Cellular Physiology and Biochemistry

Cell Physiol Biochem 2013;31:277-289
DOI: 10.1159/000343367
Published online: February 28, 2013
Accepted: February 07, 2013

1421-9778/13/0313-0277$38.00/0

Original Paper

Caspase-2 and JNK Activated by Saturated Fatty Acids are Not Involved in Apoptosis Induction but Modulate ER Stress in Human Pancreatic β-cells

Vlasta Němcová-Fürstová[a] Kamila Balušíková[a] Jan Šrámek[a] Roger F. James[b] Jan Kovář[a]

[a]Division of Cell and Molecular Biology & Center for Research of Diabetes, Metabolism and Nutrition, Third Faculty of Medicine, Charles University, Prague, [b]Department of Infection, Immunity and Inflammation, University of Leicester, Leicester

Key Words
Caspase-2 • JNK • Saturated fatty acids • Apoptosis • Endoplasmic reticulum stress • β-cells

Abstract
Background: Fatty acid-induced apoptosis and ER stress of pancreatic β-cells contribute to the development of type 2 diabetes, however, the molecular mechanisms involved are unclear. ***Aims:*** In this study we have tested the role of caspase-2 and suggested ER stress mediator JNK in saturated fatty acid-induced apoptosis of the human pancreatic β-cells NES2Y. ***Results:*** We found that stearic acid at apoptosis-inducing concentration activated ER stress signaling pathways, i.e. IRE1α, PERK and ATF6 pathways, in NES2Y cells. During stearic acid-induced apoptosis, JNK inhibition did not decrease the rate of apoptosis nor the activation of caspase-8, -9, -7 and -2 and PARP cleavage. In addition, inhibition of JNK activity did not affect CHOP expression although it did decrease the induction of BiP expression after stearic acid treatment. Caspase-2 silencing had no effect on PARP as well as caspase-8, -9 and -7 cleavage and the induction of CHOP expression, however, it also decreased the induction of BiP expression. Surprisingly, caspase-2 silencing was accompanied by increased phosphorylation of c-Jun. ***Conclusions:*** We have demonstrated that caspase-2 as well as JNK are not key players in apoptosis induction by saturated fatty acids in human pancreatic β-cells NES2Y. However, they appear to be involved in the modulation of saturated fatty acid-induced ER stress signaling, probably by a mechanism independent of c-Jun phosphorylation.

Introduction

There is convincing experimental evidence showing that saturated fatty acid exposure (in contrast to unsaturated fatty acids) is detrimental to pancreatic β-cell survival and leads to β-cell apoptosis by still unclear molecular mechanisms [1-3].

Jan Kovář

Division of Cell and Molecular Biology, Third Faculty of Medicine, Charles University, Ruská 87, 100 00 Prague 10 (Czech Republic)
Tel. +420/267102658, Fax +420/267102650, E-Mail jan.kovar@lf3.cuni.cz

As secretory cells, β-cells are especially prone to perturbations in endoplasmic reticulum (ER) function [4] and subsequent triggering of ER stress. Saturated fatty acids were shown to activate ER stress signaling in pancreatic β-cells *in vitro* [3, 5-8] and increased levels of ER stress markers have been demonstrated in β-cells of type 2 diabetes patients [9-11]. Therefore, ER stress is suggested as a likely mechanism that mediates the pro-apoptotic effect of saturated fatty acids in pancreatic β-cells [12, 13].

It has been proposed that ER stress signaling is mediated by three sensor proteins localized in the ER membrane, i.e. IRE1α (inositol-requiring protein 1α), PERK (protein kinase RNA (PKR)-like ER kinase) and ATF6 (activating transcription factor 6). Activation of IRE1α leads to JNK (c-Jun N-terminal kinase) activation by phosphorylation and to unconventional splicing of mRNA for XBP1 (X-box binding protein 1) which results in translation of active transcription factor (XBP1s). Activation of PERK results in the inhibition of protein translation via phosphorylation of eIF2α (eukaryotic initiation factor 2α). When the ATF6 pathway is activated, ATF6 translocates to nucleus where it functions as transcription factor. All this signaling is aimed primarily at the restoration of ER homeostasis, e.g. by decreasing protein translation and increasing the expression of chaperones, such as the prominent ER chaperone BiP (immunoglobulin heavy chain-binding protein) [14]. However, if this response fails, apoptosis is induced by the mechanisms that are not still completely understood. The proposed mediators are JNK and transcription factor CHOP (CCAAT-enhancer-binding protein (C/EBP) homologous protein) [15].

Within the framework of ER stress signaling, JNK is supposed to exert its effect via modulation of the activity of several Bcl-2 family proteins by phosphorylation and via phosphorylation of c-Jun that participates in formation of AP-1 (activator protein-1) complex involved in transcriptional regulation of many predominantly pro-apoptotic genes, including CHOP [16]. Fatty acid treatment activates JNK in β-cells [6, 17-19] and it is known that type 2 diabetes is associated with the activation of the JNK pathway [20], however, the contribution of JNK in fatty acid-induced apoptosis and ER stress in β-cells is not clear.

In our previous research we have newly demonstrated that caspase-2 is activated together with ER stress induction when apoptosis is induced by saturated fatty acids in human pancreatic β-cell line NES2Y [8, 21]. There are also several other lines of evidence pointing at caspase-2 as a possible transducer of pro-apoptotic ER stress signaling in various cell types [22-25] although the mode of its activation under ER stress conditions is rather speculative.

In the present study, we have tested the role of caspase-2 and suggested ER stress mediator JNK in saturated fatty acid-induced apoptosis and ER stress signaling in human pancreatic β-cells NES2Y. Here we demonstrate that caspase-2, as well as JNK, are not key players in apoptosis induction by saturated fatty acids in pancreatic β-cells. However, JNK appears to be involved in the modulation of saturated fatty acid-induced ER stress signaling.

