods”). Each column represents the mean of 4 separate cultures $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$ when comparing the effect of (A) 1 mM palmitic acid applied together with decreasing concentrations of palmitoleic acid (1–0.02 mM) and the effect of 1 mM palmitic acid alone and (B) 1 mM stearic acid applied together with decreasing concentrations of oleic acid (1–0.02 mM) and the effect of 1 mM stearic acid alone.

Fig. 2. Effect of 1 mM stearic acid (SA), 1 mM stearic acid applied together with 0.2 mM oleic acid (OA), and 0.2 mM oleic acid on the activity of caspase-3, -9, -2 and -8 in NES2Y cells. Cells incubated without fatty acids represented control cells. After 12, 24 and 36 h of incubation in media containing fatty acids or in control medium, the activity of individual caspases was measured by flow cytometry employing CaspGLOW™ Active Caspase Staining Kits (see “Materials and methods”). Each column represents the mean of two experimental values $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$ when comparing the effect of fatty acids with control cells, + $P < 0.05$, ++ $P < 0.01$ when comparing the effect of combination of stearic acid and oleic acid with the effect of stearic acid alone.

the growth was slightly slower when compared with control cells (Fig. 1).

In these experiments, similarly as in our previous studies [8], stearic acid seemed to be more effective in cell death induction than palmitic acid (Fig. 1) and therefore stearic acid was chosen for further detailed studies. We selected 0.2 mM oleic acid for the inhibition of cell death induced by 1 mM stearic acid.

Effect of stearic acid applied alone and together with oleic acid on the activity of caspases

To address the involvement of individual caspases in death induced by stearic acid and in its inhibition by oleic acid, we tested the time course of caspase-3, -9, -2 and -8 activation in NES2Y cells. Most cells were shown to be dead within 48 h after the application of 1 mM stearic acid [8] and thus we decided to test the effect of stearic and oleic acid after 12 h, 24 h and 36 h of induction. Employing commercial kits and flow cytometry (see “Materials and Methods”), we detected increases (2 to 3-fold) in the activity of caspase-9, caspase-2 and caspase-8 after 24 h of incubation with a cell death-inducing concentration (1 mM) of stearic acid. The increase for all these caspases was even more pronounced (approximately 4-fold) and significant after 36 h. The activity of caspase-9, -2 and -8 was significantly reduced by coincubation with oleic acid (0.2 mM) and did not differ significantly from the activity in control cells (Fig. 2). Even after 36-h incubation of the cells with stearic acid, no significant increase of caspase-3 activity was detected (Fig. 2).

We assessed caspase activation by the level of their cleaved forms employing western blot analysis. We detected cleaved forms of caspase-9, caspase-2 and caspase-8 after 24 h of stearic acid treatment (Fig. 3A) and thus we confirmed the data obtained by flow cytometry (see above). We also detected a slight signal as to the cleaved form of caspase-3 (Fig. 3A). Such slight cleavage corresponds to the insignificant level of activation detected by flow cytometry (Fig. 2) and also by colorimetric assay under the same experimental conditions in our previous study [8]. We also tested the cleavage of other executioner caspases, i. e. caspase-6 and caspase-7. The cleavage of both these caspases increased after 24-h stearic acid treatment and this increase was inhibited by coincubation with oleic acid (Fig. 3B).

Effect of stearic acid applied alone and together with oleic acid on mitochondrial function

The activation of caspase-9 during stearic acid-induced cell death of NES2Y cells indicated that the mitochondrial pathway of apoptosis induction could be involved. Therefore, we measured mitochondrial membrane potential ($\Delta\psi_m$) and reactive oxygen species (ROS) levels 24 h after fatty acid treatment. Flow cytometric analysis of the $\Delta\psi_m$ (see “Materials and Methods”) showed that stearic acid induced a decrease of $\Delta\psi_m$ in a minor population of cells. However, it was

Fig. 3. Effect of 1 mM stearic acid (SA), 1 mM stearic acid applied together with 0.2 mM oleic acid (OA), and 0.2 mM oleic acid on the expression of (A) cleaved caspase-3, -9, 2, -8, and (B) cleaved caspase-6, -7. Cells incubated without fatty acids represented control cells. After 24 h of incubation, the expression of tested proteins was determined by western blot analysis employing relevant antibodies (see “Materials and Methods”). Monoclonal antibody against human actin was used to confirm equal protein loading. The data shown were obtained in one representative experiment of three independent experiments.

completely abolished by the simultaneous application of oleic acid. Flow cytometric analysis of the ROS level (see “Materials and Methods”) also revealed that stearic acid treatment did not significantly change the cellular ROS levels (Fig. 4). As a positive control for the increase of ROS level, we used NES2Y cells treated with 1% hydrogen peroxide for 30 minutes (data not shown).

