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Ph.D. thesis

Molecular mechanisms of apoptosis regulation by fatty acids in pancreatic β-cells

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DECLARATION

Hereby, I declare that this thesis has been written by me and is based on my experimental work done at the Division of Cell and Molecular Biology under supervision of prof. Jan Kovář.

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1 CONTENT

2 LIST OF ABBREVIATIONS

ΔΨm	mitochondrial membrane potential
ACS	acetyl-coenzyme A synthetase
AIF	apoptosis-inducing factor
Apaf-1	apoptotic protease-activating factor 1
ARC	apoptosis repressor with caspase recruitment domain
ATF4	activating transcription factor 4
ATF6	activating transcription factor 6
ATF6f	activating transcription factor 6 fragment
ATP	adenosine triphosphate
Bad	Bcl-2-associated death promoter
Bak	Bcl-2 antagonist/killer-1
Bax	Bcl-2-associated X protein
Bcl-2	B-cell CLL/lymphoma-2
Bcl-X_L	B-cell leukemia/lymphoma-extra large
BH	Bcl-2 homology
Bid	BH3-interacting domain
Bim	Bcl-2-interacting mediator of cell death
BiP	immunoglobulin heavy chain-binding protein
BSA	bovine serum albumin
c-Myc	cellular-Myc
CARD	caspase activation and recruitment domain
CHOP	C/EBP-homologous protein
Co-A	coenzyme A
COPII	coat protein II
CPT-1	carnitine palmitoyltransferase-1
CRADD	caspase-2 and RIPK1 domain containing adaptor with death domain
DAPI	4,6-diamidino-2-phenylindole
db	*diabetic*
DD	death domain
DED	death effector domain
DIABLO	direct inhibitor of apoptosis-binding protein with low pI
DISC	death-inducing signaling complex
DMEM	Dulbecco´s Modified Eagle´s Medium
DR	death receptor
EA	elaidic acid, elaidate
eIF2α	eukaryotic initiation factor 2α
endo G	endonuclease G
ER	endoplasmic reticulum
ERAD	endoplasmic reticulum-associated degradation
fa	*fatty*
FA	fatty acid
FADD	Fas-associated death domain
FasL	Fas ligand
FAT	fatty acid translocase
FATP	fatty acid transport protein

FLICE	FADD-like IL-1β-converting enzyme
FLIP	FLICE-inhibitory protein
GADD	growth arrest and DNA damage
GPR	G protein-coupled receptor
Grp78	glucose-regulated protein 78
GSIS	glucose-stimulated insulin secretion
hIAPP	human islet amyloid polypeptide
HSP	heat shock protein
HTRA2	high temperature requirement protein A2
IAP	inhibitor of apoptosis protein
ICAD	inhibitor of caspase-dependent DNase
INF-γ	interferon-γ
iNOS	inducible nitric oxide synthase
IP3R	inositol 1,4,5-trisphosphate receptor
IRE1α	inositol-requiring protein 1α
IRE1β	inositol-requiring protein 1β
IUF1	insulin upstream factor 1
JNK	c-Jun N-terminal kinase
Kir6.2	inward rectifier potassium channel 6.2
LA	linoleic acid
LADA	latent autoimmune diabetes of adults
LC-CoA	long-chain acyl-coenzyme A
MAP	mitogen-activated protein
Mcl-1	myeloid cell leukemia-1
miRNA	microRNA
MODY	maturity onset diabetes in the young
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
NO	nitric oxide
NOS	nitric oxide synthase
OA	oleic acid, oleate
ob	*obese*
PA	palmitic acid, palmitate
PARP	poly-ADP-ribose polymerase
PDX	pancreatic duodenal homeobox
PERK	protein kinase RNA-like endoplasmic reticulum kinase
PIDD	p53-induced protein with a death domain
POA	palmitoleic acid, palmitoleate
PP	pancreatic polypeptide
PTP	permeability transition pore
Puma	p53-upregulated modulator of apoptosis
RAIDD	RIP-associated ICH-1/CED-3 homologous protein with a death domain
RIDD	regulated IRE1-dependent decay
RIP	receptor-interacting protein
RNA	ribonucleic acid
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute
SA	stearic acid, stearate
SAPK	stress-activated protein kinase

siRNA	small interfering RNA
Smac	second mitochondria-derived activator of caspase
SP600125	1,9-pyrazoloanthrone
SREBP	sterol-regulatory element- binding protein
SUR1	sulfonylurea receptor 1
SV40	simian virus 40
T1DM	type 1 diabetes mellitus
T2DM	type 2 diabetes mellitus
TAG	triacylglycerol
tBid	truncated Bid
TNF R	tumour necrosis factor receptor
TNF-α	tumour necrosis factor α
TRADD	TNF R-associated protein with a death domain
TRAF2	TNF R-associated factor 2
TRAIL R	TRAIL receptor
TRAIL	tumour necrosis factor-α-related apoptosis-inducing ligand
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling
TWEAK	tumour necrosis factor-like weak inducer of apoptosis
TWEAK R	TWEAK receptor
UPR	unfolded protein response
UV	ultraviolet
WFS1	Wolfram syndrome 1
XBP1	X-box binding protein 1
XBP1s	spliced form of XBP1
XBP1u	unspliced form XBP1
XIAP	X-linked IAP
z-VAD-fmk	N-benzyloxycarbonyl-Val-Ala-Asp(O-methyl)-fluoromethylketone
ZDF	Zucker diabetic fatty rat
ZFR	Zucker fatty rat

3 SUMMARY

The incidence of type 2 diabetes is growing rapidly and represents a big threat for the human health care and economy system as well in the 21st century. The association of type 2 diabetes with obesity is apparent and dysfunction and apoptosis of pancreatic β-cells caused by elevated levels of fatty acids in circulation are considered as an important factor contributing to the development of this disease. However, molecular mechanisms that underlie these detrimental effects of fatty acids are only partially understood.

The aim of this research project was to contribute to elucidation of mechanisms by which saturated and unsaturated fatty acids regulate viability and apoptosis induction in human pancreatic β-cells *in vitro*. Employing human pancreatic β-cell line NES2Y, we showed that increased levels of relevant dietary saturated fatty acids (palmitic and stearic acid) induce apoptosis of pancreatic β-cells, in contrast to relevant dietary unsaturated fatty acids (e.g. palmitoleic and oleic acid). We found that stearic acid-induced apoptosis is accompanied by significant activation of caspase-2, -6, -7, -8 and -9, but not by significant activation of caspase-3. Nevertheless, it was not associated with significant cytochrome c release, alteration in PIDD, Fas receptor and Fas ligand expression and activation of p53. However, stearic acid application caused rapid activation of endoplasmic reticulum (ER) stress signaling pathways (i.e. IRE1α, PERK and ATF6 pathways). In addition, we demonstrated that stearic acid-induced apoptosis is not dependent on caspase-2 and JNK activation. However, both these molecules seem to be involved in the modulation of ER stress in NES2Y cells.

Furthermore, we found that the cell death-inducing effect of saturated fatty acids is blocked by co-application of unsaturated fatty acids. We showed that the inhibitory effect of oleic acid on stearic acid-induced apoptosis occurs upstream of caspase activation and does not involve an interference with the mitochondrial pathway of apoptosis induction, p53 activation and PIDD, Fas receptor and Fas ligand expression. Oleic acid also inhibited the stearic acid-induced increase in the expression of several ER stress markers (i.e. BiP, CHOP and XBP1s). This indicates that the inhibitory effect oleic acid is exerted upstream or at the level of ER stress induction by saturated fatty acids.

In addition, we developed an alternative method for isolation of murine Langerhans islets whose novelty lies in the use of specific plastic for suspension cells for effective separation of the endocrine and exocrine tissue.

Our results contributed to the understanding of mechanisms of endoplasmic reticulum stress and apoptosis regulation by saturated and unsaturated fatty acids in pancreatic β-cells. Our findings may be useful as a basis for development of new strategies for type 2 diabetes treatment that are aimed at preservation of pancreatic β-cell function and viability.

4 SOUHRN

Incidence diabetu 2. typu velmi rychle narůstá a představuje velkou hrozbu pro zdravotnictví i ekonomiku 21. století. Diabetes 2. typu je jasně asociován s obezitou a dysfunkce a apoptóza pankreatických β buněk v důsledku zvýšené hladiny mastných kyselin v krvi je považována za důležitý faktor přispívající k rozvoji tohoto onemocnění. Molekulární mechanismy zodpovědné za neblahé účinky mastných kyselin však nejsou dostatečne známy.

Cílem tohoto výzkumného projektu bylo přispět k objasnění mechanismů, kterými nasycené a nenasycené mastné kyseliny regulují viabilitu a indukci apoptózy u lidských pankretických β buněk *in vitro*. U lidské β-buněčné linie NES2Y jsme ukázali, že zvýšené hladiny nasycených mastných kyselin přijímaných potravou (kyselina palmitová a stearová) indukují apoptózu pankreatických β buněk, narozdíl od příslušných nenasycených mastných kyselin (např. kyselina palmitolejová a olejová). Zjistili jsme, že apoptóza indukovaná kyselinou stearovou je doprovázena signifikantní aktivací kaspázy 2, 6, 7, 8 a 9, ale ne signifikantní aktivací kaspázy 3. Nebyla však spojena s vyléváním cytochromu c, změnou exprese proteinů PIDD, Fas a FasL a s aktivací proteinu p53. Působení kyseliny stearové vedlo k rychlé aktivaci signálních drah stresu endoplazmatického retikula (ER) (tj. IRE1α, PERK a ATF6 dráhy). Dále jsme ukázali, že apoptóza indukovaná působením kyseliny stearové není závislá na aktivaci kaspázy 2 a kinázy JNK. Obě molekuly se však zdají být zapojeny u buněk NES2Y do regulačních drah stresu ER.

Dále jsme zjistili, že buněčná smrt indukovaná působením nasycených mastných kyselin je blokována působením nenasycených mastných kyselin. Ukázali jsme, že inhibiční efekt kyseliny olejové na apoptózu indukovanou působením kyseliny stearové se odehrává “upstream” od aktivace kaspáz a nepůsobí skrze regulaci aktivace mitochondriální dráhy apoptózy, aktivaci proteinu p53 a změnu exprese proteinů PIDD, Fas a FasL. Kyselina olejová inhibovala také zvýšení exprese několika markerů stresu ER (tj. BiP, CHOP a XBP1s) vyvolané působením kyseliny stearové. To naznačuje, že inhibiční účinek kyseliny olejové se uplatňuje “upstream” od indukce stresu ER nebo na úrovni indukce stresu ER.

Vyvinuli jsme také vlastní metodu pro izolaci myších Langerhansových ostrůvků, jejíž inovativnost spočívá v použití plastiku určeného pro kultivaci suspenzních buněk pro efektivní separaci endokrinní a exokrinní tkáně.

Naše výsledky přispěly k porozumění mechanismům, kterými nasycené a nenasycené mastné kyseliny regulují stres endoplazmatického retikula a apoptózu u pankreatických β buněk. Tyto poznatky by mohly být prospěšné při vývoji nových postupů léčby diabetu 2. typu, zaměřených na zachování funkce a viability pankreatických β buněk.

5 INTRODUCTION

5.1 Pancreatic β-cells

5.1.1 Langerhans islets and pancreatic β-cells

The pancreas is composed of two main compartments, the endocrine and the exocrine tissue. The majority of the organ is exocrine and is responsible for the synthesis of digestive enzymes and for their transport *via* an intricate ductal system into the duodenum. The endocrine tissue is organized into functional units called islets of Langerhans. They comprise α-, β-, δ- and PP-cells producing the hormones glucagon, insulin, somatostatin and pancreatic polypeptide (PP), respectively [1]. The insulin secreting β-cells make up of about 65-90% of all islet cells [2]. It is estimated that adult humans have approximately 1-2 million islets which measure about 0.2 mm in diameter and make up about 2% of the pancreas weight [3, 4]. There are approximately 3.000 β-cells per islet [5, 6]. The total mass of β-cells in healthy humans is approximately 0.8 g [4].