Materials and Methods

Materials

All chemicals were from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise indicated. For western blot analysis and fluorescence microscopy, the following primary and secondary antibodies were used: anti-BiP (#3177), anti-CHOP (#2895), anti-cleaved caspase-7 (#9491), anti-cleaved caspase-8 (#9496), anti-cleaved caspase-9 (#9505), anti-phospho eIF2α (#9721), anti-phospho-c-Jun (#9261), anti-c-Jun (#9165), anti-phospho-SAPK/JNK (#4668), anti-SAPK/JNK (#9258) and anti-PARP (#9542) from Cell Signaling Technology (Danvers, MA, USA), anti-actin (clone AC-40) from Sigma-Aldrich (St. Louis, MO, USA), anti-ATF6 (ab 11909) and anti-caspase-2 (ab 32021) from Abcam (Cambridge, UK), HRP-linked goat anti-mouse and goat anti-rabbit antibody from Santa Cruz (Santa Cruz, CA, USA) and AlexaFluor 488-conjugated goat anti-mouse antibody from Invitrogen (Invitrogen-Molecular Probes, Eugene, OR, USA).

Cells and culture conditions

The human pancreatic β-cell line NES2Y [21, 26] was used. NES2Y are proliferating insulin-secreting cells with a defect in glucose responsiveness. Cells were routinely maintained in an RPMI 1640 based culture medium [27]. In experiments, a defined serum-free medium [28] supplemented with stearic acid bound to 2% fatty-acid free bovine serum albumin (BSA) was used [21]. Stock solution containing stearic acid bound to the 10% BSA in the serum-free medium was prepared as described previously [21] and 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 [29]. In some experiments, thapsigargin at a concentration of 1 μM in serum-containing culture medium was used for ER stress induction.

Flow cytometric assessment of cell viability

Cells (approximately $5x10^5$ cells per sample) were seeded and stearic acid or control media were applied after 24-h preincubation as described above (see "Western blot analysis"). After 18 and 24-h incubation, the percentage of viable, apoptotic and necrotic cells was determined employing flow cytometry and Annexin V-FITC Apoptosis Detection Kit (Abcam, Cambridge, UK) according to manufacturer´s instructions. Apoptotic cells were defined as annexin V-FITC positive cells. Necrotic cells were defined as propidium iodide-positive, annexin V-FITC negative cells.

Western blot analysis

Cells (approximately $1-3x10^6$ 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 stearic acid or thapsigargin at required concentration or by control medium. After required period of incubation, both adherent and floating cells were harvested and western blot analysis was performed as described previously [8]. All primary antibodies were used in 1:1 000 dilution with exception of anti-CHOP antibody that was diluted 1:500. The chemiluminiscent signal was detected using Carestream Gel Logic 4000 PRO Imaging System equipped with Carestream Molecular Imaging Software (Carestream Health, New Haven, CT, USA) for image acquisition and analysis.

Assessment of XBP1 mRNA splicing

Cells (approximately $1x10^6$ cells per sample) were seeded and stearic acid and thapsigargin were applied after 24-h preincubation as described above (see "Western blot analysis"). After 3, 6, 12 and 24-h incubation, the cells were harvested and the splicing of XBP1 mRNA was assessed by RT-PCR as described previously [8, 30] with GAPDH as a housekeeping gene [31].

Confocal microscopy analysis of ATF6 translocation

Cells were seeded onto coverslips (approximately 2×10^5 cells per coverslip) and stearic acid or thapsigargin were applied after 24-h preincubation as described above (see "Western blot analysis"). After desired period of induction, coverslips were fixed with 4% paraformaldehyde for 20 min at 37°C. After washing with PBS, cells were permeabilized with 0.1% Tween in PBS for 10 min and blocked with FX Enhancer (Invitrogen-Molecular Probes, Eugene, OR, USA). ATF6 was stained employing primary antibody against ATF6 (Abcam, Cambridge, UK) (1:50 in PBS at 4°C overnight) and AlexaFluor 488-conjugated goat anti-mouse secondary antibody (Invitrogen-Molecular Probes, Eugene, OR, USA) (1:100 in PBS for 2 hour in the dark at room temperature). Stained cells on coverslips were transfered onto a droplet of Vectashield® Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA) or ProLong Gold with DAPI (Invitrogen-Molecular Probes, Paisley, UK) 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.

Inhibition of JNK kinase activity

Cells (approximately $1x10^6$ cells per sample) were seeded and after a 23-h preincubation period allowing cells to attach, the culture medium was replaced by the serum-free medium (for stearic acid treatment) or serum-containing medium (for thapsigargin treatment) with JNK inhibitor SP600125 (Enzo Life Sciences, Farmingdale, NY, USA) at desired concentration, or by control medium with the vehiculum

only (DMSO). After 1 h of pretreatment with JNK inhibitor, stearic acid and BSA or thapsigargin were added to achieve the required concentrations. After 18 h and 24 h of incubation the cells were harvested and lysates were prepared for Western blot analysis.

Assessment of the effect of JNK inhibition on growth and viability

Cells were seeded at 2 x 10^4 cells/100 µl of culture media (see above) into wells of 96-well plate. The JNK inhibitor SP600125 and stearic acid were applied in the same way as described in „Inhibition of JNK kinase activity". After 48 h-incubation, the number of living cells was determined by hemocytometer counting after staining with trypan blue.

Silencing of caspase-2 expression by siRNA

For the inhibition of caspase-2 expression, caspase-2 specific siRNA (Applied Biosystems, Foster City, CA, USA) and INTERFERin (PolyPlus-Transfection, Illkirch, France) as transfection reagent, were used according to manufacturer instructions. Nonspecific siRNA (Applied Biosystems, Foster City, CA, USA) was used as negative control. The efficiency of inhibition of caspase-2 expression at the protein level was tested after 72 or 96 h of siRNA treatment in each experiment by Western blot analysis of caspase-2 expression.

For caspase-2 silencing, 1.5 x 10^5 cells were seeded into Petri dishes (Ø 6 cm). After 24 h allowing cells to attach, the media was changed for media containing caspase-2 specific siRNA or nonspecific siRNA at final concentration of 5 nM and INTERFERin transfection reagent at 1:300 dilution. After 72 h of incubation with siRNA, cells were harvested into their cultivation media and seeded in the same media into 6-well plates in concentration of 1 x 10^6 cells/2.5 ml per well. After 24 h allowing cells to attach, the media was replaced by relevant media for the treatment with stearic acid or thapsigargin, containing in addition fresh siRNA and transfection reagent at the same concentration as used for the initial inhibition of caspase-2 expression. Stearic acid- or thapsigargin-induced and control cells were harvested after 6, 18 and 24 h of treatment as described in „Western blot analysis". The level of caspase-2 expression during each experiment was tested after 72 h of induction with siRNA, i.e. at the time of seeding cells for stearic acid or thapsigargin application, and at the end of experiment, i.e. 24 h after the application.