Fig. 4. Effect of 1 mM stearic acid (SA), 1 mM stearic acid applied together with 0.2 mM oleic acid (OA), and 0.2 mM oleic acid on the mitochondrial membrane potential ($\Delta\psi_m$), reactive oxygen species (ROS) level and the cytochrome c release from mitochondria in NES2Y cells. Cells incubated without fatty acids represented control cells. After 24 h of incubation, the mitochondrial membrane potential was assessed by flow cytometric analysis of cells after staining with 20 nM [DiOC₆(3)] (see “Materials and Methods”). The collapse of $\Delta\psi_m$ caused by the treatment of cells with 100 μ M CCCP is shown (blank areas in $\Delta\psi_m$ part). After 24 h of incubation, the ROS level was assessed by flow cytometric analysis of cells after staining with 5 μ M dihydroethidium (see “Materials and Methods”). The analysis of cells without staining with dihydroethidium is shown (blank areas in ROS part). After 24 h of incubation, the release of cytochrome c was assessed by flow cytometric analysis of cells, employing a commercial cytochrome c release kit (see “Materials and Methods”). Cells without staining for cytochrome c are also shown (blank areas in cytochrome c part). Data obtained in one representative experiment of three independent experiments are shown.

Effect of stearic acid applied alone and together with oleic acid on cytochrome c release

Cytochrome c release from mitochondria in NES2Y cells was assessed after fatty acid treatment by flow cytometric analysis (see “Materials and Methods”). We found no change in cytochrome c release from mitochondria after stearic acid treatment for 24 h (Fig. 4). This finding was confirmed using confocal microscopy (see “Materials and Methods”) which showed the same pattern of cytochrome c mitochondrial staining for both control and stearic acid-treated cells. It again suggested that cytochrome c was not released from mitochondria (Fig. 5). However, we observed a slight change in mitochondrial morphology after stearic acid treatment. Mitochondria became more globular than under control

conditions. As a positive control for cytochrome c release, staurosporine treated cells were used (Fig. 5).

Effect of stearic acid applied alone and together with oleic acid on p21^{WAF1/CIP1}, PIDD, Fas receptor and Fas ligand expression

In order to contribute to elucidation of the mechanism of apoptosis induction by stearic acid and its inhibition by oleic acid, we assessed the expression of p21^{WAF1/CIP1}, a protein known to be transcriptionally upregulated by activated p53 [41], in NES2Y cells using western blot analysis (see “Materials and Methods”). We found that under control conditions the cells expressed p21^{WAF1/CIP1} in a small but still detectable amount and that the expression of

Fig. 5. Effect of 1 mM stearic acid (SA), 1 mM stearic acid applied together with 0.2 mM oleic acid (OA), and 0.2 mM oleic acid alone on cytochrome c release from mitochondria in NES2Y cells. Cells incubated without fatty acids represented control cells. After 24 h of incubation, mitochondria (red), cytochrome c (green) and nuclei (blue) within the cells were stained and representative images were acquired with Leica confocal scanning microscope (see „Materials and Methods“). The merge of mitochondria, cytochrome c and DAPI staining is shown. For mitochondria and cytochrome c staining, a graph displaying signal intensity in representative region of interest („ROI“, indicated in the image by a green line) is also shown. Data obtained in one representative experiment of three independent experiments are shown. As a positive control, staining of cells after 24-h incubation with 1 μ M staurosporine leading to cytochrome c release was used.

p21^{WAF1/CIP1} was not affected by a 24-h incubation with stearic acid (Fig. 6A).

To shed some light on the mechanism of caspase-2 activation during stearic acid-induced apoptosis, we assessed the expression of PIDD, which is known to be involved in caspase-2 activation, using western blot analysis. PIDD has been reported to be also transcriptionally upregulated by activated p53 [19]. Although the high molecular weight form of PIDD (approximately 100 kDa) was not found in NES2Y cells, we did detect a cleaved form of PIDD (approximately 50 kDa) which results from constitutive cleavage [42]. However, there was no difference in PIDD expression after stearic acid treatment (Fig. 6A).

The expression of Fas receptor and Fas ligand, which are involved in caspase-8 activation, were also tested. However, western blot analysis did not show any increase in Fas receptor or Fas ligand expression. In fact, the expression of Fas receptor seemed to be slightly decreased after stearic acid treatment of NES2Y cells (Fig. 6A).

Effect of stearic acid applied alone and together with oleic acid on ER stress markers

It has been suggested that endoplasmic reticulum (ER) stress represents one of the possible mechanisms involved in fatty acid induced apoptosis of pancreatic β -cells [26]. Therefore, we tested the expression of ER

Fig. 6. Effect of 1 mM stearic acid (SA), 1 mM stearic acid applied together with 0.2 mM oleic acid (OA), and 0.2 mM oleic acid on (A) the expression of PIDD, Fas receptor (FasR), Fas ligand (FasL) and p21^{WAF1/CIP1} and (B) the expression of BiP and CHOP and (C) XBP1 mRNA splicing in NES2Y cells. Cells incubated without fatty acids represented control cells. After 24 h of incubation, the expression of proteins was determined by western blot analysis employing relevant antibodies and XBP1 splicing was assessed by RT-PCR using relevant primers (see „Materials and Methods“). Monoclonal antibody against human actin was used to confirm equal protein loading. GAPDH was used as a control gene for RT-PCR. In the case of p21^{WAF1/CIP1} analysis, MCF-7 cells treated with taxane SB-T-1216 were used as a positive control. For XBP1 splicing analysis, NES2Y cells treated with 0.1 μ M thapsigargin were used as a positive control. The data shown were obtained in one representative experiment of three independent experiments.