5.1.2 Diabetes mellitus

Diabetes mellitus is a metabolic disorder defined by the presence of chronic hyperglycemia. Based on the cause of this hyperglycemia, several types of diabetes are distinguished.

In type 1 diabetes mellitus (T1DM), formerly known as insulin-dependent or childhood-onset diabetes, hyperglycemia is a consequence of body failure to produce insulin due to the loss of β-cell mass. In most cases of T1DM, this loss is the result of apoptosis induced by autoimmune attack of T-lymphocytes against the β-cells. T1DM is usually diagnosed in childhood and therefore it is also called juvenile diabetes. T1DM is typically treated with insulin or synthetic insulin analogs to achieve normal level of blood glucose. Transplantation of pancreas or Langerhans islets is also possible to enable independence on external insulin supply, although it is not the common therapeutical approach.

In type 2 diabetes mellitus (T2DM), formerly called non-insulin-dependent diabetes mellitus (NIDDM) or adult-onset diabetes, the hyperglycemia results from the insufficiency of β-cells to produce enough insulin to match the metabolic needs of the body. The inadequacy of insulin production stems from two basic causes – insulin resistance of peripheral tissues and β-cell dysfunction. Insulin resistance is typically present for some years before diagnosis, manifested as diminished stimulation of glucose transport in muscle and adipose tissue and inadequate suppression of glucose production in liver in response to insulin. However, euglycemia is maintained as long as β-cells secrete higher amount of insulin. Over time, insulin levels decline because of the decreased number of β-cells and their diminished secretory capacity [7]. Based on longitudinal studies there is at least a 50% loss of maximal β-cell function at the time of diagnosis of T2DM [8, 9] and the critical role of β-cell dysfunction and failure in the development of T2DM is largely accepted nowadays [10-17]. Recently, it has been estimated that approximately 350 million people worldwide have diabetes, approximately 90% of them have T2DM (www.who.int).

The β-cell failure in T2DM is mediated by combination of genetic factors, exposure to elevated levels of glucose and free fatty acids (FAs) in blood (see 5.3, p. 23), increased demands to secret more insulin in response to ambient hyperglycemia and possibly also deposition of amyloid fibrils in islets (see 5.4.2, p. 37) [17]. The management of T2DM focuses primarily on maintaining blood glucose levels in the normal range, on lowering the risk factors of other complications, e.g. cardiovascular and microvascular, and on lifestyle interventions.

Other less frequent types of diabetes are gestational diabetes, caused by insufficiency of β-cells to produce enough insulin during increased demands in pregnancy, LADA (latent autoimmune diabetes of adults) which is an autoimmune form of diabetes that occurs in adult age, and a monogenic form of diabetes called MODY (maturity onset diabetes in the young).

To date, mutations in at least nine different genes were identified that result in the MODY phenotype primarily due to the effect of mutations on proper β-cell function [18]. However, for a classical T2DM no such clear causality between the specific genotype and the development of T2DM was found despite intensive research. Instead, a number of risk genotypes (38 individual susceptibility loci) was revealed that predispose their carriers to T2DM development when other behavioral and

environmental triggering factors are present. Majority of such loci code for proteins involved in β-cell function, including glucose-sensing, proinsulin processing and insulin secretion [19].

Because studying the effects of FAs on pancreatic β-cells has relevance only to development of T2DM, most parts of this Ph.D. thesis will deal exclusively with this type of diabetes.

5.1.3 Role of obesity and fatty acids in the pathogenesis of type 2 diabetes

The incidence of obesity worldwide is growing to pandemic proportions. The reason is modern lifestyle, characterized by high food consumption combined with low physical activity. As the weight gain progresses, there are increasing demands on insulin production by β-cells. Development of obesity is also associated with increased insulin resistance [20] which leads to further increase in insulin requirements by the body. When β-cells are unable to compensate for this gradually increasing request for insulin production, T2DM develops as a result of β-cell exhaustion, dysfunction and subsequent apoptosis. Clear evidence linking obesity to insulin resistance and T2DM exists [21, 22]. Notably, 44% of the diabetes cases are attributable to overweight and obesity (www.who.int). A common characteristics of obese people is increased level of circulating FAs [22]. This factor, besides the increased level of blood glucose due to hyperglycemia, is regarded as very important trigger of pancreatic β-cell dysfunction and subsequent apoptosis (see 5.3, p. 23).

5.1.4 Experimental models used for studies concerning processes associated with type 2 diabetes

Diabetes is multifactorial disease, however, its research in humans is impeded by obvious ethical considerations, because provocation of the disease is strictly impermissible in man. Animal models of diabetes are therefore highly useful and advantageous, although not all conclusions from experiments on animals are directly applicable to humans. Most of the available models are based on rodents because of their small size, short generation interval, good availability and economic considerations [23]. Isolated Langerhans islets of human and animal origin are also used for research, however, their use is limited by the availability of endocrine tissue

(especially in humans) and demandingness of the isolation protocol (see 5.1.5, p. 15). Therefore, β-cell lines are also commonly used as a source of homogenic and inexhaustible experimental material to study the processes that occur in β-cells at the cellular and especially at the molecular level. Nevertheless, it must be noted that despite the obvious convenience of the work with cell lines, they are far away from being ideal experimental model as they represent solely population of β-cellls that is taken out of the complex regulation that exists at the level of the whole body.

Animal and β-cell line models commonly used for studying of pathogenesis of T2DM [23, 24] that are relevant to the topic of this Ph.D. thesis are listed below.

5.1.4.1 Animal models

Several rodent model organisms exist that develop diabetes spontaneously. The ***ob/ob* mouse** develops obesity and diabetes because of an autosomal recessive mutation in the *ob (obese)* gene that codes for leptin [25]. The ***db/db* mouse** possess an autosomal recessive mutation in the *db (diabetes)* gene that encodes receptor for leptin [26]. **Zucker fatty rat (ZFR, *fa/fa* rat)** carries, similarly to *ob/ob* mouse, a mutation in gene coding for leptin receptor, named the *fa* (*fatty*) gene [27]. **Zucker diabetic fatty rat (ZDF rat)** is a substrain of ZFR selectively inbred for hyperglycemia. Male ZDF rats spontaneously develop diabetes usually at 7-10 weeks of age. These rats are less obese but more insulin resistant than ZFR. Interestingly, female littermates are obese, insulin resistant but do not develop diabetes and serve as control [23].

T2DM can be also induced in experimental animals by diet composition. **Mice of C57BL/6J strain** develop T2DM **after feeding high fat diet** [28]. ***Psammomys obesus* (*P. obesus*; Sand rat)** retains normal weight in its natural habitat but develops obesity and diabetes in captivity when fed on standard laboratory chow (high energy diet) instead of its usual low energy vegetable diet [29].

5.1.4.2 Pancreatic β-cell lines

Several pancreatic β-cell lines are commonly used in research of T2DM. **RIN lines** (RIN-r and RIN-m) were derived from a transplantable islet cell tumour that was induced by high-dose X-ray irradiation in an inbred NEDH rat strain [30]. The RIN-m5F line was derived from the RIN-m line and secretes also glucagon and somatostatin together with insulin [31]. The line RIN1046-38 is a subclone of RIN-r line that secretes

insulin but exhibits no co-secretion of somatostatin and glucagon, compared to its parental line RIN-r [32].

The **BRIN-BD11** line arose from electrofusion of normal NEDH rat pancreatic β-cells with immortal RINm5F cells [33].

INS-1 cell line was also derived [34] from the original radiation-induced tumour described by Chick et al. [30]. INS-1 cells do not produce glucagon, somatostatin and PP. Several subclones were established that exhibit higher glucose responsiveness than the original cell line, e.g. INS-832/13 [35] and INS-1E [36]. The glucose-stimulated insulin secretion (GSIS) of INS-1E cells is similar to that of rat islets.

HIT-T15 cells were produced by SV40-mediated transformation of isolated Syrian hamster pancreatic β-cells followed by serial cloning and selection for high insulin content [37]. The GSIS of HIT-T15 cells is similar to normal hamster islets.

βTC class of insulinoma cell lines was established from transgenic mice that developed insulinomas due to targeted expression of SV40 large T antigen under the control of rat insulin II promoter [38]. Several cell lines were derived from such insulinomas, e.g. βTC3, βTC6 and βTC7, that differ in their insulin secretory characteristics. Some of them were also shown to produce glucagon and somatostatin [39].

MIN6 line was also derived from tumours arising in transgenic mice expressing the SV40 T antigen under control of insulin promoter and exhibits GSIS similar to normal islet β-cells [40].

NES2Y cell line was established from a patient with persistent hyperinsulinemic hyperglycemia of infancy. The cells secrete insulin constitutively due to loss of function of ATP-regulated potassium channel [41]. By transfection of SUR1 (sulfonylurea receptor 1) and Kir6.2 (inward rectifier potassium channel 6.2) components of this channel and the transcription factor PDX (pancreatic duodenal homeobox), glucose-responsiveness can be introduced [42].

5.1.5 Isolation of Langerhans islets

Intact and functional Langerhans islets can be isolated from animal and human pancreas employing several protocols and can then be used for both therapeutical and experimental purposes. Therapeutical use of isolated human Langerhans islets is mainly for transplantations to substitute for the islets damaged by autoimmune reaction in

patients with T1DM. This therapeutical approach is not common due to shortage of suitable donors that is even worse comparing to other organ transplantation because islets from more than one donor are usually necessary for successful transplantation of Langerhans islets [43]. For experimental purposes, usually rat and mouse islets are used, as these represent the most commonly used experimental animals for the diabetes research (see 5.1.4, p. 13). Human islet for experimental research came mainly from isolations performed at transplantation centers that failed to provide a sufficient amount of islets for transplantation purposes.

Langerhans islets are commonly isolated after enzymatic and mechanical dispersal of the pancreas tissue. After enzymatic digestion, islets are separated from the rest of the pancreas tissue usually by Ficoll gradient centrifugation or by size-filtration employing cell strainers with appropriate pore size, followed by time-consuming hand-picking of individual islets under a stereomicroscope. Then, islets are assessed for purity and yield, eventually also for their functionality [44-46].

5.2 Apoptosis of β-cells

5.2.1 Basic molecular mechanisms of apoptosis

Apoptosis, a type of programmed cell death, is a physiological ”cell-suicide” whose main goal is to remove useless, damaged, potentially dangerous or just no longer wanted cells from the organism. Among other processes, it is essential for embryonic development, functioning of the immune system and maintenance of tissue homeostasis. Dysregulation of apoptosis has been implicated in numerous pathological conditions, including neurodegenerative diseases, autoimmunity and cancer [47].

During apoptosis, the cell undergoes many morphological changes. The cell shrinks, plasma membrane blebbs, chromatin condenses and nucleus is fragmented. Usually chromosomal DNA is cleaved into oligonucleosomal fragments. As a result of successful apoptosis, the cell is fragmented into a number of membrane-bound vesicles called apoptotic bodies that are eliminated by neighbouring cells or professional phagocytes without any release of the cellular content that could eventually cause inflammation in adjacent tissue. In contrast, necrosis, a non-physiological form of cell death, is accompanied by rupturing of plasma membrane and release of cellular content which leads to the tissue damage and inflammation. A typical biochemical hallmark of

apoptosis is exposure of phosphatidylserin in the outer leaflet of plasma membrane which serves as an “eat-me-signal” for phagocytosis to remove the dying cell [48].

5.2.1.1 Caspases

At the molecular level, apoptosis is mediated by a family of cysteine proteases known as caspases. So far, 14 mammalian caspases have been recognized [49]. Under normal conditions, caspases are present in the cell as inactive precursors called procaspases, consisting of a large subunit, a small subunit and a prodomain that serves as the inhibitory subunit of the protein. When apoptosis is induced, caspases are rapidly activated by proteolytic cleavage that removes the prodomain and small and large subunit can form a tetramer which is the active caspase capable of proteolytic activity on its substrates. Based on the structure, mode of activation and function in apoptosis, caspases are divided into three classes: (1) initiator caspases, (2) executioner caspases and (3) inflammatory caspases that play no role in apoptosis but regulate inflammatory processes [50].