Assessment of the effect of caspase-2 silencing on growth and viability

The experiment was set up exactly in the same way as described in „Inhibition of caspase-2 expression by siRNA", but after 72 h-incubation with siRNA, nontransfected and both caspase-2 siRNA and nonspecific siRNA treated cells were seeded at 2 x 10^4 cells/100 µl of relevant media (see above) into wells of 96-well plate and after 48 h-incubation with stearic acid, the number of living cells was determined by hemocytometer counting after staining with trypan blue.

Statistical analysis

Statistical significance of differences was determined by Student´s *t*-test. $P < 0.05$ was considered statistically significant.

Results

Assessment of the mode of stearic acid-induced cell death

Our previous studies showed that stearic acid at a concentration of 1 mM induces cell death in most of NES2Y cells within 48 h after stearic acid application. However, apoptotic cells as well as caspase-2 activation appeared within 24 h after the application [8, 21, our unpublished data]. Therefore, all effects were tested until 24 h after fatty acid application. Under the experimental conditions used, application of stearic acid (1 mM) led to apoptosis in 16.2 ± 0.8 % of cells and necrosis in 3.2 ± 0.2 % of cells within 18 h after the application. In corresponding controls it was 2.9 ± 0.1 % and 0.8 ± 0.1 %, respectively. Within 24 h after the application, it led to apoptosis in 28.3 ± 7.6 % of cells and necrosis in 6.3 ± 0.2 % cells. In corresponding controls it was 3.8 ± 0.1 % and 1.6 ± 0.1 %, respectively, as assessed by flow cytometry after annexin V-propidium iodide staining (data not shown).

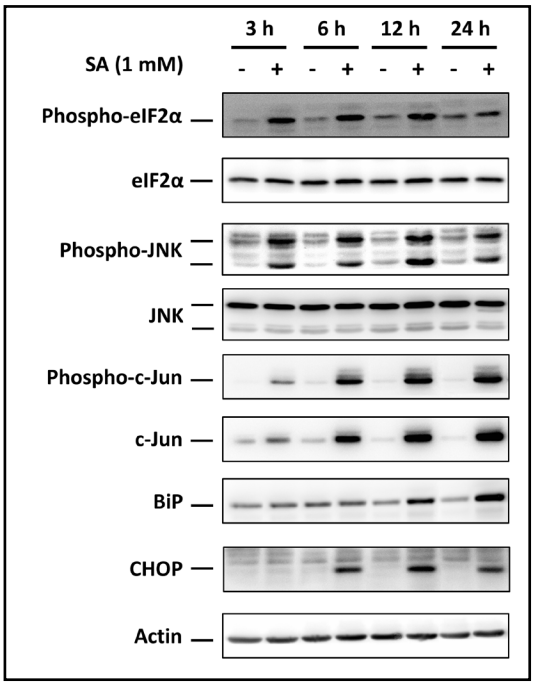


Fig. 1. Effect of 1 mM stearic acid (SA) on the expression of phospho-eIF2α, eIF2α, phospho-JNK, JNK, phospho-c-Jun, c-Jun, BiP and CHOP in NES2Y cells. Cells incubated without stearic acid represented control cells. After 3, 6, 12 and 24 h of incubation, the expression of proteins was determined by 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 at least three independent experiments.

Activation of ER stress signaling by stearic acid

We tested the level of expression of molecules known to be involved in ER stress signaling (phospho-elF2a, phospho-JNK, phospho-c-Jun, Bip and CHOP) at 3, 6, 12 and 24 h after stearic acid (1 mM) treatment of NES2Y cells. Interestingly, we found that JNK (IRE1α pathway of ER stress signaling) was phosphorylated and thus activated as early as 3 h after stearic acid application. This activation was accompanied by increased expression and phosphorylation of transcription factor c-Jun, i.e. the target of JNK activity. Phosphorylation of both these molecules lasted until 24 h after the application (Fig. 1). Stearic acid also induced early splicing (3 h after the application) of XBP1 (Fig. 2).

To assess the involvement of PERK pathway of ER signaling, we tested the phosphorylation status of the translation factor eIF2α. Stearic acid treatment resulted in early phosphorylation (3 h after the application) of eIF2α that lasted at least for the first 12 hours of treatment but then decreased to the level comparable with control cells in contrast to the molecules involved in IRE1α signaling, i.e. JNK and c-Jun.

We employed confocal microscopy in order to assess ATF6 translocation to the nucleus after its activation by cleavage, as it has been demonstrated after palmitate application in pancreatic β-cells previously [11]. As a positive control, cells were treated with thapsigargin, a synthetic inductor of ER stress. We were able to detect ATF6 translocation due to stearic acid application as early as 3 h after the application (Fig. 3). This activation lasted at least 12 h. After 24 h of treatment, the assessment of the extent of ATF6 translocation after stearic acid application was very unreliable due to undergoing apoptosis.

As to the downstream effector molecules we found CHOP expression to be induced 6 h after stearic acid application that lasted for 24 h (Fig. 1). In contrast, we did not observe any change in BiP level until 12 h of stearic acid treatment. Significant increase in BiP level was detected 12 h and 24 h after stearic acid application (Fig. 1).

Effect of JNK inhibition on ER stress

We assessed the role of JNK in stearic acid-induced ER stress and apoptosis in NES2Y β-cells employing SP600125, a specific inhibitor of JNK. The concentration of inhibitor, which was necessary for efficient JNK inhibition, was determined by testing the effect of several inhibitor concentrations on the level of phosphorylated c-Jun (data not shown).

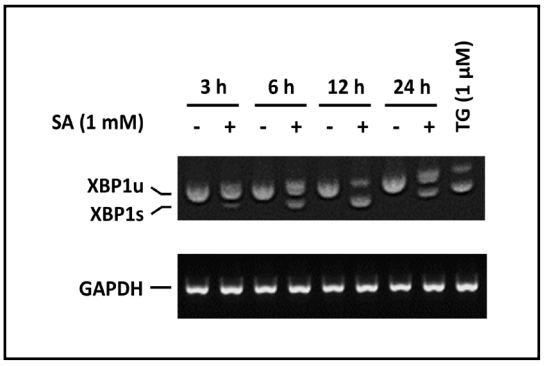


Fig. 2. Effect of 1 mM stearic acid (SA) on XBP1 mRNA splicing in NES2Y cells. Cells incubated without stearic acid represented control cells. After 3, 6, 12 and 24 h of incubation, the XBP1 splicing was assessed by RT-PCR using relevant primers (see „Materials and Methods"). As a positive control of XBP1 splicing, NES2Y cells were treated with 1 μM thapsigargin (TG). GAPDH was used as a control gene for RT-PCR. The data shown were obtained in one representative experiment of three independent experiments.