stress markers BiP and CHOP in NES2Y cells employing western blot analysis (see “Materials and Methods”). BiP (also known as Grp78 or HSPA5) functions as an ER chaperone and protein misfolding sensor [43]. CHOP (C/EBP homologous protein, also known as GADD153, i.e. growth arrest and DNA damage-inducible protein) is a transcription factor known to mediate ER stress-induced apoptosis [44]. After 24-h incubation with stearic acid, we detected a significant increase in the BiP protein level. This increase was significantly reduced by coincubation with oleic acid. BiP expression in cells treated with oleic acid alone was unchanged compared to control cells (Fig. 6B). CHOP expression was almost undetectable under control conditions and after oleic acid treatment but was strongly induced by stearic acid treatment. This increase was inhibited by coincubation with oleic acid (Fig. 6B). Next, we assessed whether XBP1 mRNA underwent splicing resulting in the translation of active transcription factor. Such splicing also points at the presence of ER stress [45]. Stearic acid treatment led to XBP1 mRNA splicing that was reduced by coapplication of oleic acid (Fig. 6C).

Discussion

In this study, we have tested how unsaturated fatty acids affect cell death induction by saturated fatty acids in human pancreatic β -cells. Experiments were performed with the human pancreatic β -cell line NES2Y in a chemically defined serum-free medium allowing growth of the cells and precise control of fatty acid

concentrations.

We found that unsaturated fatty acids (palmitoleic acid, oleic acid) were able to block completely cell death induced by saturated fatty acids (palmitic acid, stearic acid). The effect of unsaturated fatty acids was achieved using concentrations 5 times lower than the concentrations of saturated fatty acid used for death induction (see Fig. 1). These findings are consistent with experiments using rat β -cell lines [6, 23, 24, 46] and rat islet cells [5] as well as isolated human islets [6, 7]. Despite intensive research, the underlying mechanisms of unsaturated fatty acid effect are yet to be fully understood. The present study is newly addressing the underlying mechanisms in pancreatic β -cells of human origin.

After inducing cell death with stearic acid, we detected a significant increase of caspase-9 activity which was inhibited by coincubation with oleic acid (see Fig. 2). However, we were unable to detect cytochrome c release from mitochondria (see Figs. 4 and 5) or changes in reactive oxygen species (ROS) levels and mitochondrial membrane potential ($\Delta\psi_m$) (see Fig. 4). ROS level was shown to be increased in oleate-treated human pancreatic islets by Bikopoulos et al. [47]. However, they employed method assessing different type of ROS. The level of ROS in cells under our experimental serum-free conditions was even slightly lower than the level of ROS in cells cultured in medium containing FBS (data not shown) in contrast to data of Maestre et al. [16]. This discrepancy may result from the use of defined serum-free medium in our experiments (see "Materials and Methods") which allows growth of the cells in contrast to medium simply depleted of FBS used by Maestre et al. [16] that can serve as a stress signal itself. Our findings concerning ROS level, mitochondrial membrane potential ($\Delta\psi_m$) and cytochrome c release suggest that the mitochondrial pathway is not involved in either apoptosis induction by stearic acid or the inhibitory intervention of unsaturated fatty acids. While caspase-9 activation is normally associated with the apoptosome complex as a consequence of cytochrome c release from mitochondria, caspase-9 activation without cytochrome c release has been demonstrated in several cell types employing various apoptotic stimuli, e.g. dexamethasone [48], cephalostatin [49] and paclitaxel [37]. Thus, the results presented here and the data of others suggest that an alternative pathway for caspase-9 activation, bypassing apoptosome formation, may exist in at least some cell types for certain apoptotic stimuli.

Caspase-3 activation has been demonstrated during fatty acid-induced apoptosis in several models [7, 9-14].

However, using flow cytometric analysis we have confirmed our previous finding [8] that caspase-3 was only slightly, but not significantly activated when cell death was induced by saturated fatty acid treatment (see Fig. 2). We further corroborated this finding by western blot analysis employing a specific antibody against cleaved caspase-3 (Fig. 3). The discrepancy in the degree of caspase-3 activation between our data and the data presented in other studies can be explained by the fact that most lines of evidence concerning caspase-3 activation in fatty acid-induced cell death come from animal cells. Only Maedler et al. [7] has reported caspase-3 activation in human β -cells, although the level of caspase-3 activation was not fully documented in their paper. As to our best knowledge, there is no other data in the literature concerning caspase-3 activation in human β -cells following treatment with saturated fatty acids. Our findings may therefore suggest that there are perhaps differences in the role of caspase-3 activation in fatty acid-induced apoptosis between rodent cells and human cells, or particularly human NES2Y cells. Another possibility is that NES2Y cells may possess functionally defective caspase-3 because we were unable to detect significant caspase-3 activity even after employing staurosporine and doxorubicine as apoptosis inducers. Another possible explanation for the discrepancy is that caspase-3 activation described by others in rodent islet cells is a consequence of some other cell death-inducing pathway. Taken together, our data suggest that the activation of caspase-3 does not appear to be essential for saturated fatty acid-induced apoptosis and that an interference with caspase-3 activation is not involved in the protective effect of unsaturated fatty acids.