Initiator caspases possess a long prodomain. They are activated in response to apoptotic stimuli in large multiprotein complexes and function at the apex of their respective signaling cascades to promote activation of executioner caspases either directly or indirectly. Caspase-2, caspase-8, caspase-9 and caspase-10 belong to this group [51, 52].

Executioner caspases, e.g. caspase-3, caspase-6 and caspase-7, contain a short prodomain and are activated by proteolytic cleavage executed by initiator caspases. After activation, executioner caspases cleave a wide variety of cellular proteins called death substrates which enables execution of apoptosis and also underlies the typical morphological changes observable on apoptotic cells. Importantly, various non-caspase proteases are also involved in apoptosis [53-55].

5.2.1.2 Receptor-mediated pathway of apoptosis induction

Apoptosis can be triggered by a number of various endogenic and exogenic stimuli. Based on the nature of the cell death stimulus, principally two basic pathways of apoptosis can be triggered: (1) receptor-mediated pathway of apoptosis induction, also called extrinsic pathway, usually activated by an external signaling molecule, e.g. TNF-α (tumour necrosis factor-α) and IFN-γ (interferon-γ), or by an immune system

cell, and (2) mitochondrial pathway of apoptosis induction, also known as intrinsic pathway, that is initiated by starvation, irreparable DNA damage, increase in reactive oxygen species (ROS) level, exposure to various cytotoxic agents or by other factors that interfere with proper cellular functioning [56].

Receptor-mediated pathway is triggered from the cell surface after ligation of a class of receptors called death receptors with their relevant ligands. These receptors are trimeric molecules, e.g. receptors for TNF-α, FasL (Fas ligand) and TRAIL (tumour necrosis factor-α-related apoptosis-inducing ligand). After binding of their respective ligands, called death ligands, receptors undergo a conformational change in the cytosolic part that enables binding of the adaptor proteins (e.g. FADD and TRADD) and subsequent recruitment of initiator caspase-8 or caspase-10. The assembly of this multiprotein complex called DISC (death-inducing signaling complex) results in the mutual proteolytic activation of caspase molecules. Active caspase-8 or -10 then cleave and thus activate the executioner caspases [51].

5.2.1.3 Mitochondrial pathway of apoptosis induction

When mitochondrial pathway is triggered, cytochrome c is released from mitochondrial intermembrane space into cytosol enabling assembly of a multiprotein complex called apoptosome that functions as activating platform for caspase-9. Active caspase-9 then activates executioner caspases-3, -6 and -7 by proteolytic cleavage [51].

Cytochrome c release is controlled at the level of mitochondrial membrane permeability by proteins of Bcl-2 family. Members of this evolutionary conserved family of proteins are divided into pro- and antiapoptotic group, according to their action in regulation of apoptosis induction. Antiapoptotic members, e.g. Bcl-2, Mcl-1 and Bcl-X_L, act to stabilize the mitochondrial membrane and prevent cytochrome c release. Proapoptotic members are further distinguished into two groups, based on their structure in respect to the content of so called BH (Bcl-2 homology) domains: (1) the Bax/Bak-like proteins and (2) the BH3 only proteins. The Bax/Bak-like proteins, e.g. Bax and Bak, are able to form pores in the outer mitochondrial membrane (permeability transition pore, PTP) and thus enable cytochrome c release. Under normal conditions, Bax/Bak-like proteins are sequestered in inactive state bound to antiapoptotic proteins of Bcl-2 family [57]. BH3-only proteins, e.g. Bid, Bad, Bim, Puma and Noxa, contain only one BH domain named BH3 and function during apoptosis *via* forming complexes

with antiapoptotic proteins of Bcl-2 family and thus liberating the Bax/Bak-like proteins to assembly PTP [58].

Mitochondrial pathway can also serve as an amplification loop of apoptosis triggered by the death receptors. This occurs *via* caspase-8- and caspase-10-mediated cleavage of Bid protein resulting in the formation of Bid fragment called tBid (truncated Bid) that is able to induce cytochrome c release [57, 59].

5.2.1.4 Regulation of apoptosis

Regulation of apoptosis can occur at many levels. For example, caspase activation can be regulated *via* activity of a family of proteins referred to as inhibitor of apoptosis proteins (IAPs). These include, among others, IAP1, IAP2, XIAP and Survivin. In general, they bind to caspases and prevent their activation (caspase-9) or inhibit their effector function (caspases-3 and -7). Binding of IAPs to caspases is competitively inhibited by Smac/DIABLO (second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI) and HTRA2 (high temperature requirement protein A2)/Omi) released from mitochondria together with cytochrome c [60]. Similarly, initiator caspase-8 activation is inhibited by FLIP (FLICE-inhibitory protein) that blocks the recruitment of caspase-8 and -10 into DISC complex [61].

Another level of regulation is through the change in the level of pro- and antiapoptotic proteins of Bcl-2 family and thus inhibiting or activating the release of cytochrome c from mitochondria through PTP. Such regulation of Bcl-2 family proteins can occur at the transcriptional level or posttranslational level *via* phophorylation or degradation [62]. Potent transcriptional inducer of several proapoptotic members of Bcl-2 family (e.g. Bax, Bak, Bid, Puma and Noxa) is the protein p53 which is activated by many proapoptotic stimuli, e.g. DNA damage by ionizing and UV radiation, cytotoxic drugs, hypoxia, and also some extrinsic signals [63]. Activity of several members of the Bcl-2 family can be regulated by phosphorylation, e.g. phosphorylation of Bcl-2 and Bad by JNK (c-Jun N-terminal kinase, also known as SAPK, stress-activated protein kinase) or phosphorylation of Bad through MAP (mitogen-activated protein) kinase signaling pathway [64].

Besides cytochrome c, other proapoptotic factors reside in mitochondrial intermembrane space and can be released during apoptosis, e.g. apoptosis-inducing factor (AIF) or endonuclease G (endo G). Upon an apoptotic insult, they translocate to the nucleus where they trigger chromatin condensation and DNA degradation in a caspase-independent manner [65, 66].

The current knowledge concerning the mechanisms of apoptosis induction and regulation is summarized in Figure 5-1 (see p. 21).

5.2.1.5 Caspase-2

Caspase-2 is the most evolutionary conserved caspase. Although there is a wealth of literature on the role of caspase-2 in apoptosis, there is much controversy regarding this protein, making it difficult to correctly place caspase-2 in the apoptotic cascade and its role in apoptosis remain rather unclear [52].

The proposed model of caspase-2 activation is shown on Figure 5-2 (see p. 22). The signaling platform for activation of caspase-2 is PIDDosome that consists of caspase-2, PIDD (p53-induced protein with a death domain) and RAIDD (RIP-associated ICH-1/CAD-3 homologous protein with a death domain) [67] (see Figure 5-2A, p. 22). Procaspase-2 molecules exist as monomers at normal physiological concentrations. In response to a proapoptotic signal, procaspase-2 molecules are recruited into close proximity *via* interactions mediated through the prodomain and an adaptor molecule such as RAIDD. When brought into close proximity, the protease domains of caspase-2 can interact to form a transient dimeric complex, which is sufficient to impart some intrinsic catalytic activity to the unprocessed zymogens. Dimerization is followed by cleavage between the large and small subunits of the protease domains of each participating monomer leading to formation of an active stable tetrameric complex, which attains maximal catalytic competence. Further processing, possibly by caspase-3, generates the fully mature $(p19+p12)_2$ caspase-2 enzyme (see Figure 5-2B, p. 22).

Caspase-2 was shown to be activated in response to DNA damage and it appears to be involved in regulation of cell cycle arrest and cell death by mitotic catastrophe as well. Relatively newly, it has been also linked with apoptosis induction by ER stress (see also 5.4.1.5, p. 36) [68-72].

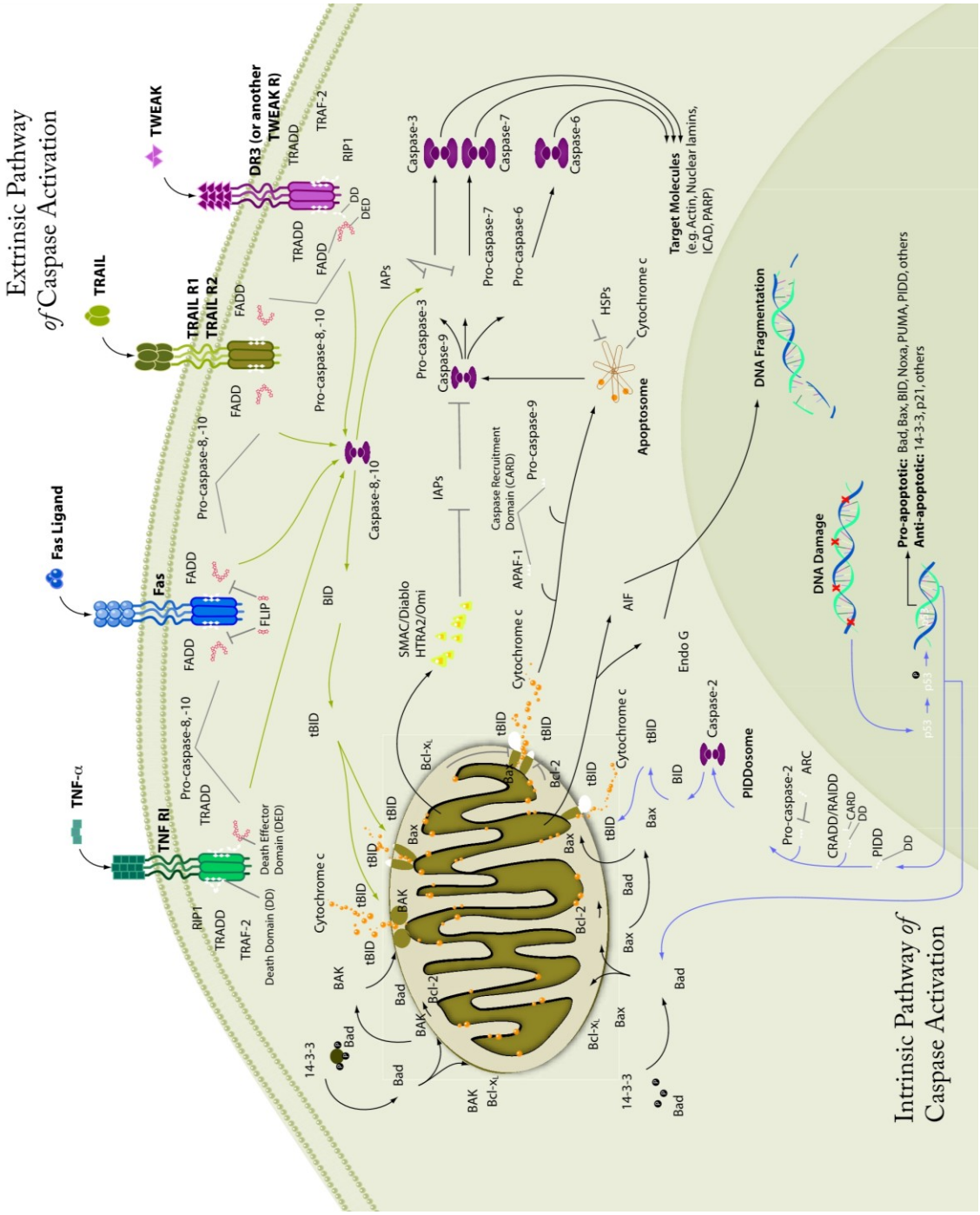


Figure 5-1: Summary of the current knowledge about apoptosis induction and regulation. Extrinsic and intrinsic mechanisms of caspase activation are shown. Mechanism of caspase-2 activation is also depicted. For explanation of abbreviations, see "List of abbreviations" on p. 4. Adopted from R&D Systems, Apoptosis Catalogue 2010, p. 17.