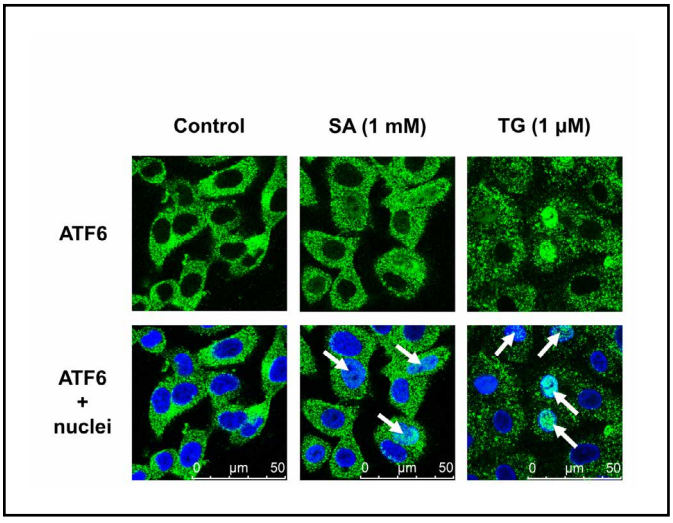


Fig. 3. Effect of 1 mM stearic acid (SA) on ATF6 translocation into nucleus in NES2Y cells. Cells incubated without stearic acid represented control cells. After 3 h of incubation, ATF6 (green) and nuclei (blue) within the cells were stained and representative images were acquired with Leica confocal scanning microscope (see „Materials and Methods"). As a positive control of ATF6 translocation, NES2Y cells were treated with 1 μM thapsigargin (TG). ATF6 staining and the merge of ATF6 and nuclear signal is shown. Data obtained in one representative experiment of three independent experiments are shown.

Using 50 and 100 μM SP600125 together with stearic acid, we achieved 61±5% and 82±7% decrease of phosphorylated c-Jun level, respectively, after 24 h of treatment compared to cells treated with stearic acid alone. Inhibition of JNK activity in stearic acid-treated cells resulted in significant decrease of BiP level after 18 h and 24 h of treatment. However, surprisingly CHOP expression induced by stearic acid was not affected by JNK inhibition (Fig. 4A, only data for 18 h are shown). To determine the role of JNK inhibition in the regulation of BiP expression, we assessed the effect of JNK inhibition on BiP upregulation induced by another ER stressor, i.e. thapsigargin. There was only slight effect of JNK inhibition on BiP expression after 18 h of treatment with 1 μM thapsigargin, however, the inhibitory effect on BiP expression was clearly detectable after shorter period (6 h) of treatment. Similarly to the effect of JNK inhibition after stearic acid application, CHOP expression induced by thapsigargin was not affected by JNK inhibition, (Fig. 5).

Effect of JNK inhibition on caspase activation and apoptosis induction

We showed previously that caspase-7, caspase-9, caspase-8 and caspase-2 are activated during stearic acid-induced apoptosis in NES2Y β-cells, in contrast to caspase-3. Therefore, we tested the effect of inhibition of JNK activity on activation (cleavage) of these caspases and also on the cleavage of PARP (common marker of apoptosis) after 18 and 24 h of treatment. However, the level of cleaved caspase-7, caspase-9, caspase-8 and PARP and the

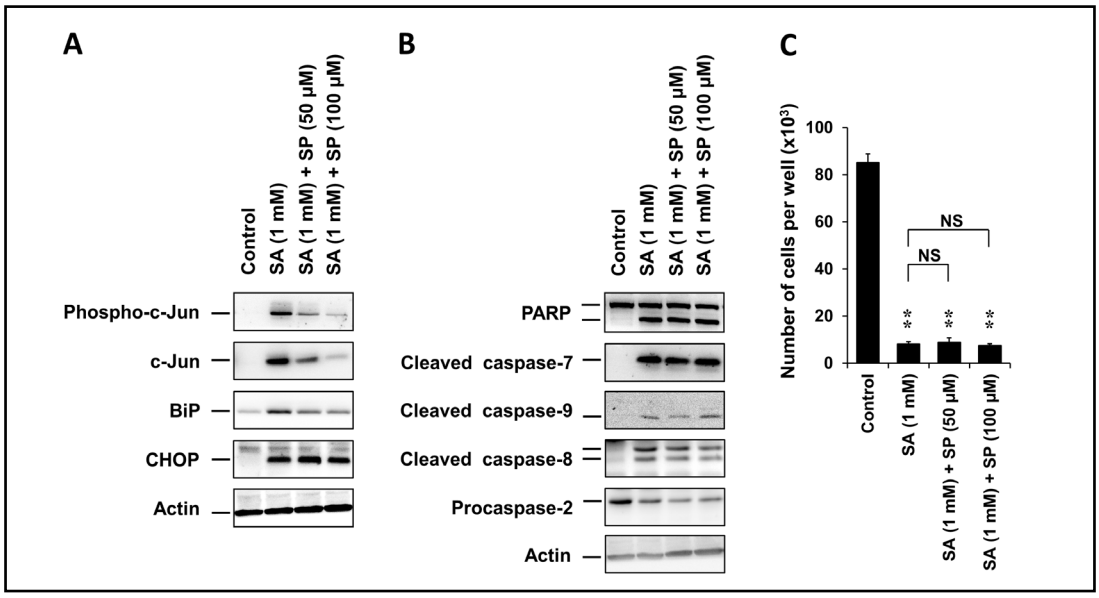
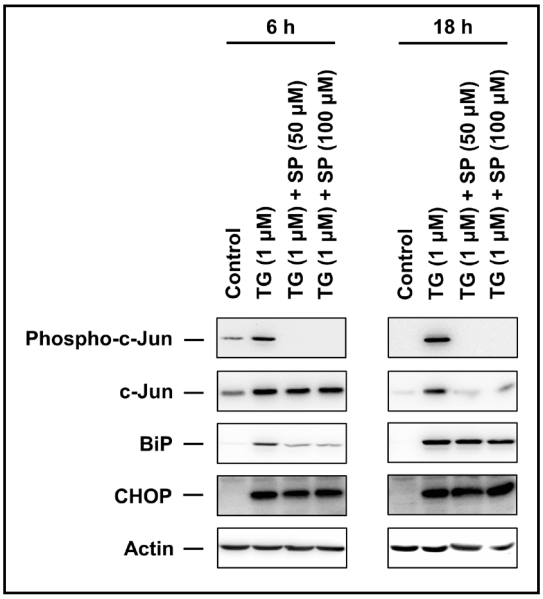