However, as we detected the activation of executioner caspases caspase-6 and caspase-7 after stearic acid treatment (see Fig. 3), it is very likely that the role of caspase-3 can be at least partially substituted by these caspases. The activation of caspase-6 was also shown by Hirota et al. [12] in the case of palmitate induced apoptosis in murine β -cells. As to our best knowledge, the involvement of caspase-7 activation per se in fatty acid-induced apoptosis of human β -cells was not demonstrated till now.

In our previous study [8] we demonstrated newly that saturated fatty acid-induced apoptosis was also associated with the activation of caspase-2. In this study we have demonstrated that caspase-2 activation after stearic acid application was inhibited by coincubation with oleic acid (see Fig. 2). Furthermore, we have demonstrated that stearic acid-induced apoptosis was not

associated with p53 activation and a change in PIDD expression (see Fig. 6A). The expression of PIDD, and also the expression of Fas receptor, is known to be regulated by p53 [50]. These findings demonstrate that caspase-2 activation does not concurrently involve p53 activation as well as increased PIDD expression and thus caspase-2 activation in human NES2Y cells does not probably involve PIDDosome formation. These findings also suggest that p53 activation and subsequent PIDD expression are not involved in the inhibitory intervention of unsaturated fatty acids. The existence of an alternative mechanism of caspase-2 activation, circumventing PIDDosome formation, has already been shown since caspase-2 processing was not affected in mice deficient in PIDD [51, 52]. Recently DISC was reported as an alternative activating platform for caspase-2 [53]. However, whether this can play a role in stearic acid-induced caspase-2 activation, remains to be elucidated.

The activation of caspase-8 following stearic acid treatment (see Fig. 2 and 3) implies that Fas receptor-Fas ligand interaction and a receptor-mediated pathway may be involved in saturated fatty acid-induced apoptosis although this is not a generally accepted mechanism for pancreatic β -cell lipotoxicity. Caspase-8 activation was also inhibited by coincubation with oleic acid as was found for caspase-9 and caspase-2 (see Fig. 2). Fas receptor was constitutively expressed on NES2Y cells even under control conditions possibly due to the hyperglycemic conditions (11 mM glucose) of the RPMI 1640 culture medium [54]. However, the expression of Fas receptor nor the expression of Fas ligand were not changed by the application of stearic acid. This implies that caspase-8 activation does not result from increased Fas receptor and/or Fas ligand expression and that the antiapoptotic effect of unsaturated fatty acids is not mediated by the modulation of Fas receptor and/or Fas ligand expression. However, we cannot exclude the possibility that the effect of unsaturated fatty acids is exerted by regulation of the expression of other proteins involved in caspase-8 activation, e.g. FLIP (FLICE-inhibitory protein) [55]. Despite the unknown mechanism of its activation, a role for caspase-8 in saturated fatty acid-induced apoptosis in the development of type 2 diabetes is likely as has been demonstrated in animal models [56] and individuals with type 2 diabetes [15]. In contrast, Fas receptor *per se* does not seem to play a decisive role in saturated fatty acid-induced apoptosis as demonstrated by our data as well as by the fact that β -cell specific Fas receptor deletion is ineffective in protecting mice against a high fat diet-induced type 2 diabetes [57].

Summarizing, stearic acid-induced activation of caspase-6, -7, -9, -2 and -8 in NES2Y cells is almost completely inhibited by the application of oleic acid. This supports a suggestion that oleic acid interferes with the cell death-inducing effect of stearic acid upstream of caspase activation. However, detailed mechanisms of the activation of caspases and the order of their activation, when cell death is induced by saturated fatty acids, remain unclear. An upstream activator may be another protease such as calpain-10 [58].