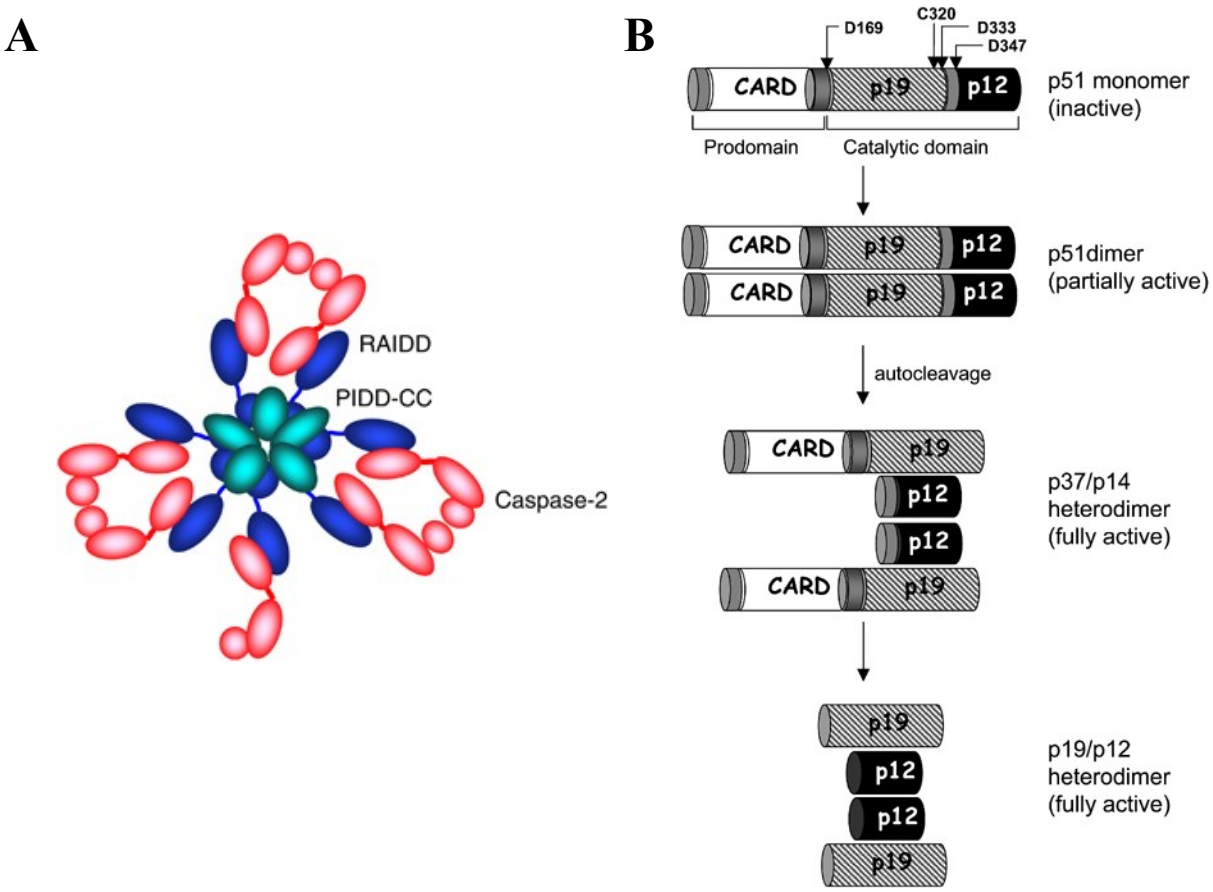


Figure 5-2: (A) A proposed model of the PIDDosome. The schematic model is based on the crystal structure of the PIDD-DD:RAIDD-DD core complex, which forms the center rings mediating oligomerization. The RAIDD CARD occupies the outer part of the PIDDosome bringing seven caspase-2 molecules into proximity facilitating dimerization. Adopted from Bouchier-Hayes et Green 2012 [52], **(B) A proposed model for the mechanism of caspase-2 activation.** For explanation, see 5.2.1.5, p. 20. In the three-dimensional structure, the two caspase-2 monomers will be oriented in an antiparallel manner. For simplicity, this is not depicted in this model. Adopted from Baliga et al. 2004 [73].

5.2.2 Apoptosis and loss of β-cell mass in the pathogenesis of type 2 diabetes

There are no doubts that pancreatic β-cell death is crucially involved in the pathogenesis of T1DM. Here, β-cell apoptosis is induced by immunological mechanisms [74]. However, for a long time there was a debate whether pancreatic β-cell death also occurs in T2DM and if so, to what extent it is involved in its pathogenesis. Nevertheless, reduction of β-cell mass in patients with T2DM was observed in several studies [75-79] and clear evidence for decreased β-cell mass in T2DM by apoptotic

mechanism came from the study by Butler et al. [5]. They examined autopsy samples from lean and obese subjects with and without T2DM and reported increased frequency of β-cell apoptosis in both lean and obese cases of T2DM compared with the non-diabetic control cases. Similarly, Marchetti et al. [80] also demonstrated significantly higher percentage of apoptotic cells in Langerhans islets isolated from individuals with T2DM than in those isolated from non-diabetics.

Therefore, pancreatic β-cell death is considered as an important factor contributing to the development of T2DM [10-12, 81-86].

5.3 Dysfunction and apoptosis of β-cells induced by fatty acids

5.3.1 Fatty acids and their structure

Fatty acids (FAs) are carboxylic acids with an aliphatic chain. Most naturally occurring FAs have a chain consisting of an even number of carbon atoms ranging from 4 to 28. Concerning the intake of dietary FAs and their effect on cells including pancreatic β-cells, so called long-chain FAs that have 14-22 carbons in their chain are the most important. For convenience the term „fatty acids“ (FAs) is used in this Ph.D. thesis to designate “long-chain fatty acids”, unless otherwise indicated.

According to the level of saturation, FAs are divided to saturated FA species with no double bond in the molecule and unsaturated FAs species that have one (monounsaturated FAs) or more double bonds (polyunsaturated FAs) in their molecule. In unsaturated FAs, the double bond can exist in two conformations, *cis* or *trans*. The *cis* configuration means that hydrogen atoms adjacent to the double bond are on the same side of the double bond. If the double bond exists in the *trans* configuration, it means that the two hydrogen atoms next to the double bond are bound to opposite sides of the bond. The most naturally occuring unsaturated FAs have double bonds in *cis* configuration.

The level of saturation and the configuration of double bonds in FAs also affect their melting point and rigidity which has in turn great impact on properties of cellular membranes, e.g. their fluidity. Besides the structural functions in membranes, FAs are also involved in regulation of many cellular processes, e.g. *via* palmitoylation and

myristoylation of proteins [87], *via* formation of various signaling molecules, e.g. ceramides [88-90], and *via* regulation of gene transcription [91, 92].

The list of the most common FAs present in the diet and thus the most commonly occuring *cis* and *trans* FAs in plasma [93-95] is presented in Table 5-1 (see p. 25). Chemical structure, carbon chain length and configuration of the double bond are indicated.

5.3.2 Transport of fatty acids across the membrane and their metabolism

Long-chain FAs can be transported into the cell by free diffusion with no requirement for active transport due to their hydrophobic nature [96], however, several membrane proteins were identified that seem to facilitate the cellular entry of FAs [97], some of them were also demonstrated to expressed in β-cells (FATP-1, FATP-4, FAT/CD36 and caveolin-1) [98-100]. Islets express low-density lipoprotein receptors and lipoprotein lipase as well and can acquire FAs from circulating lipoproteins [101].

Metabolism of FAs in the cell starts with their conversion to long-chain acyl-coenzyme A (LC-CoA) by the activity of the enzyme acetyl-coenzyme A synthetase (ACS). LC-CoA then may be esterified to triacylglycerol (TAG) in the presence of glycerol 3-phosphate provided by glucose metabolism or may be alternatively oxidized in mitochondrial matrix to provide acetyl-CoA when the rate of glucose uptake and metabolism is low. The transport of LC-CoA into mitochondria is accomplished by the enzyme carnitine palmitoyltransferase-1 (CPT-1), found on the outer mitochondrial membrane [101]. LC-CoA can also directly promote insulin secretion *via* facilitation of the fusion of insulin secretory granules with the plasma membrane of β-cells [102].

When both FAs and glucose are available for oxidative metabolism, glucose metabolism is favoured at the expense of FA oxidation. This metabolic regulation is accomplished, besides glucose regulation of lipogenesis gene expression, *via* glucose conversion to malonyl-CoA. Malonyl-CoA inhibits CPT-1 which in turn leads to a marked rise in the cytoplasmic content of LC-CoA that are incorporated into TAGs or other derivates, e.g. ceramides that can also exert deleterious effects on cells (see Figure 5-3, p. 26) [14, 103].

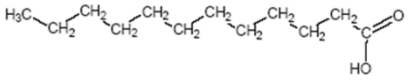
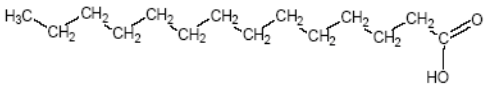
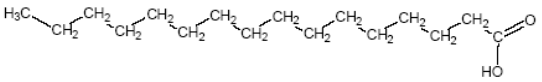
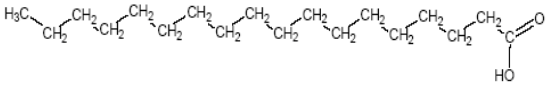
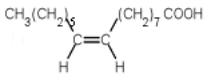
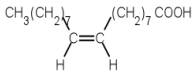
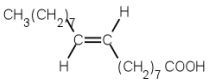
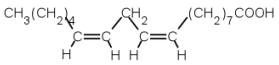
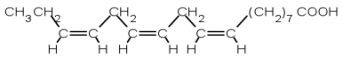
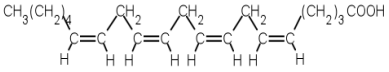
Trivial nomenclature	C:D	Δ^x	ω-x	Chemical formula
Saturated fatty acids				
Lauric acid	12:0			
Myristic acid	14:0			
Palmitic acid	16:0			
Stearic acid	18:0			
Unsaturated fatty acids				
Monounsaturated fatty acids				
Palmitoleic acid	16:1	cis-Δ^9	n-7	
Oleic acid	18:1	cis-Δ^9	n-9	
Elaidic acid	18:1	trans-Δ^9	n-9	
Polyunsaturated fatty acids				
Linoleic acid	18:2	cis,cis-Δ^9,Δ^{12}	n-6	
α-Linolenic acid	18:3	cis,cis,cis-Δ^9,Δ^{12},Δ^{15}	n-3	
Arachidonic acid	20:4	cis,cis,cis,cis-Δ^5,Δ^8,Δ^{11},Δ^{14}	n-6	

Table 5-1: Overview of the structure of the most common dietary long-chain saturated and unsaturated FAs .
C:D indicates the number of carbons (C) and the number of double bonds (D) in the molecule.
Δ^x refers to the Δ^x nomenclature of FAs; each double bond is indicated by Δ^x where the double bond is located on the x^{th} carbon–carbon bond, counting from the carboxylic acid end. Each double bond is preceded by a *cis*- or *trans*- prefix, indicating the conformation of the molecule around the bond.
ω–*x* refers to the ω–*x* nomenclature of FAs; a double bond is located on the x^{th} carbon–carbon bond, counting from the terminal methyl carbon (designated as ω) toward the carbonyl carbon.