Fig. 4. Effect of specific JNK inhibitor SP600125 (SP) on the effect of 1 mM stearic acid (SA) on (A) the expression of phospho-c-Jun, c-Jun, BiP and CHOP, on (B) the cleavage of PARP, caspase-7, caspase-9, caspase-8 and caspase-2 in NES2Y cells and on (C) the cell growth and viability of NES2Y cells. Cells incubated without stearic acid represented control cells. After 18 h (A) or 24 h (B) of incubation, the expression or cleavage of 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. (C) Cells were seeded at $2x10^4$ cells/100 µl of culture medium per well of 96-well plate and stearic acid and SP600125 were applied as described in "Materials and Methods". The number of living cells was determined after 48 h of incubation. Each column represents the mean of 4 separate cultures ± SEM. ** $P<0.01$ when comparing the effect of stearic acid with or without SP600125 and control cells, nonsignificant (NS) when comparing the effect of stearic acid applied together with SP600125 and the treatment with stearic acid alone.

Fig. 5. Effect of specific JNK inhibitor SP600125 (SP) on the effect of 1 µM thapsigargin (TG) on the expression of phospho-c-Jun, c-Jun, BiP and CHOP in NES2Y cells. Cells incubated without thapsigargin represented control cells. After 6 h and 18 h of incubation, the expression of 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.



level of procaspase-2 were not influenced by JNK inhibition during stearic acid treatment (Fig. 4B, only data for 18 h are shown) as well as the cell growth and viability within 48 h of treatment (Fig. 4C).

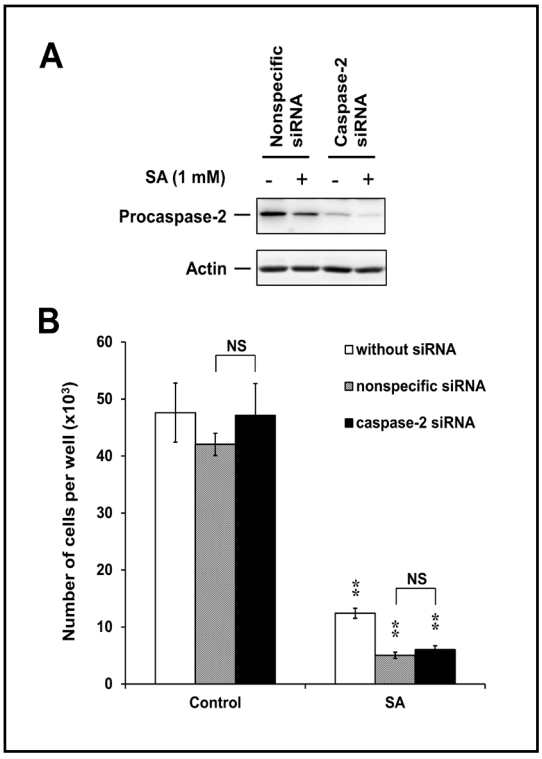


Fig. 6. Effect of caspase-2 silencing by specific siRNA on (A) caspase-2 expression and on (B) the effect of 1 mM stearic acid (SA) on the cell growth and viability of NES2Y cells. Cells incubated without stearic acid represented control cells. (A) The expression of caspase-2 after 24 h of incubation with stearic acid was determined by western blot analysis using caspase-2 specific antibody (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. (B) Cells treated with nonspecific siRNA, caspase-2 specific siRNA and without siRNA as described in „Materials and Methods" were seeded at $2x10^4$ cells/100 µl of culture medium per well of 96-well plate and stearic acid was applied (see "Materials and Methods"). The number of living cells was determined after 48 h of incubation. Each column represents the mean of 4 separate cultures ± SEM. ** $P<0.01$ when comparing the effect of stearic acid with respective control cells, nonsignificant (NS) when comparing the effect of caspase-2 and nonspecific siRNA ($P \geq 0.05$).

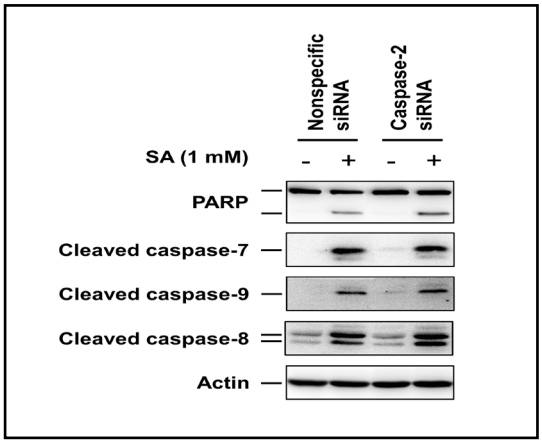


Fig. 7. Effect of caspase-2 silencing by specific siRNA on the effect of 1 mM stearic acid (SA) on the cleavage of PARP, caspase-7, caspase-9 and caspase-8 in NES2Y cells. Cells incubated without stearic acid represented control cells. After 18 h of incubation, the cleavage of proteins was determined 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.