The integrity and function of pancreatic β -cells is particularly sensitive to maintaining endoplasmic reticulum (ER) homeostasis due to the high rate of insulin synthesis. ER stress and subsequent apoptosis is now considered to play an important role in fatty acid-induced cytotoxicity [28, 45]. Increased expression of ER stress markers BiP and CHOP was demonstrated in human islets after fatty acid treatment [21, 59] as well as in β -cells of type 2 diabetic patients [26]. Increased expression of BiP (also known as Grp78 or HSPA5), which functions as an ER chaperone and protein misfolding sensor [43], was recently shown to be involved in the inhibition of fatty acid-induced apoptosis by GLP-1 agonists [60]. The upregulation of the ER chaperone BiP and ER stress-induced transcription factor CHOP as well as XBP1 splicing, which were detected in this study (see Fig. 6B and 6C), documented the existence of ER stress after stearic acid treatment in NES2Y cells. The fact, that BiP and CHOP upregulation and XBP1 splicing induced by stearic acid was inhibited by the coincubation with oleic acid, could indicate that the inhibition occurs either at the level or upstream of ER stress induction. According to the data presented by Diakogiannaki et al. [27], different regulation of the phosphorylation of eIF2 α (a molecule involved in PERK pathway of ER stress signaling) by saturated and unsaturated fatty acids could be a candidate mechanism involved in the protective effect of unsaturated fatty acids. Such an explanation would be in agreement with a previously postulated suggestion that a signaling event initiated by unsaturated fatty acids may be responsible for cell death inhibition by unsaturated fatty acids rather than a metabolic interference [14, 23, 46]. However, there is a possibility that other mechanisms can also contribute to the protective effect of unsaturated fatty acids, e. g. alterations in endogenous lipid partitioning [61].

Execution of ER stress-induced apoptosis in rodent models was shown to be dependent on caspase-12 [62]. However, except of specific populations of African descent, human population do not possess functional

caspase-12 [63]. However, both caspase-2 and caspase-9, which were found in our study to be activated by stearic acid treatment (see Fig. 3), have been previously shown to participate in ER stress-induced apoptosis [64]. Caspase-2 can also be activated by caspase-8 as demonstrated recently [53]. Nevertheless, whether any of these or some unidentified alternative mechanism is involved remains to be elucidated.

Taken together, we found that unsaturated fatty acids (palmitoleic and oleic acid) are able to completely inhibit cell death induced by saturated fatty acids (palmitic and stearic acid) in the human pancreatic β -cell line NES2Y. The cell death induced by stearic acid was accompanied by significant activation of caspase-6, -7, -9, -2 and -8, but not by significant caspase-3 activation. Stearic acid treatment did not result in any significant changes of mitochondrial membrane potential ($\Delta\psi_m$), reactive oxygen species (ROS) levels or in cytochrome c release from mitochondria. We suggest that oleic acid interferes with the pathway of apoptosis induction by stearic acid

upstream of caspase activation and ER stress induction. We can conclude that the antiapoptotic effect of oleic acid is not based on its interference with the mitochondrial pathway of apoptosis induction or p53 activation, and it is not associated with changes in PIDD as well as Fas receptor and Fas ligand expression.

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References

- 1 Sakuraba H, Mizukami H, Yagihashi N, Wada R, Hanyu C, Yagihashi S: Reduced beta-cell mass and expression of oxidative stress-related DNA damage in the islet of Japanese Type II diabetic patients. *Diabetologia* 2002;45:85-96.
- 2 Butler AE, Janson J, Bonner-Weir S, Ritzel R, Rizza RA, Butler PC: β -cell deficit and increased β -cell apoptosis in humans with type 2 diabetes. *Diabetes* 2003;52:102-110.
- 3 Chang-Chen KJ, Mullur R, Bernal-Mizrachi E: Beta-cell failure as a complication of diabetes. *Rev Endocr Metab Disord* 2008;9:329-343.
- 4 Morgan NG, Dhayal S, Diakogiannaki E, Welters HJ: The cytoprotective actions of long-chain mono-unsaturated fatty acids in pancreatic beta-cells. *Biochem Soc Trans* 2008;36:905-908.
- 5 Maedler K, Spinas GA, Dyntar D, Moritz W, Kaiser N, Donath MY: Distinct effects of saturated and monounsaturated fatty acids on β -cell turnover and function. *Diabetes* 2001;50:69-76.
- 6 Eitel K, Staiger H, Brendel MD, Brandhorst D, Bretzel RG, Haring HU, Kellerer M: Different role of saturated and unsaturated fatty acids in β -cell apoptosis. *Biochem Biophys Res Commun* 2002;299:853-856.
- 7 Maedler K, Oberholzer J, Bucher P, Spinas GA, Donath MY: Monounsaturated fatty acids prevent the deleterious effects of palmitate and high glucose on human pancreatic β -cell turnover and function. *Diabetes* 2003;52:726-733.
- 8 Fürstová, V, Kopska T, James RF, Kovár J: Comparison of the effect of individual saturated and unsaturated fatty acids on cell growth and death induction in the human pancreatic β -cell line NES2Y. *Life Sci* 2008;82:684-691.
- 9 Eitel K, Staiger H, Rieger J, Mischak H, Brandhorst H, Brendel MD, Bretzel RG, Haring HU, Kellerer M: Protein kinase C delta activation and translocation to the nucleus are required for fatty acid-induced apoptosis of insulin-secreting cells. *Diabetes* 2003;52:991-997.
- 10 El-Assaad W, Buteau J, Peyot ML, Nolan C, Roduit R, Hardy S, Joly E, Dbaibo G, Rosenberg L, Prentki M: Saturated fatty acids synergize with elevated glucose to cause pancreatic β -cell death. *Endocrinology* 2003;144:4154-4163.
- 11 Rakatzi I, Mueller H, Ritzeler O, Tennagels N, Eckel J: Adiponectin counteracts cytokine- and fatty acid-induced apoptosis in the pancreatic beta-cell line INS-1. *Diabetologia* 2004;47:249-258.
- 12 Hirota N, Otabe S, Nakayama H, Yuan X, Yamada K: Sequential activation of caspases and synergistic β -cell cytotoxicity by palmitate and anti-Fas antibodies. *Life Sci* 2006;79:1312-1316.
- 13 Choi SE, Kim HE, Shin HC, Jang HJ, Lee KW, Kim Y, Kang SS, Chun J, Kang Y: Involvement of Ca^{2+} -mediated apoptotic signals in palmitate-induced MIN6N8a beta cell death. *Mol Cell Endocrinol* 2007;272:50-62.
- 14 Dhayal S, Welters HJ, Morgan NG: Structural requirements for the cytoprotective actions of mono-unsaturated fatty acids in the pancreatic beta-cell line, BRIN-BD11. *Br J Pharmacol* 2008;153:1718-1727.
- 15 Marchetti P, Del Guerra S, Marselli L, Lupi R, Masini M, Pollera M, Bugliani M, Boggi U, Vistoli F, Mosca F, Del Prato S: Pancreatic islets from type 2 diabetic patients have functional defects and increased apoptosis that are ameliorated by metformin. *J Clin Endocrinol Metab* 2004;89:5535-5541.
- 16 Maestre I, Jordan J, Calvo S, Reig JA, Cena V, Soria B, Prentki M, Roche E: Mitochondrial dysfunction is involved in apoptosis induced by serum withdrawal and fatty acids in the β -cell line INS-1. *Endocrinology* 2003;144:335-345.