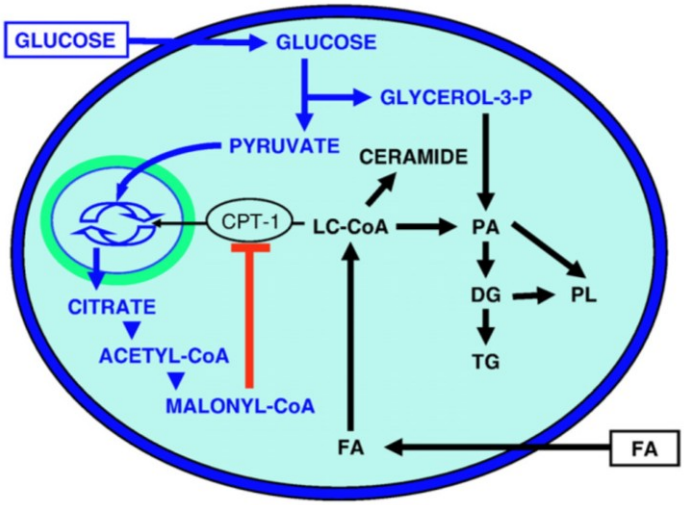


Figure 5-3: Effects of glucose on lipid partitioning in the beta cell. In the presence of simultaneously elevated levels of glucose and fatty acids (FAs), the increase in cytosolic malonyl-CoA resulting from glucose metabolism inhibits the enzyme carnitine palmitoyltransferase-1 (CPT-1). Transport of long-chain acyl-CoA (LC-CoA) in the mitochondria is reduced, and the esterification pathway is preferentially activated, leading to cytosolic accumulation of lipid-derived signaling molecules such as ceramide, diglycerides (DG), phosphatidic acid (PA), phospholipids (PL), and triglycerides (TG). Adopted from Poitout et al. 2010 [14].

5.3.3 Regulation of pancreatic β-cell functions by fatty acids

5.3.3.1 Effect on insulin secretion

Insulin secretion is regulated predominantly by the blood glucose and by the prevailing FAs in the circulation [104]. Nevertheless, when β-cells are challenged with FAs, the final outcome is highly influenced by the length of treatment. The acute effect of FAs on β-cells is stimulation of GSIS as shown in both *in vitro* experiments [105, 106] and *in vivo* studies on animal models [106-108]. Potentiation of GSIS by FAs was also observed in humans [109-112]. The potency of FAs to promote GSIS increases with the carbon chain length and the degree of saturation [113, 114].

In contrast, longer treatment with high FA concentrations leads to a decrease in GSIS, as demonstrated *in vitro* in both pancreatic β-cell lines [115-118] and animal [116, 119, 120] and human islets [121] and also *in vivo* in rats [116, 122] and humans [109, 112, 123].

5.3.3.2 Proapoptotic effect of saturated fatty acids

Another impact of longer incubation (in the range of several hours up to few days) of β-cells with higher concentration of FAs is negative effect on their viability. In experimental animals, high fat diet was shown to cause reduction in functional β-cell mass by apoptosis [124-127]. Such studies provided very important evidence for the existence of FA-induced β-cell apoptosis *in vivo* and legitimize the intensive research aimed at understanding of molecular mechanisms of the deleterious effect of FAs of β-cells *in vitro*.

From this research, it is becoming increasingly evident that all FA species do not exert equal effect on β-cells and that there are marked differences in respect to FA carbon chain length and the degree of saturation [85, 128-130]. This phenomenon was studied in detail using isolated animal and human islets and animal β-cell lines treated with individual FA species. The most studied FAs in this respect were palmitic acid (PA), stearic acid (SA), palmitoleic acid (POA) and oleic acid (OA) because these are the prevalent FAs in the blood circulation [93, 94]. Due to their inherent insolubility in water, FAs are applied in experiments in complex with bovine serum albumin (BSA).

In primary rat β-cells [131] as well as in isolated rat [115, 132] and human islets [132-136], saturated PA and SA cause β-cell death, in contrast to unsaturated POA and OA. In human islets, PA was usually tested as the representative saturated FA [132, 134-136]. However, according to the results of Eitel et al. [133], SA seems to be more effective than PA in human islets.

The cell death-inducing effect of saturated FA species contrasting with lower or no cell-death inducing effect of unsaturated FA species was also demonstrated in MIN6 [117, 135, 137-140], β-TC [141], RIN1046-38 [133, 142], RINm5F [143], BRIN-BD11 [128, 144, 145], HIT-T15 [146, 147] and INS-1 [134, 148, 149] rodent β-cell lines. Surprisingly, saturated FAs having chain length shorter than 16 carbons, e.g. myristate with 14 carbons and octanoate with 8 carbons, are ineffective as inducers of cell death [144].

Unfortunately, in the pioneering era of the research of FA-mediated effects in β-cells, FAs were administered by some researchers in the form of OA:PA mixture (in molar ratio 2:1) [150-153] that was commercially available from Sigma-Aldrich at that time but its production was discontinued later. Although these studies brought some

interesting findings, they do not offer the possibility of detailed analysis concerning the contribution of either saturated or unsaturated FA.

However, it is important to note that some studies detected the deleterious effect in case of unsaturated FAs as well [131, 135, 154-161], but mostly this cytotoxic effect was less pronounced. Such variance against other experimental evidence is striking but may stem from different experimental settings in individual studies, e.g. using of FAs complexed with BSA at different ratios or use of serum-free vs. serum-reduced medium that could have different impact on cell viability *per se* and serve as an additive stress factor in such experiments.

5.3.3.3 Antiapoptotic effect of unsaturated fatty acids

Despite some inconsistency in experimental evidence (see above), there is general consensus concerning higher detrimental potential of saturated FAs in β-cells in comparison with unsaturated FAs [85, 128-130, 162]. Moreover, there is growing body of experimental data showing the ability of unsaturated FAs to inhibit the deleterious effects of saturated FAs. Such effects were demonstrated in INS-1 [130], INS-1E [159], BRIN-BD11 [128, 130, 144, 145], RIN1046-38 [133] and also rat [115, 163] and human islets [132, 133].

Interestingly, only the long chain unsaturated FAs (C16:1 and C18:1, for explanation of these abbreviations, see Table 5-1, p. 25) exert the inhibitory effect, monounsaturated FAs with shorter chain (C14:1 and C8:1) are completely ineffective [144] or significantly less effective in comparison with POA and OA [130]. Notably, similar trend in respect to carbon chain length was also observed for toxicity of saturated FAs (see 5.3.3.2, p. 27). Polyunsaturated FAs, e.g. linoleic acid (C18:2) and γ-linolenic acid (C18:3), or variants of OA (C18:1, n-9) where the double bond is located at different positions, e.g. vaccenic (C18:1, n-11) and petroselinic (C18:1, n-6) acid, were cytoprotective as well [164]. The impact of *cis* and *trans* configuration on the inhibitory effect of unsaturated FAs was also examined. In both BRIN-BD11 and INS-1 cells, palmitelaidic and elaidic acid (EA), *trans* counterparts of POA and OA, were cytoprotective but were less effective in its protective effect on viability compared to their respective *cis* isomers [130].

It was also demonstrated that even many times lower concentrations of unsaturated FAs (e.g. 50x) are sufficient, when compared with concentrations of

saturated FAs used for cell death induction, to significantly inhibit this apoptosis [130, 144]. Moreover, unsaturated FAs were shown to decrease the cell death rate even when applied several hours later after the addition of saturated FA and were effective against various non-FA stimuli as well, e.g. serum withdrawal or exposure to proinflammatory cytokines [130, 144, 165].

These findings underlie the hypothesis that the inhibitory effect of unsaturated FAs on saturated FA-induced apoptosis is mediated by activation of some so far unidentified signaling pathway rather than by their direct interference with metabolism of saturated FAs [85, 129, 144].

5.3.4 Molecular mechanisms of apoptosis induced by FAs in β-cells

5.3.4.1 Activation of caspases

The occurrence of apoptosis after treatment with FAs is usually defined by the activation of caspases (see below) or presence of other typical apoptotic hallmarks (see 5.2.1, p. 16), e.g. detection of phosphatidylserin exposure on the outer surface of the plasma membrane, increased permeability of plasma membrane, chromatin condensation and demonstration of DNA fragmentation by the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling) assay or presence of the DNA ladder [115, 132, 134, 138, 146, 150, 156, 166, 167].

The most intensively studied executioner caspase (see 5.2.1.1, p. 17) in context of FA-induced apoptosis is caspase-3. It was reported to be activated by PA and/or SA in MIN6 [138, 166, 168, 169], INS-1 [138, 167], INS-1E [170, 171], INS 832/13 [134], RIN1046-38 [142] and β-TC1 [141] cell lines and also in isolated rat [138] and human islets [132]. However, some experimental methods do not allow to distinguish between activation of caspase-3 and caspase-7 and their combined activity was demonstrated in BRIN-BD11 cells [130] and in primary rat β-cells [159].

Activation of caspase-6 by PA was also demonstrated and surprisingly, it preceded the activation of caspase-3 [141]. Besides the activation of the executioner caspases, PA treatment of primary rat β-cells resulted in the activation of caspase-12 that is very likely to play role in apoptosis induction downstream of ER stress (see 5.4.1.5, p. 36) [159].

The question was also addressed whether inhibition of caspases is able to prevent FA-induced apoptosis. To answer this, the induction with FAs was done in the

presence of wide spectrum caspase inhibitor z-VAD-fmk [145, 151]. The results confirmed an important role of caspases in FA-induces apoptosis but indicated involvement of non-caspase proteases in FA-induced cell death as well.

5.3.4.2 Activation of mitochondrial pathway of apoptosis

Involvement of mitochondrial pathway of apoptosis (see 5.2.1.3, p. 18) in the FA-induced apoptosis was also studied. The release of cytochrome c from mitochondria was detected after PA treatment in isolated rat [115, 138] and human islets [132] and rodent β-cell lines [138, 156, 171].

FAs were also shown to regulate the expression of proteins of Bcl-2 family (see 5.2.1.3, p. 18) in islets and β-cell lines. However, a big drawback of some of these studies is that the expression of tested molecules was assessed only at the mRNA level. Nevertheless, based on the available data, PA treatment tends to increase the mRNA or protein expression of proapoptotic Bcl-2 family members (e.g. Bax) [156, 169]. On the other hand, the level of antiapoptotic members of Bcl-2 family (e.g. Bcl-2) stays unchanged [156] or decreases during PA treatment [132, 171]. Unsaturated POA and OA were shown to have contradictory effects to PA [132]. Importantly, increased expression of Bax in pancreatic sections from patients with T2DM compared to non-diabetic controls was also documented [139].

The levels of mitochondrial membrane potential ($\Delta\Psi_m$) and ROS as markers of mitochondrial apoptotic pathway activation were assessed by Maestre et al. [156] and a decrease of $\Delta\Psi_m$ and an increase in ROS after PA treatment in INS-1 cells was shown.

Taken together, change in the ratio between pro- and antiapoptotic members of Bcl-2 family and triggering the mitochondrial pathway of apoptosis seems to be a likely mechanism by which FAs regulate the viability of β-cells.

5.3.4.3 Activation of receptor pathway of apoptosis

Several findings suggest that receptor pathway of apoptosis may also play an important role in the pathogenesis of T2DM. It was shown that high fat diet-fed mice lacking caspase-8 lose less β-cell mass and are protected from development of T2DM [172]. Moreover, increased caspase-8 activation was shown in islets from T2DM patients compared to non-diabetic controls [173]. However, Fas receptor is very likely not involved in caspase-8 activation and T2DM pathogenesis because β-cell specific

deletion of Fas receptor is not able to protect mice against high-fat diet induced T2DM. Surprisingly, the possible involvement of other death receptors and death ligands and their activation by individual FA species was not studied yet.

5.3.4.4 Role of fatty acid metabolisms in apoptosis regulation

One of the fundamental questions concerning the mechanisms of pro- and antiapoptotic action of FAs in β-cells is whether they are toxic or protective *per se* or whether they act *via* some intermediates of their metabolism. Therefore, very important findings arose from studies that compared the effect of FAs with the effect of their derivatized counterparts that can not be metabolized by cells, e.g alkyl esters and 2-bromo-derivatives of FAs. In alkyl esters of FAs, the carboxy group is unavailable for esterification and as a result formation FA-CoA is disabled. On the contrary, bromo-derivates of FAs can be esterified to CoA but can not be oxidized further [129].

Experimental data showed that methyl esters of saturated FAs are not effective in apoptosis induction in INS-1E [159], similarly to bromo-derivates of saturated FAs in MIN6 [140] and BRIN-BD11 cells [128]. Surprisingly, methyl esters of monounsaturated FAs were equipotent as cytoprotective agents with their underivatized counterparts [128, 130] and were also effective against non-FA stimuli [130]. Moreover, comparison of the *cis* and *trans* form of methyl-palmitoleate revealed similar difference in the inhibitory potential to that seen when non-methylated, parental molecules were used (see 5.3.3.3, p. 28) [130].