Effect of caspase-2 silencing on apoptosis induction and caspase activation

To assess the role of caspase-2 in apoptosis induced by stearic acid in NES2Y cells, we employed siRNA approach to silence caspase-2 expression. Using caspase-2 specific siRNA, we achieved 81±4% inhibition of caspase-2 expression in the latest time point tested, i.e. 24 h of treatment (Fig. 6A). First, we assessed the effect of caspase-2 silencing on the growth and viability of NES2Y cells during the treatment with stearic acid. After 48 h of treatment, there was no significant difference between the number of cells with down-regulated expression of caspase-2 and cells treated with control nonspecific siRNA. We also did not observe any significant effect of the transfection procedure per se on the growth and viability of NES2Y cells (Fig. 6B). Next, we tested the effect of inhibition of caspase-2 expression on PARP cleavage and the activation of caspase-7, -9 and -8. Activation of all caspases tested was readily detectable after 18 h of treatment, however, we did not see any inhibitory effect of caspase-2 silencing on the activation of caspases tested as well as on PARP cleavage (Fig. 7).

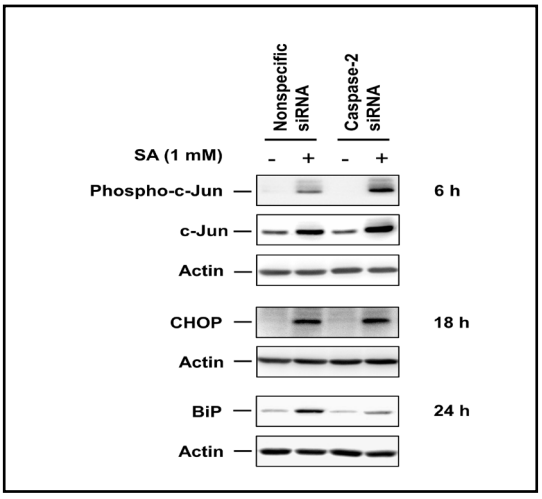


Fig. 8. Effect of caspase-2 silencing by specific siRNA on the effect of 1 mM stearic acid (SA) on the expression of phospho-c-Jun, c-Jun, CHOP and BiP in NES2Y cells. Cells incubated without stearic acid represented control cells. After 6 h (phospho-c-Jun and c-Jun), 18 h (CHOP) and 24 h (BiP) of incubation, the expression of 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.

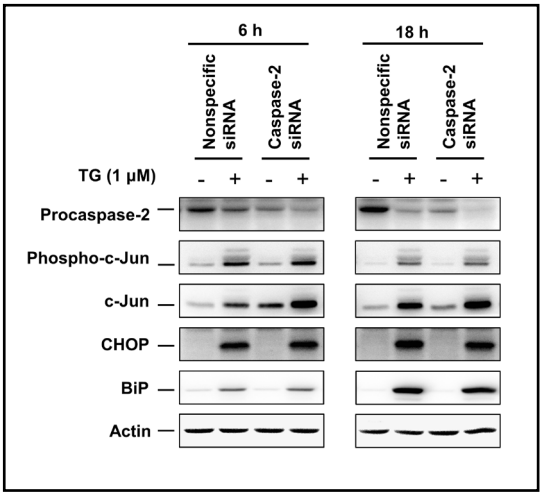


Fig. 9. Effect of caspase-2 silencing by specific siRNA on the effect of 1 µM thapsigargin (TG) on the expression of procaspase-2, phospho-c-Jun, c-Jun, CHOP and BiP in NES2Y cells. Cells incubated without thapsigargin represented control cells. After 6 h and 18 h of incubation, the expression of proteins 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.

Effect of caspase-2 silencing on ER stress

We did not detect any effect of caspase-2 silencing on the induction of CHOP expression after 18 and 24 h of stearic acid treatment (Fig. 8, only data for 18 h are shown). However, caspase-2 silencing led, similarly like the inhibition of JNK activity, to the decrease of BiP upregulation induced by stearic acid treatment for 18 h and 24 h (Fig. 8, only data for 24 h are shown). Surprisingly, we also found increased level of phosphorylated c-Jun in cells with silenced caspase-2 expression after 6 h, 18 h and 24 h of treatment with stearic acid (Fig. 8, only data for 6 h are shown). In order to compare the involvement of caspase-2 in the upregulation of BiP expression and c-Jun phosphorylation after stearic acid application (Fig. 8) with the involvement after ER stress induction, we tested the effect of caspase-2 silencing after ER stress induction by thapsigargin. However, we found no effect of caspase-2 silencing on thapsigargin-induced upregulation of BiP expression and c-Jun phoshorylation (Fig. 9).

Discussion

Our previous studies showed that saturated fatty acids (palmitic acid and, more efficiently, stearic acid) induce apoptosis associated with caspase-2 activation in human pancreatic β-cells NES2Y [8, 21]. These studies also pointed at a possible role of ER stress in apoptosis induction by saturated fatty acids [8]. Thus in this study we decided to test the role of caspase-2 and the ER stress mediator JNK in stearic acid-induced apoptosis of NES2Y cells considering their possible role in ER stress signaling.

NES2Y cells respond to fatty acids similarly like primary human β-cells [2, 6, 7, 32], as we also found in our previous experiments [8, 21]. Thus it may be expected that the responses to saturated fatty acid seen in this study with NES2Y cells are comparable to the effects on human β-cells *in vivo*. Currently, there is a limited number of studies investigating the effect of fatty acids on ER stress signaling in relation to apoptosis in β-cells of human origin [6, 7, 32]. Moreover, the effect of stearate is studied only rarely despite it appears to be more effective than palmitate in human β-cells [1, 8].

We demonstrated activation of all known pathways of ER stress signaling, i.e. IRE1α, PERK and ATF6 pathway, by saturated stearic acid at a cell death-inducing concentration (1 mM) in NES2Y cells as soon as 3 h after the treatment. After that we detected upregulation of the expression of ER stress markers CHOP and BiP 6 h and 12 h after the treatment, respectively.

JNK is considered as one of the most possible mediators that connect excessive ER stress with apoptosis induction. However, in human NES2Y cells, inhibition of JNK activity did not lead to significant changes in the level of stearate-induced apoptosis (see Fig. 4C). In the study of Cunha et al. with rat β-cells INS-1E [6] inhibition of palmitate-induced JNK activation resulted in a partial reduction of apoptosis. In another study with INS-1E cells, JNK inhibition was showed to be anti-apoptotic [18]. In NES2Y cells, JNK activation by stearic acid was accompanied with intensive and fast c-Jun phosphorylation (see Fig. 1A). However, there are conflicting data concerning the question whether palmitate-induced JNK activation in INS-1E cells also leads to phosphorylation of c-Jun [18, 33]. Therefore it is somewhat difficult to compare the data. Nevertheless, none of the studies showed complete inhibition of fatty acid-induced apoptosis by JNK inhibition. It indicates that some other mediator(s) or pathway(s) different from JNK signaling should be involved in saturated fatty acid-induced apoptosis.