- 17 Wrede CE, Dickson LM, Lingohr MK, Briaud I, Rhodes CJ: Protein kinase B/Akt prevents fatty acid-induced apoptosis in pancreatic beta-cells (INS-1). *J Biol Chem* 2002;277:49676-49684.
- 18 Lovis P, Roggli E, Laybutt DR, Gattesco S, Yang JY, Widmann C, Abderrahmani A, Regazzi R: Alterations in microRNA expression contribute to fatty acid-induced pancreatic beta-cell dysfunction. *Diabetes* 2008;57:2728-2736.
- 19 Tinel A, Tschopp J: The PIDDosome, a protein complex implicated in activation of caspase-2 in response to genotoxic stress. *Science* 2004;304:843-846.
- 20 Cnop M, Hannaert JC, Hoorens A, Eizirik DL, Pipeleers DG: Inverse relationship between cytotoxicity of free fatty acids in pancreatic islet cells and cellular triglyceride accumulation. *Diabetes* 2001;50:1771-1777.
- 21 Cunha DA, Hekerman P, Ladrière L, Bazarra-Castro A, Ortis F, Wakeham MC, Moore F, Rasschaert J, Cardozo AK, Bellomo E, Overbergh L, Mathieu C, Lupi R, Hai T, Herchuelz A, Marchetti P, Rutter GA, Eizirik DL, Cnop M: Initiation and execution of lipotoxic ER stress in pancreatic β -cells. *J Cell Sci* 2008;121:2308-2318.
- 22 Beechey N, Chambers JA, Green IC: Fatty acid protection from palmitic acid-induced apoptosis is lost following PI3-kinase inhibition. *Apoptosis* 2004;9:599-607.
- 23 Welters HJ, Tadayyon M, Scarpello JH, Smith SA, Morgan NG: Mono-unsaturated fatty acids protect against β -cell apoptosis induced by saturated fatty acids, serum withdrawal or cytokine exposure. *FEBS Lett* 2004;560:103-108.
- 24 Welters HJ, Diakogiannaki E, Mordue JM, Tadayyon M, Smith SA, Morgan NG: Differential protective effects of palmitoleic acid and cAMP on caspase activation and cell viability in pancreatic β -cells exposed to palmitate. *Apoptosis* 2006;11:1231-1238.
- 25 Karaskov E, Scott C, Zhang L, Teodoro T, Ravazzola M, Volchuk A: Chronic palmitate but not oleate exposure induces endoplasmic reticulum stress, which may contribute to INS-1 pancreatic beta-cell apoptosis. *Endocrinology* 2006;147:3398-3407.
- 26 Laybutt DR, Preston AM, Akerfeldt MC, Kench JG, Busch AK, Biankin AV, Biden TJ: Endoplasmic reticulum stress contributes to beta cell apoptosis in type 2 diabetes. *Diabetologia* 2007;50:752-763.
- 27 Diakogiannaki E, Welters HJ, Morgan NG: Differential regulation of the endoplasmic reticulum stress response in pancreatic beta-cells exposed to long-chain saturated and monounsaturated fatty acids. *J Endocrinol* 2008;197:553-563.
- 28 Diakogiannaki E, Morgan NG: Differential regulation of the ER stress response by long-chain fatty acids in the pancreatic beta-cell. *Biochem Soc Trans* 2008;36:959-962.
- 29 Macfarlane WM, Cragg H, Docherty HM, Read ML, James RF, Aynsley-Green A, Docherty K: Impaired expression of transcription factor IUF1 in a pancreatic beta-cell line derived from a patient with persistent hyperinsulinaemic hypoglycaemia of infancy (nesidioblastosis). *FEBS Lett* 1997;413:304-308.
- 30 MacFarlane WM, Chapman JC, Shepherd RM, Hashmi MN, Kamimura N, Cosgrove KE, O'Brien RE, Barnes PD, Hart AW, Docherty HM, Lindley KJ, Aynsley-Green A, James RF, Docherty K, Dunne MJ: Engineering a glucose-responsive human insulin-secreting cell line from islets of Langerhans isolated from a patient with persistent hyperinsulinemic hypoglycemia of infancy. *J Biol Chem* 1999;274:34059-34066.
- 31 Musilková J, Kovár J: Additive stimulatory effect of extracellular calcium and potassium on non-transferrin ferric iron uptake by HeLa and K562 cells. *Biochim Biophys Acta* 2001;1514:117-126.