Another approach that addressed this question employed different chemicals in order to block or modulate different steps in FA metabolism, e.g. triacsin-C, which blocks the esterification of FAs to CoA, or etomoxir, inhibitor of CPT-1 that governs the rate-limiting step of β-oxidation of FAs [174].

Results obtained in these [128, 134, 138, 150] and the above mentioned experiments indicate that β-oxidation of FAs *per se* is not involved in the molecular mechanism by which FAs induce apoptosis and suggest that rather glucose inhibition of fat oxidation and increased accumulation of acyl-CoA and/or metabolites derived from them in cytosol have detrimental consequences. The importance of β-oxidation as a protective mechanism against FA toxicity is supported by the finding that increased capacity for oxidation of FAs was demonstrated in a PA-resistant subclone of MIN-6 cells [137]. In a marked contrast, unsaturated FAs do not need to be esterified to CoA to

exert the antiapoptotic effect even when used against non-FA stimuli, e.g. serum withdrawal [144].

The question whether FA conversion to TAGs is detrimental or protective in β-cells was also intensively studied, however, conflicting data were obtained [128, 148, 163, 175, 176]. Thus, this issue requires further clarification.

5.3.4.5 Ceramide formation

Increased supply of saturated FAs in β-cells might also lead to *de novo* ceramide formation. Ceramides can activate various signaling pathways, including apoptosis [88-90, 177]. For example, ceramides inhibit protein kinase B (PKB) [178, 179] and activate JNK [177]. In β-cells, activity of both PKB [138] and JNK [159] is known to be altered by PA treatment as well.

Experimental evidence strogly argues for the role of ceramide in FA-induced apoptosis. The important contribution of ceramide to FA-induced apoptosis was documented in islets of ZDF rats [150]. Fuminisin B1, a ceramide synthase inhibitor, reduced the effect of PA on β-cell apoptosis in INS-1 cells and isolated rat as well as human islets and, importantly, the deleterious effect of ceramide on β-cell viability was reproduced by exposure of islets to exogenous ceramide [115, 132, 180]. Another inhibitor of ceramide synthesis, myriocin, was also shown to ameliorate survival after the treatment with FAs [151].

These data are noteworthy because formation of ceramide would well explain the diference in the toxicity of saturated and unsaturated FA species as only saturated FAs are substrates for ceramide production [181].

5.3.4.6 iNOS activation and nitric oxide production

Nitric oxide (NO) is a short-lived messenger with pleiotropic roles in various physiological and pathophysiological processes, including survival, death, proliferation and differentiation [182]. NO is generated from arginine by the activity of nitric oxide synthase (NOS). Low concentrations of NO produced by constitutive form of NOS in pancreatic β-cells have been suggested as physiological regulator of insulin secretion. In contrast, excess of NO produced by inducible form of NOS (iNOS) is a known mediator of β-cell apoptosis [183].

The induction of iNOS mRNA and/or NO production was shown in fat-laden islets of ZDF rats [150] and PA-treated HIT-T15 cells [146]. However, these results were highly discussed as several other studies failed to demonstrate iNOS activation and NO production by FAs in BRIN-BD11 cells [144] and human [151] and rat islets [163]. These contradictory results can be explained, at least partly, by the extreme trickiness of the methods for NO assessment [184]. Nevertheless, the involvement of NO in FA-induced β-cell apoptosis remains an open question.

5.3.4.7 Glucolipotoxicity, glucotoxicity vs. lipotoxicity

A debate is also running concerning the interplay between high glucose and high FAs levels in the development of T2DM and several hypotheses exist as to the importance of either of these two factors in triggering β-cell dysfunction and cell death. The concept of glucolipotoxicity refers to the combined, deleterious effect of elevated glucose and FA levels on pancreatic β-cell function and survival [14, 185, 186]. The other concepts accentuate the critical role of either increased glucose levels (glucotoxicity) or increased FA levels (lipotoxicity). Both excess of glucose [187-190] and FAs *per se*, as documented in this chapter, have been demonstrated to have detrimental effects on β-cell function and viability in many experimental systems. However, the combination of both nutrients resulted in synergistic proapoptotic effect in some studies [134, 170, 175, 191] whereas not in others [115, 132, 159].

Importantly, the increased levels of FAs precede, by many years, the onset of hyperglycemia in the course of diabetes development. Moreover, increased expression of markers of the endoplasmic reticulum (ER) stress that accompany the dysfunction of β-cells in T2DM [139] (see 5.4.2, p. 37), can be reproduced *in vitro* by exposure of human islets to FAs but not to high glucose levels [159]. These facts suggest that lipotoxicity may be more relevant than glucolipotoxicity for β-cell loss in the pathogenesis of T2DM [162].

5.3.4.8 Other mechanisms and signaling pathways possibly involved in fatty acid-induced apoptosis

Based on the above presented and other available experimental evidence so far, saturated FA-induced apoptosis and its inhibition by unsaturated FAs appear to be complex processess where a very complicated cross-talk of multiple signaling pathways ocurrs and decides about the cell fate. In the last years, the role of ER stress signaling in

the regulation of β-cell function and survival by FAs rapidly emerges (see 5.5, p. 39). In addition, contribution of many other mechanisms and signaling pathways were proposed, however, these were not systematically studied and contradictory data were very often obtained. The mechanisms speculated to play role in regulation of β-cell viability by saturated and unsaturated FAs include: activation of protein kinase Cδ [142], degradation of carboxypeptidase E [140], calpain-10 activation [166], activation of transcription factor NFκB [157, 167], inhibition of PKB [138] and the level of stearoyl-CoA desaturase-1 expression [135, 137].

5.4 Endoplasmic reticulum stress in β-cells

5.4.1 Basic overview of ER stress signaling

Proteins must be folded into a proper three-dimensional structure in order to carry out their cellular functions. One site where protein folding in the cell occurs is endoplasmic reticulum (ER). Besides being involved in the assembly, folding and post-translational modification of proteins, ER serves also many other vital functions, e.g. lipid biosynthesis and maintaining of cellular Ca^{2+} homeostasis. Therefore, proper functioning of ER is essential to cell survival and any perturbations in its function can lead to cellular damage and subsequently result is cell death induction. Various conditions can affect the ER performance, e.g. inhibition of protein glycosylation or accumulation of misfolded proteins, and cause perturbations of ER function collectively termed “ER stress”. In order to survive under ER stress conditions, cells have evolved a self-protective mechanism termed “ER stress response” or “unfolded protein response” (UPR) [192].

The sensing of ER stress in vertebrates is carried out by three ER membrane-resident proteins: IRE1, PERK and ATF6 which transduce the information about the protein folding status of the ER to the cytosol and nucleus to restore protein folding capacity. Under normal conditions, all of these three proteins are kept in inactive state, probably by interaction with one of the major ER chaperones, BiP (immunoglobulin binding protein, also known as glucose-regulated protein, Grp78) [193].

5.4.1.1 IRE1α pathway

IRE1 exists in mammals in two isoforms: (1) IRE1α which is expressed in all cells, and (2) IRE1β which is restricted to the gastrointestinal and respiratory tract. Under ER stress conditions, BiP dissociates from IRE1α. Consequently, IRE1α dimerizes which leads to auto-transphophorylation and activation of the cytosolic endoribonuclease and kinase domain [192]. Active IRE1α processes the mRNA encoding the transcription factor XBP1 (X-box binding protein 1), excising 26 nucleotide-long intron that shifts the coding reading frame of this mRNA [194]. This results in expression of an active and stable transcription factor, termed spliced XBP1 (XBP1s). XBP1s translocates to the nucleus and upregulate expression of genes whose protein products operate in the ER-associated degradation (ERAD), in regulation of protein entry into the ER and in protein folding, among other functions [195]. XBP1 also modulates phospholipid synthesis which is required for ER membrane expansion under ER stress conditions. In addition, active IRE1α is capable of selective degradation of mRNAs encoding for certain ER-located proteins; this process is called IRE1α-dependent decay (RIDD) [196]. Notably, in β-cells, insulin mRNA was shown to be targeted for RIDD under certain circumstances [197-199].

Active IRE1α also recruits the adaptor protein TRAF2 (TNFR-associated factor 2) which leads to activation of JNK [200]. JNK is proposed to transmit the proapoptotic signaling downstream of ER stress (see 5.4.1.5, p. 36) [192].

5.4.1.2 ATF6 pathway

ATF6 associates with BiP under normal conditions. Under ER stress, BiP dissociates from ATF6 which results in unmasking of the ATF6 Golgi localization signal [201]. ATF6 then translocates v*ia* COPII (coat protein II) vesicles to Golgi where it is processed by site-1 and site-2 proteases [202]. This leads to the release of the cytosolic part of ATF6 termed ATF6 fragment (ATF6f) that functions as a transcription factor in the nucleus and directly controls expression of genes encoding chaperones, ERAD components and XBP1 [192].

5.4.1.3 PERK pathway

Similarly to IRE1α, PERK is also activated after BiP dissociation by autophosphorylation and homomultimerization. Activated PERK phosphorylates the α

subunit of translation initiation factor eIF2 (eIF2α) at serin 51. This leads to attenuation of general translation initiation. However, translation of several mRNAs is selectively enhanced by this phosphorylation, e.g. mRNA encoding the transcription factor ATF4 which controls the level of prosurvival genes that are related to the redox balance, amino acid metabolism, protein unfolding and autophagy [192]. ATF4 is also potent inducer of CHOP (C/EBP-homologous protein) expression that is engaged in apoptosis induction by ER stress [203]. Important transcriptional target of both ATF4 and CHOP is GADD34 (growth arrest and DNA damage-inducible protein) which promotes dephosphorylation of phosphorylated eIF2α. This restores global protein translation and supresses ATF4 translation to basal levels [204]. This branch of UPR also regulates the expression of several miRNAs which can contribute to the attenuation of protein expression [205].

5.4.1.4 Functional consequences of ER stress signaling

The initial response to induction of ER stress culminates in the temporary inhibition of general protein synthesis which provides the cell with a window of opportunity during which it may recover from stress stimulus and thereby restore normal homeostasis. By the various mechanisms described above, the UPR response is intended primarily to favour cell survival, however, if this is not effective, cell death is induced. To summarize, four distinct cellular responses are activated by the ER stress in the cell: (1) transcriptional induction of ER chaperones to increase protein folding activity and prevent protein aggregation, (2) translational attenuation to reduce the load of new protein synthesis and prevent further accumulation of unfolded proteins, (3) activation of the ER-associated degradation (ERAD) pathway to eliminate misfolded proteins by the ubiquitin-proteasome system, (4) induction of apoptosis, when functions of the ER stress are severely impaired, to eliminate the cell [206].

5.4.1.5 Mechanisms of apoptosis induction by ER stress

The mechanisms of apoptosis induction downstream of ER stress are largely speculative, but the role of transcription factor CHOP, proteins of Bcl-2 family and JNK and caspase activation is assumed [207, 208].

CHOP expression is induced predominantly by the PERK-ATF4 axis. The proposed mechanism of CHOP-mediated apoptosis is transcriptional regulation of

several proteins of Bcl-2 family (see 5.2.1.3, p. 18), e.g. it induces expression of proapoptotic Bim and suppresses expression of antiapoptotic Bcl-2. IRE1α was shown to interact directly with Bax and Bak, proapoptotic proteins of Bcl-2 family [209]. Moreover, phosphorylation of eIF2α was shown to block the expression of Mcl-1, an antiapoptotic protein of Bcl-2 family, and promote the cell death [171]. JNK can be activated downstream of ER stress through the IRE1α-TRAF2-JNK axis [200]. The proposed mechanism of its proapoptotic action is also *via* the regulation of the pro- and antiapoptotic activity of Bcl-2 family proteins, e.g. phosphorylation of Bad and Bcl-2 [64, 210].