Several lines of evidence indicate that fatty acid-induced activation of JNK in β-cells is very fast and precedes the activation of ER stress response and therefore could not result solely from IRE1α activation [17, 19]. JNK can also be activated by MAP kinase kinases MKK4 and MKK7 and thus its activation by saturated fatty acids can result also from the involvement of signaling pathways that are not related to ER stress induction [16]. Our results do not contradict this hypothesis since we observed full JNK activation after 3 h but splicing of XBP1 reached its maximum at 6 h. To sum up, our data suggest that JNK signaling in NES2Y cells is not a mediator of stearic acid-induced apoptosis regardless of whether its activation is solely the result of ER stress induction or whether also other signaling pathways contribute to its activation.

In rodent cells, the activation of caspase cascade in response to ER stress is initiated by the activation of caspase-12 [34]. However, except of specific population of African descendents, the human population does not possess functional caspase-12 [35]. Several lines of evidence indicate that caspase-2 [22-25] or caspase-4 [36, 37] could substitute for caspase-12 in human cells but this hypothesis has not yet been tested in pancreatic β-cells. Our preliminary experiments did not point at a significant role of caspase-4 activation in stearic acid-induced apoptosis of NES2Y cells (data not shown). However, we previously identified that caspase-2 was significantly activated by palmitate and stearate in NES2Y cells [8, 21]. Caspase-2 was also shown to play the key role in GTP-depletion-induced β-cell apoptosis [38]. In this study we show not only that caspase-2 is dispensable for fatty acid-induced apoptosis in NES2Y but we also found no influence of caspase-2 silencing on the activation of other caspases previously shown to be associated with stearate-induced apoptosis [8].

During stearic acid-induced apoptosis in NES2Y cells, in contrast to JNK inhibition, caspase-2 silencing was accompanied by the increased phosphorylation of c-Jun. However, both caspase-2 silencing and JNK inhibition had similar effect on CHOP and BiP expression after stearic acid treatment (see Figs. 4A and 8). This indicates that CHOP and BiP expression in NES2Y cells are not regulated by c-Jun. This is consistent with the finding that dimers of transcription factor AP-1 that contribute to CHOP induction by palmitate in INS-1E β-cells are composed preferentially of c-Fos and jun-B, but not c-Jun [33] and the activity

of jun-B is not controlled by JNK-mediated phosphorylation [39]. However, the mechanism mediating the decrease of BiP expression downstream of JNK inhibition (see Fig. 4A) and the mechanism responsible for increased c-Jun phosphorylation after caspase-2 silencing (see Fig. 8) remain obscure. Nevertheless, some regulatory role of JNK in BiP expression is likely, as demonstrated by the decrease of BiP expression by JNK inhibition during thapsigargin-induced ER stress (see Fig. 5) [40].

Downregulation of BiP expression due to caspase-2 silencing after stearic acid-induced apoptosis (see Fig. 8) is unexpected. As we did not observe this downregulation during thapsigargin-induced ER stress, the downregulation by caspase-2 silencing seems to be restricted to specific ER stress stimuli, such as saturated fatty acids. Caspase-2 was shown to be transcriptionally regulated by the sterol regulatory element binding protein 2 (SREBP2) and to participate together with this transcription factor in the activation of endogenous cholesterol and triacylglycerol synthesis [41, 42]. In this way, caspase-2 silencing might be associated with lowering of intracellular lipid levels which could be reflected in turn by the alleviation of ER stress and reduction in BiP expression induced by stearic acid.

Taken together, in this study we assessed the role of caspase-2 and JNK in stearic acid-induced apoptosis and ER stress in NES2Y cells. Stearic acid activates IRE1α, PERK and ATF6 pathways of ER stress signaling. Neither caspase-2 nor JNK activation is involved in apoptosis induction by saturated fatty acids. However, both caspase-2 and JNK modulate stearic acid-induced ER stress in NES2Y cells.

Acknowledgements

This work was supported by research program MSM0021620814 of the Third Faculty of Medicine, Charles University in Prague, provided by Ministry of Education, Youth and Sports of the Czech Republic and by research projects SVV-2010-260704, SVV-2011-262706, UNCE 204015 and PRVOUK P31 from the Charles University in Prague, Czech Republic. We would like to thank Dr. Lenka Rossmeislová for her technical assistance concerning confocal microscopy.

References

1 Eitel K, Staiger H, Brendel MD, Brandhorst D, Bretzel RG, Haring HU, Kellerer M: Different role of saturated and unsaturated fatty acids in beta-cell apoptosis. Biochem Biophys Res Commun 2002;299:853-856.

2 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 beta-cell turnover and function. Diabetes 2003;52:726-733.

3 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.

4 Harding HP, Zeng H, Zhang Y, Jungries R, Chung P, Plesken H, Sabatini DD, Ron D: Diabetes mellitus and exocrine pancreatic dysfunction in perk-/- mice reveals a role for translational control in secretory cell survival. Mol Cell 2001;7:1153-1163.

5 Kharroubi I, Ladriere L, Cardozo AK, Dogusan Z, Cnop M, Eizirik DL: Free fatty acids and cytokines induce pancreatic beta-cell apoptosis by different mechanisms: role of nuclear factor-kappaB and endoplasmic reticulum stress. Endocrinology 2004;145:5087-5096.

6 Cunha DA, Hekerman P, Ladriere 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 beta-cells. J Cell Sci 2008;121:2308-2318.