- 32 Kovár J, Franek F: Growth-stimulating effect of transferrin on a hybridoma cell line: relation to transferrin iron-transporting function. *Exp Cell Res* 1989;182:358-369.
- 33 Koc M, Nad'ová Z, Truksa J, Ehrlichová M, Kovár J: Iron deprivation induces apoptosis via mitochondrial changes related to Bax translocation. *Apoptosis* 2005;10:381-393.
- 34 Castedo M, Ferri K, Roumier T, Metivier D, Zamzami N, Kroemer G: Quantitation of mitochondrial alterations associated with apoptosis. *J Immunol Methods* 2002;265:39-47.
- 35 Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC: Measurement of protein using bicinchoninic acid. *Anal Biochem* 1985;150:76-85.
- 36 Ehrlichová M, Koc M, Truksa J, Nadová Z, Václavíková R, Kovár J: Cell death induced by taxanes in breast cancer cells: cytochrome C is released in resistant but not in sensitive cells. *Anticancer Res* 2005;25:4215-4224.
- 37 Voborilová J, Nemcová-Fürstová V, Neubauerová J, Ojima I, Zanardi I, Gut I, Kovár J: Cell death induced by novel fluorinated taxanes in drug-sensitive and drug-resistant cancer cells. *Invest New Drugs* 2011;29:411-423.
- 38 Yan Y, Gao YY, Liu BQ, Niu XF, Zhuang Y, Wang HQ: Resveratrol-induced cytotoxicity in human Burkitt's lymphoma cells is coupled to the unfolded protein response. *BMC Cancer* 2010;10:445-454.
- 39 Balusikova K, Neubauerova J, Dostalíková-Cimburova M, Horak J, Kovar J: Differing expression of genes involved in non-transferrin iron transport across plasma membrane in various cell types under iron deficiency and excess. *Mol Cell Biochemistry* 2009;321:123-133.
- 40 Lagerstedt SA, Hinrichs DR, Batt SM, Magera MJ, Rinaldo P, McConnell JP: Quantitative determination of plasma c8-c26 total fatty acids for the biochemical diagnosis of nutritional and metabolic disorders. *Mol Genet Metab* 2001;73:38-45.
- 41 El-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW, Vogelstein B: WAF1, a potential mediator of p53 tumor suppression. *Cell* 1993;75:817-825.
- 42 Tinel A, Janssens S, Lippens S, Cuenin S, Logette E, Jaccard B, Quadroni M, Tschopp J: Autophagy of PIDD marks the bifurcation between pro-death caspase-2 and pro-survival NF-kappaB pathway. *EMBO J* 2007;26:197-208.
- 43 Bertolotti A, Zhang Y, Hendershot LM, Harding HP, Ron D: Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. *Nat Cell Biol* 2000;2:326-332.
- 44 Oyadomari S, Mori M: Roles of CHOP/GADD153 in endoplasmic reticulum stress. *Cell Death Differ* 2004;11:381-389.
- 45 Eizirik DL, Cardozo AK, Cnop M: The role for endoplasmic reticulum stress in diabetes mellitus. *Endocr Rev* 2008;29:42-61.
- 46 Diakogiannaki E, Dhayal S, Childs CE, Calder PC, Welters HJ, Morgan NG: Mechanisms involved in the cytotoxic and cytoprotective actions of saturated versus monounsaturated long-chain fatty acids in pancreatic beta-cells. *J Endocrinol* 2007;194:283-291.
- 47 Bikopoulos G, da Silva Pimenta A, Lee SC, Lakey JR, Der SD, Chan CB, Ceddia RB, Wheeler MB, Rozakis-Adcock M: *Ex vivo* transcriptional profiling of human pancreatic islets following chronic exposure to monounsaturated fatty acids. *J Endocrinol* 2008;196:455-464.
- 48 Chauhan D, Hideshima T, Rosen S, Reed JC, Kharbanda S, Anderson KC: Apaf-1/cytochrome c-independent and Smac-dependent induction of apoptosis in multiple myeloma (MM) cells. *J Biol Chem* 2001;276:24453-24456.