Concerning the involvement of caspases and their specific activation in response to the ER stress, the experimental evidence is not very rich. Activation of caspase-12 is supposed to be the mediator of ER stress-induced apoptotic signaling in rodent cells [211]. However, except of specific population of African descendents, human population does not possess functional caspase-12 [212] and its role as the sole transducer of ER stress-mediated apoptosis may therefore be excluded. In several model systems, caspase-4 was shown to be activated by ER stress [213, 214]. Caspase-2 activation, subsequent cleavage of Bid and activation of mitochondrial pathway of apoptosis was also linked with ER stress under different experimental conditions [68-72]. However, the indispensability of both caspase-4 and caspase-2 for ER stress-induced apoptosis has not been convincingly proved yet.

Simplified overview of the current knowledge concerning the mechanisms of UPR signaling and ER stress-induced apoptosis is provided in Figure 5-4 (see p. 38).

5.4.2 Involvement of ER stress in the pathogenesis of type 2 diabetes

Pancreatic β-cells are, due to their specialization on insulin secretion, especially sensitive to any perturbations in the ER functioning and belong to most susceptible tissues for ER stress [206]. It is suggested that pro-insulin mRNA represents 20% of the total β-cell mRNA expression [216] and glucose-regulated insulin translation can account for up to 50% of total protein synthesis in β-cells [217].

Clear evidence for involvement of impaired protein folding and ER stress in pathogenesis of T2DM came from animal studies and rare human genetic disorders. In Akita mouse, diabetes develops spontaneously accompanied by hyperglycemia, reduction of β-cell mass (without insulitis) and obesity due to mutation in Ins2 gene.

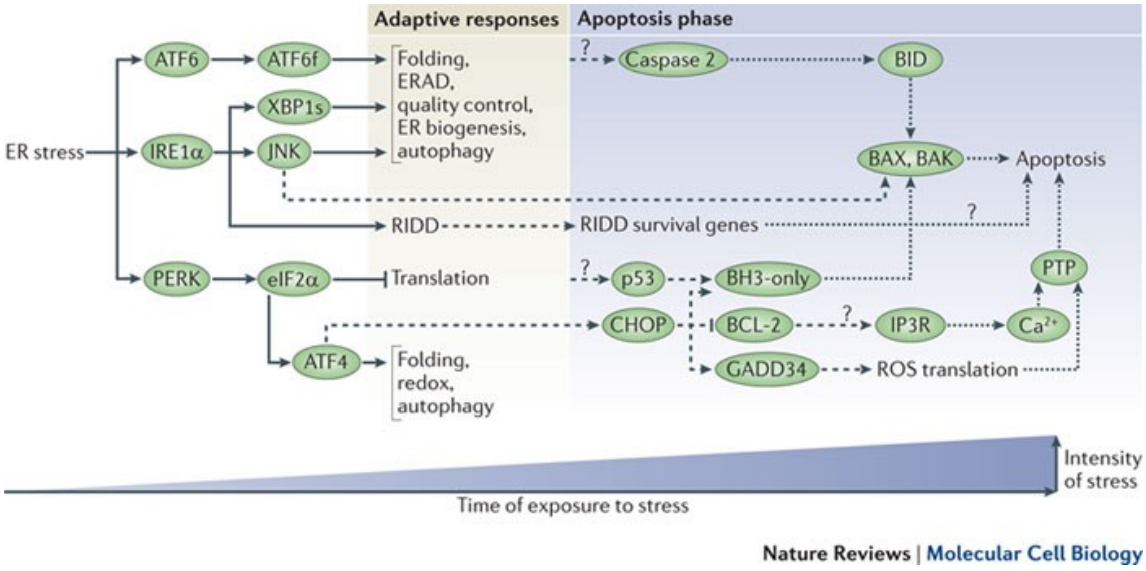


Figure 5-4. Overview of UPR-related signaling.
The adaptive responses downstream of ATF6, IRE1α and PERK activation that work to restore ER function and maintain cell survival are listed. The molecules involved in ER stress-induced apoptosis and their mutual relationships are also shown. Dashed arrows exemplify transition steps from adaptive responses to apoptosis. Dotted arrows indicate events mediating apoptosis. Question marks indicate where the mechanism responsible for the depicted step is unclear. For explanation of abbreviation, see „List of abbreviations“ on p. 4. Adopted from Hetz 2012 [215]

This mutation (Cys96Tyr) changes cystein to tyrosine in the proinsulin molecule and thus disables the disulfide bond formation between A and B chains of proinsulin which leads to production of mutant protein that is degraded [218]. During the development of diabetes in Akita mouse, BiP and CHOP expression was detected in pancreas, indication of the ER stress triggered by the mutant insulin [219].

The Wolcott-Rallison syndrome is caused by mutations in the PERK gene and subsequent lack of the functional PERK-eIF2α pathway [220]. The affected people develop severe diabetes in early age and have reduced number of β-cells [221]. Wolfram syndrome, another genetic disease associated with non-autoimmune loss of β-cells, results from mutations in WFS1 (Wolfram syndrome 1) gene. Protein WFS1 is localized in the ER membrane and was shown to be a negative regulator of ATF6 signaling. Mutations in WFS1 result in hyperactivation of ATF6 pathway regardless of ER stress conditions which leads to β-cell death [222].

The direct evidence for the presence of ER stress in the islets of T2DM patients came from the studies of Laybutt et al. [139] and Huang et al. [223] who clearly demonstrated higher expression of BiP and CHOP in pancreas sections of T2DM

patients compared to the non-diabetic controls. In β-cells of T2DM patients, expansion of ER surface also occurs indicating ER stress [80]. Furthermore, human islet amyloid polypeptide (hIAPP), which is responsible for formation of amyloid deposites commonly found in islets of T2DM patients [6], was shown to induce ER stress-mediated apoptosis of β-cells through formation of ER-damaging sheets [224]. All these findings argue strongly for the important contribution of dysregulated ER stress signaling and subsequent induction of β-cell apoptosis in the development of T2DM.

5.5 ER stress of β-cells induced by fatty acids

5.5.1 Morfological changes of ER

Similarly to apoptosis, ER stress signaling also appears to be activated differentially by saturated and unsaturated FAs. Treatment with saturated FAs was shown to cause remarkable changes of ER morphology in β-cells [135, 148, 158, 165]. For example, widely distended intracellular membranes, including the ER membrane, were described in BRIN-BD11 cells after PA treatment. The affected cells also displayed very frequently the condensation and margination of the nuclear chromatin, indicating apoptotic cell death in progress. In contrast, POA and combination of PA and POA induced only mild alteration in ER morphology compared to control cells [165]. These results point to the fact that saturated and unsaturated FAs exert different effect also at the level of ER stress induction and that the inhibitory effect of unsaturated FAs on saturated FAs-induced signalling is not limited to apoptotic pathways.

5.5.2 Activation of PERK pathway

The ER stress response and involvement of individual branches of ER stress signaling after FA treatment was studied most intensively in INS-1E and BRIN-BD11 pancreatic β-cell lines but data from primary rat and human β-cells also exist. However, very often these studies provided conflicting data, especially concerning the IRE1α and ATF6 pathway, despite using the same experimental model.The discrepancies may result, at least partially, from the fact that only mRNA level of tested molecules was assessed in some studies [131, 136, 159] which must not always correlate with the level and activity of the respective protein product.

The highest concordance in experimental evidence so far was reached for the activation of PERK-eIF2α-ATF4-CHOP pathway by saturated FAs, however, there are some conflicting data whether and to which extent is this signaling axis also activated by unsaturated FAs. In INS-1 and INS-1E cells, several studies demonstrated activation of this pathway by PA, but not OA or combination of PA and OA [131, 158, 159, 225]. However, in another study on INS-1E cells both PA and OA induced expression of transcription factors CHOP and ATF4 and surprisingly, the response to OA was more pronounced than to PA [157].

Activation of PERK pathway only by PA but not by POA alone or in combination with PA was shown also in BRIN-BD11 cells [165]. PA induces phosphorylation of PERK and eIF2α and expression of CHOP also in MIN6 cells [139, 168, 169, 226, 227]. In rat primary β-cells [159] and in human islets [136, 159, 227] is this pathway activated only by saturated FAs.

5.5.3 Activation of IRE1α pathway

Markedly more equivocal data were obtained concerning the activation of IRE1α pathway of ER stress signaling. Activation of this ER stress signaling arm is usually demonstrated at the level of XBP1 mRNA splicing. Spliced form of XBP1 mRNA was found after PA treatment in INS-1 [157, 158], INS-1E [131, 159] and MIN6 [139, 226]. However, OA was shown to induce XBP1 splicing as well although the extent of this response differed among studies [131, 157, 159]. In a marked contrast, Karaskov et al. did not find OA to induce XBP1 splicing in INS-1E [158], similarly to Laybutt et al. in MIN6 cells [139]. In BRIN-BD11 cells, IRE1α pathway is claimed to be not activated, however, no experimental evidence for such conclusion is provided by the authors [165].

In rat primary β-cells, only PA but not OA and combination of PA and OA were reported to induce significant XBP1 splicing. In human islets, XBP1 splicing activation by PA, but not OA was shown in one study [159] but surprisingly, no splicing after PA treatment was detected in another study from the same experimental group [136].

The activation of the second arm of ER stress signaling, i.e. activation of JNK, was studied only rarely. JNK was reported to be activated by PA [159, 170], but not OA [159].

5.5.4 Activation ATF6 pathway

Experimental evidence concerning activation of ATF6 signaling is also not unambiguous. Triggering of this pathway by PA, but not OA was reported in MIN6 cells [139]. No involvement of ATF6 pathway in FA-induced signaling was declared in BRID-BD11 cells but convincing data for this statement are missing, similarly to data concerning IRE1α pathway activation from the same authors [165]. On the contrary, activation by both PA and OA was detected in INS-1 cells [157]. The results of other studies that addressed this experimental question are not easily interpretable, since reporter construct with both ATF6 and XBP1s binding sites was used [131, 159] and its activation by both PA and OA demonstrated thus results from cumulative effect of ATF6 and IRE1α pathway activation.

5.5.5 Effect on BiP expression

Increased expression of ER chaperone BiP is considered as a typical ER stress marker. However, variance in experimental evidence also exists as to the induction of BiP expression by FA treatment in β-cells. In several studies on pancreatic β-cell lines, no induction of BiP expression by any of the FAs tested was observed [135, 158, 165] whereas increased expression by both PA and OA was reported by others [131, 157]. BiP induction was found in PA- but not OA-treated rat and human islets [136, 159] and MIN6 cells [139].

Increased expression of BiP is also regarded as an important antiapoptotic mechanism. Overexpression of BiP was found protective against PA-induced apoptosis in MIN6 cells [139] whereas not in INS-1 cells [135]. Altogether, these results indicate that the role of BiP in FA-induced apoptosis of β-cells remains to be elucidated.

5.5.6 The role of fatty acid metabolism in ER stress induction

In several studies, the mechanisms that lie behind the regulation of ER stress induction by FAs were addressed. Similarly to their inability to induce apoptosis, methylesters of saturated FAs were shown to be ineffective in induction of expression of ER stress-related molecules [159, 164], indicating that conversion of saturated FA into FA-CoA is necessary for triggering of ER stress. In contrast, but in line with the observed effect on apoptosis induction, methylesters of unsaturated FAs exerted

identical inhibitory response on ER stress induction by saturated FAs as their parental molecules [164, 165]. These findings indicate that conversion of saturated FA into FA-CoA is necessary for ER stress triggering whereas no metabolic conversion of unsaturated FAs is needed for the inhibitory effect on ER stress signalling activated by saturated FAs (see also 5.3.4.431, p. 31).

5.5.7 Mediators of ER-stress induced apoptosis

The experimental evidence concerning mechanisms by which FA-induced ER stress triggers apoptosis in β-cells is very weak and concerned only CHOP, JNK and caspase-12 as possible mediators. In INS-1E cells, PA treatment was associated with caspase-12 activation. However, the siRNA-mediated knockdown of CHOP only delayed but not inhibited the PA-induced apoptosis, in contrast to inhibition of JNK activity that partially reduced the apoptosis [159]. These results point to the fact that both CHOP and JNK play role in apoptosis activation downstream of ER stress but some other molecules must be involved as well.