7 Ladriere 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.
8 Nemcova-Furstova V, James RF, Kovar J: Inhibitory effect of unsaturated fatty acids on saturated fatty acid-induced apoptosis in human pancreatic beta-cells: activation of caspases and ER stress induction. Cell Physiol Biochem 2011;27:525-538.
9 Hartman MG, Lu D, Kim ML, Kociba GJ, Shukri T, Buteau J, Wang X, Frankel WL, Guttridge D, Prentki M, Grey ST, Ron D, Hai T: Role for activating transcription factor 3 in stress-induced beta-cell apoptosis. Mol Cell Biol 2004;24:5721-5732.
10 Huang CJ, Lin CY, Haataja L, Gurlo T, Butler AE, Rizza RA, Butler PC: High expression rates of human islet amyloid polypeptide induce endoplasmic reticulum stress mediated beta-cell apoptosis, a characteristic of humans with type 2 but not type 1 diabetes. Diabetes 2007;56:2016-2027.
11 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.
12 Cnop M, Ladrière L, Igoillo-Esteve M, Moura RF, Cunha DA: Causes and cures for endoplasmic reticulum stress in lipotoxic β-cell dysfunction. Diabetes Obes Metab 2010;12:S76-82.
13 Fonseca SG, Gromada J, Urano F: Endoplasmic reticulum stress and pancreatic β-cell death. Trends Endocrinol Metab 2011;22:266-74.
14 Hetz C: The unfolded protein response: controlling cell fate decisions under ER stress and beyond. Nat Rev Mol Cell Biol 2012;13:89-102.
15 Tabas I, Ron D: Integrating the mechanisms of apoptosis induced by endoplasmic reticulum stress. Nat Cell Biol 2011;13:184-190.
16 Dhanasekaran DN, Reddy EP: JNK signaling in apoptosis. Oncogene 2008;27:6245-6251.
17 Martinez SC, Tanabe K, Cras-Meneur C, Abumrad NA, Bernal-Mizrachi E, Permutt MA: Inhibition of Foxo1 protects pancreatic islet beta-cells against fatty acid and endoplasmic reticulum stress-induced apoptosis. Diabetes 2008;57:846-859.
18 Bachar E, Ariav Y, Ketzinel-Gilad M, Cerasi E, Kaiser N, Leibowitz G: Glucose amplifies fatty acid-induced endoplasmic reticulum stress in pancreatic beta-cells via activation of mTORC1. PLoS One 2009;4:e4954.
19 Tanabe K, Liu Y, Hasan SD, Martinez SC, Cras-Meneur C, Welling CM, Bernal-Mizrachi E, Tanizawa Y, Rhodes CJ, Zmuda E, Hai T, Abumrad NA, Permutt MA: Glucose and fatty acids synergize to promote B-cell apoptosis through activation of glycogen synthase kinase 3beta independent of JNK activation. PLoS One 2011;6:e18146.
20 Ozcan U, Cao Q, Yilmaz E, Lee AH, Iwakoshi NN, Ozdelen E, Tuncman G, Gorgun C, Glimcher LH, Hotamisligil GS: Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. Science 2004;306:457-461.
21 Furstova V, Kopska T, James RF, Kovar J: Comparison of the effect of individual saturated and unsaturated fatty acids on cell growth and death induction in the human pancreatic beta-cell line NES2Y. Life Sci 2008;82:684-691.
22 Cheung HH, Lynn KN, 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.
23 Gu H, Chen X, Gao G, Dong H: Caspase-2 functions upstream of mitochondria in endoplasmic reticulum stress-induced apoptosis by bortezomib in human myeloma cells. Mol Cancer Ther 2008;7:2298-2307.
24 Upton JP, Austgen K, Nishino M, Coakley KM, Hagen A, Han D, Papa FR, Oakes SA: Caspase-2 cleavage of BID is a critical apoptotic signal downstream of endoplasmic reticulum stress. Mol Cell Biol 2008;28:3943-3951.
25 Huang WC, Lin YS, Chen CL, Wang CY, Chiu WH, Lin CF: Glycogen synthase kinase-3beta mediates endoplasmic reticulum stress-induced lysosomal apoptosis in leukemia. J Pharmacol Exp Ther 2009;329:524-531.
26 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.

27 Musilkova J, Kovar 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.
28 Kovar 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.
29 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.
30 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.
31 Balusikova K, Neubauerova J, Dostalikova-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 Biochem 2009;321:123-133.
32 Lai E, Bikopoulos G, Wheeler MB, Rozakis-Adcock M, Volchuk A: Differential activation of ER stress and apoptosis in response to chronically elevated free fatty acids in pancreatic beta-cells. Am J Physiol Endocrinol Metab 2008;294:E540-E550.
33 Pirot P, Ortis F, Cnop M, Ma Y, Hendershot LM, Eizirik DL, Cardozo AK: Transcriptional regulation of the endoplasmic reticulum stress gene chop in pancreatic insulin-producing cells. Diabetes 2007;56:1069-1077.
34 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.
35 Martinon F, Tschopp J: Inflammatory caspases and inflammasomes: master switches of inflammation. Cell Death Differ 2007;14:10-22.
36 Hitomi J, Katayama T, Eguchi Y, Kudo T, Taniguchi M, Koyama Y, Manabe T, Yamagishi S, Bando Y, Imaizumi K, Tsujimoto Y, Tohyama M: Involvement of caspase-4 in endoplasmic reticulum stress-induced apoptosis and Abeta-induced cell death. J Cell Biol 2004;165:347-56.
37 Yamamuro A, Kishino T, Ohshima Y, Yoshioka Y, Kimura T, Kasai A, Maeda S: Caspase-4 directly activates caspase-9 in endoplasmic reticulum stress-induced apoptosis in SH-SY5Y cells. J Pharmacol Sci 2011;115:239-43.
38 Huo J, Luo RH, Metz SA, Li G: Activation of caspase-2 mediates the apoptosis induced by GTP-depletion in insulin-secreting (HIT-T15) cells. Endocrinology 2002;143:1695-1704.
39 Kallunki T, Deng T, Hibi M, Karin M: c-Jun can recruit JNK to phosphorylate dimerization partners via specific docking interactions. Cell 1996;87:929-939.
40 Raciti M, Lotti LV, Valia S, Pulcinelli FM, Di Renzo L: JNK2 is activated during ER stress and promotes cell survival. Cell Death Dis 2012,3:e429.
41 Logette E, Le Jossic-Corcos C, Masson D, Solier S, Sequeira-Legrand A, Dugail I, Lemaire-Ewing S, Desoche L, Solary E, Corcos L: Caspase-2, a novel lipid sensor under the control of sterol regulatory element binding protein 2. Mol Cell Biol 2005;25:9621-9631.
42 Logette E, Solary E, Corcos L: Identification of a functional DNA binding site for the SREBP-1c transcription factor in the first intron of the human caspase-2 gene. Biochim Biophys Acta 2005;1738:1-5.