- 49 Dirsch, VM, Müller IM, Eichhorst ST, Pettit GR, Kamano Y, Inoue M, Xu JP, Ichihara Y, Wanner G, Vollmar AM: Cephalostatin 1 selectively triggers the release of Smac/DIABLO and subsequent apoptosis that is characterized by an increased density of the mitochondrial matrix. *Cancer Res* 2003;63:8869-8876.
- 50 Yu J, Zhang L, Hwang PM, Rago C, Kinzler KW, Vogelstein B: Identification and classification of p53-regulated genes. *Proc Natl Acad Sci USA* 1999;96:14517-14522.
- 51 Kim IR, Murakami K, Chen NJ, Saibil SD, Matysiak-Zablocki E, Elford AR, Bonnard M, Benchimol S, Jurisicova A, Yeh WC, Ohashi PS: DNA damage- and stress-induced apoptosis occurs independently of PIDD. *Apoptosis* 2009;14:1039-1049.
- 52 Manzl C, Krumschnabel G, Bock F, Sohm B, Labi V, Baumgartner F, Logette E, Tschopp J, Villunger A: Caspase-2 activation in the absence of PIDDosome formation. *J Cell Biol* 2009;185:291-303.
- 53 Olsson M, Vakifahmetoglu H, Abruzzo PM, Högstrand K, Grandien A, Zhivotovsky B: DISC-mediated activation of caspase-2 in DNA damage-induced apoptosis. *Oncogene* 2009;28:1949-1959.
- 54 Maedler K, Spinas GA, Lehmann R, Sergeev P, Weber M, Fontana A, Kaiser N, Donath MY: Glucose induces beta-cell apoptosis via upregulation of the Fas receptor in human islets. *Diabetes* 2001;50:1683-1690.
- 55 Maedler K, Fontana A, Ris F, Sergeev P, Toso C, Oberholzer J, Lehmann R, Bachmann F, Tasinato A, Spinas GA, Halban PA, Donath MY: FLIP switches Fas-mediated glucose signaling in human pancreatic beta cells from apoptosis to cell replication. *Proc Natl Acad Sci USA* 2002;99:8236-8241.
- 56 Liadis N, Salmena L, Kwan E, Tajmir P, Schroer SA, Radziszewska A, Li X, Sheu L, Eweida M, Xu S, Gaisano HY, Hakem R, Woo M: Distinct *in vivo* roles of caspase-8 in beta-cells in physiological and diabetes models. *Diabetes* 2007;56:2302-2311.
- 57 Choi D, Radziszewska A, Schroer SA, Liadis N, Liu Y, Zhang Y, Lam PP, Sheu L, Hao Z, Gaisano HY, Woo M: Deletion of Fas in the pancreatic beta-cells leads to enhanced insulin secretion. *Am J Physiol Endocrinol Metab* 2009;297:E1304-1312.
- 58 Johnson JD, Han Z, Otani K, Ye H, Zhang Y, Wu H, Horikawa Y, Misler S, Bell GI, Polonsky KS: RyR2 and calpain-10 delineate a novel apoptosis pathway in pancreatic islets. *J Biol Chem* 2004;279:24794-24802.
- 59 Ladrière L, Igoillo-Esteve M, Cunha DA, Brion JP, Bugliani M, Marchetti P, Eizirik DL, Cnop M: Enhanced signaling downstream of ribonucleic Acid-activated protein kinase-like endoplasmic reticulum kinase potentiates lipotoxic endoplasmic reticulum stress in human islets. *J Clin Endocrinol Metab* 2010;95:1442-1449.
- 60 Cunha DA, Ladrière L, Ortis F, Igoillo-Esteve M, Gurzov EN, Lupi R, Marchetti P, Eizirik DL, Cnop M: Glucagon-like peptide-1 agonists protect pancreatic β -cells from lipotoxic endoplasmic reticulum stress through upregulation of BiP and JunB. *Diabetes* 2009;58:2851-2862.
- 61 Busch AK, Gurisik E, Cordery DV, Sudlow M, Denyer GS, Laybutt DR, Hughes WE, Biden TJ: Increased fatty acid desaturation and enhanced expression of stearoyl coenzyme A desaturase protects pancreatic beta-cells from lipopoptosis. *Diabetes* 2005;54:2917-2924.
- 62 Szegezdi E, Fitzgerald U, Samali A: Caspase-12 and ER-stress-mediated apoptosis: the story so far. *Ann N Y Acad Sci* 2003;1010:186-194.
- 63 Martinon F, Tschopp J: Inflammatory caspases and inflammasomes: master switches of inflammation. *Cell Death Differ* 2007;14:10-22.
- 64 Cheung HH, Lynn Kelly N, Liston P, Korneluk RG: Involvement of caspase-2 and caspase-9 in endoplasmic reticulum stress-induced apoptosis: a role for the IAPs. *Exp Cell Res* 2006;312:2347-2357.