Taken together, the research in the field of ER stress induction in β-cells was not able to provide us with a clear view of the mechanisms of ER stress regulation by saturated and unsaturated FAs so far. Nevertheless, the role of ER stress in physiological and pathological functioning of β-cells is accepted without any doubts today and at least some efforts were made towards its understanding.

6 AIMS OF THE PROJECT

- **to develop an alternative method for the isolation of Langerhans islets from mice**
  - the method without the use of Ficoll gradient should provide viable and functional islets

- **to contribute to our understanding of molecular mechanisms of apoptosis and ER stress induction by saturated fatty acids and their regulation by unsaturated fatty acids in human pancreatic β-cells NES2Y**

  In particular, our studies were focused on:

  - the effect of individual fatty acids on the growth and viability
  - basic mechanisms of saturated fatty acid-induced apoptosis
  - the inhibitory effect of unsaturated fatty acids on apoptosis induction by saturated fatty acids
  - ER stress induction and regulation by fatty acids
  - the role of caspase-2 in saturated fatty acid-induced apoptosis and ER stress

7 COMMENTS ON PAPERS AND DISCUSSION OF RESULTS

7.1 List of papers

- **Development of an alternative method for the isolation of Langerhans islets from mice**

PAPER 1:

Kopska T., **Fürstova V.**, Kovar J.:
Modified method for isolation of Langerhans islets from mice.
Transplantation Proceedings 40(10): 3611-4, 2008
IF = 1.055 (2008)

- **Effect of fatty acids on apoptosis and ER stress in pancreatic β-cells**

PAPER 2:

Fürstova V., Kopska T., James R.F., Kovar 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 Sciences 82(13-14): 684-91, 2008
IF = 2.583 (2008)

PAPER 3:

Němcová-Fürstová V., James R.F., Kovář J.:
Inhibitory effect of unsaturated fatty acids on saturated fatty acid-induced apoptosis in human pancreatic β-cells: activation of caspases and ER stress induction.
Cellular Physiology and Biochemistry 27(5): 525-38, 2011
IF = 3.585 (2010)

PAPER 4:

Němcová-Fürstová V., Šrámek J., Balušíková K., James R.F., Kovář J.:
Caspase-2 and JNK activated by saturated fatty acids are not involved in apoptosis induction but modulate ER stress in human pancreatic β-cells.
Cellular Physiology and Biochemistry, submitted
IF = 3.585 (2010)

7.2 Development of an alternative method for the isolation of Langerhans islets from mice

Nowadays, isolation of Langerhans islets is well described but technically very demanding and time-consuming procedure whose results are, despite being performed usually only at specialized laboratories, not always satisfactory in respect to the number and quality of isolated islets. However, transplantation of Langerhans islets has a great potential for therapy of type 1 diabetes (T1DM) and therefore, there is a high pressure on the availability of suitable and reproducible methods for islets isolation. In addition, isolated islets of both human and animal origin can serve as experimental material for research purposes that can provide, in respect to its higher resemblance with conditions *in vivo*, significantly more relevant data than experiments on pancreatic β-cell lines.

The most commonly used protocols for isolation of Langerhans islets are based on the protocol described by Lacy and Kostianovsky in 1967 [228]. However, besides other modification, the most important improvement done since then in the original protocol is the substitution of the sucrose gradient used for separation of Langerhans islets from the endocrine tissue that caused hyperosmotic injury of the isolated islets, for the Ficoll gradient. Ficoll is high molecular weight polymer of sucrose (40 kDa) and permitts the recovery of functionally viable islets [229]. However, Ficoll was shown later to affect islet yield, viability and function to certain extent [230-234]. This implies the need of less toxic, cheaper and technically less demanding approaches.

From time to time, improvements of already established methods or new protocols for islet isolation employing quite original approaches are reported [233-236]. For example, recently, an enzyme-free method of islet isolation based on cryopreservation of islets with simultaneous freeze-destruction of acinar tissue was published [237]. Because of planned studies on Langerhans islets, we tried to develop our own method whose novelty would lie in employment of specific type of cultivation plastic during the isolation procedure.

Laboratory plastic that is commonly used for cell culture is available in two options: for suspension cells and for adherent cells. Plastic for suspension cells is hydrophobic which results from inherent characteristics of polystyrene material they are made from. In contrast, the surface of plastic for adherent cells has to undergo special treatment to make it more hydrophilic to enable efficient cell attachement.

Islets do not have an inherent tendency to attach to plastic for suspension cells during cultivation after isolation, in contrast to the exocrine tissue cells. So we tried to use this difference between the endocrine and exocrine tissue for their effective separation during the isolation of murine Langerhans islets. Similar approach was used successfully many years ago by Hegre et al. [238] to circumvent the use of exogenous proteolytic enzymes in the isolation of Langerhans islets from the perinatal rodent pancreas. However, the potential benefit provided by the employement of this type of plastic during separation of endocrine and exocrine tissue after enzymatic digestion has not been tested yet.

Taking advantage of the use of the plastic for suspension cells combined with differential sedimentation rate of islets and the contaminating material, we were able to separate effectively islets from the exocrine tissue (for the complete protocol, see p. 62). Also the subsequent hand-picking of islets from suspension before functional testing was facilited by the relative purity of isolated islets (see Fig 1, p. 63). Islets isolated by our modification of the isolation method were viable and insulin-producing and the yield was comparable to the yield reported in other commonly used protocols [239-241].

The advantage of our modified protocol for murine islet isolation is that it is relatively easy and time- and cost-efficient. As it omits the use of Ficoll gradient centrifugation without affecting the yield and viability of islets, it would be particularly suitable for experiments where the possible negative impact of Ficoll on islet function is considered undesirable. Also, minimum of laboratory equipment and previous technical experience with islet isolation is required for successful isolation by this protocol. We have not tested the efficacy of this protocol for isolation of islets from other species, however, we believe it is applicable to other species as well. The use of specific plastic for suspension cells could be useful also for isolation or purification of various other cell types where different cell adhesiveness to cultivation surfaces can be expected.

7.3 Effect of fatty acids on apoptosis and ER stress in pancreatic β-cells

The need for effective cure of type 2 diabetes (T2DM) is of high importance because of the increasing number of patients with this disease and growing cost of the medication. Taking into account the simultaneous rise in obesity worldwide, further

increase in the number of people affected with T2DM is unfortunatelly guaranteed. Therefore, the profound knowledge of mechanisms involved in the pathogenesis of this disease is highly desirable as it can provide new therapeutical approaches for the treatment. It may also delineate the ways for prevention of the disease or at least for delaying its onset.

The research in our laboratory is focused on molecular mechanisms that underlie β-cell dysfunction and apoptosis in T2DM. In particular, our experimental data should contribute to the understanding of mechanisms involved in the regulation of pancreatic β-cell viability and apoptosis by fatty acids (FAs) that are only partially understood so far.

Because of the lack of ideal human β-cell line, most experiments in the field of β-cell apoptosis research are carried out with rat and mouse β-cell lines or islets; human Langerhans islets are used only sporadically due to their preferential use for transplantations. In our experimental setup, we took the advantage of one of the two existing human β-cell lines, the NES2Y line, established in 1997 at University of Leicester, UK from a patient with persistent hyperinsulinemic hypoglycemia of infancy [41]. The major weak side of these cells as a model system for study of diabetes mellitus-associated processes is their incomplete β-cell characteristics, as they are not able to respond to glucose due to impaired expression of the transcription factor IUF1 (insulin upstream factor 1). This feature excludes the NES2Y cells from studies concerning processes related to insulin secretion, however, we believe that this does not discriminate them as plausible model system for research of apoptosis, induced e.g. by FAs. Previously, this cell line was used by other investigators as well for studies concernig apoptosis induction [242, 243].

7.3.1 The effect of individual fatty acids on the growth and viability

We determined the time-course effect of several FAs species on the growth and survival of NES2Y cells. The FA species tested were saturated palmitic acid (PA) and stearic acid (SA) and unsaturated palmitoleic acid (POA), oleic acid (OA), elaidic acid (EA) and linoleic acid (LA). These FAs differ in the carbon chain length, level of saturation (none, one or two double bonds) and space conformation of the double bond (*cis* versus *trans*). For the structure and chemical formula of the FAs used see Table 5-1, p. 25. This spectrum of FAs was selected because they are the most abundant FAs in the

blood (PA, SA, POA, OA, LA) [93, 94, 244]. EA was chosen as the most common *trans* FA that is generated and introduced into food by industrial oil hardening process [95, 245].

All FAs were administered in experiments bound to bovine serum albumin (BSA) because they are naturally water-insoluble. This also mimics the situation in *in vivo* system where FAs are transferred by the blood stream in complex with BSA. The range of FA concentrations tested in experiments (0-3 mM) are within the physiologically available range [94] and in the range of concentrations used in similar experiments by other researchers, e.g. [132, 133, 151]. As fetal bovine serum, routine culture media supplement, contains undefined spectrum of FAs in unknown concentrations, we performed our experiments in RPMI 1640-based chemically-defined serum-free culture media [246, 247] (see also "Cells and culture conditions", p. 67) allowing precise control of individual FA concentrations in experiments. These media enabled the cell growth during experiments in contrast to classical RPMI 1640 media simply depleted of serum used by many other investigators in this field of research, e.g. [115, 128, 130, 145, 156].

We revealed that the effect of saturated and unsaturated FAs on the growth and viability of NES2Y pancreatic β-cells is strikingly different. Saturated FAs, PA and SA affected dose-dependently the viability of cells after 48 h of treatment; the significant decrease in the number of living cells after the treatment was apparent already at 0.3 mM concentration and concentrations 1 mM and higher led to the death of almost all cells in the culture. In contrast, their *cis* unsaturated counterpart FAs, POA and OA, were well tolerated by β-cells and only the highest concentration used (3 mM) caused decrease in the cell number but did not lead to cell death. Moreover, the proliferation of β-cells was even increased after treatment with low concentration of OA (see Figs 1 and 2, p. 68). Such difference in the effect of saturated and unsaturated FAs is consistent with the results obtained by other researchers with a number of animal β-cell lines and with isolated animal and human islet β-cells as well (see also 5.3.3.2, p. 27). The rather proliferative effect of unsaturated FAs in human and animal β-cells was also noticed by others [115, 132].

When comparing OA to its *trans* counterpart EA, we found that significant growth-inhibiting effect of EA was present at concentration of 0.1 mM, in contrast to OA that exhibited similar effect at 10x higher concentration, i.e. 1 mM (see Fig. 3., p.

69). Although both OA and EA exerted similar toxicity at 3 mM concentration, this different behaviour in lower concentrations indicates higher deleterious effect of *trans* FA species that could have physiological relevance. Our finding is consistent with the observation that *trans* FAs are effective but less potent in inhibition of saturated FA-induced apoptosis than their *cis* counterparts [130].

The more deleterious effect of *trans* FAs in comparison with *cis* FAs was also found in other cell types [248-251]. Furthermore, based on the results of several epidemiological studies, the adverse effects of *trans* FAs has already started to be discussed in relation to various health problems and diseases, including coronary heart disease and T2DM [252-254].

The reason for more detrimental effects of *trans* unsaturated FAs compared to *cis* unsaturated FAs could lie in their different physical properties, e.g. melting point, that are more similar to properties of saturated FAs than that of *cis* unsaturated FAs. The composition of FAs in membrane phospholipids can influence, through the physical properties of incorporated FAs, the membrane fluidity and affect various membrane functions including signal transduction [255-257]. For example, induction of proinflammatory response was shown in endothelial cells after incorporation of *trans* FAs into membrane phospholipids recently [258].

We also investigated whether the cell death-inducing effect of saturated FAs can be regulated by unsaturated FAs. We found, that both unsaturated FAs, POA and OA, were able to inhibit the decrease in β-cell viability induced by saturated FAs, PA and SA, and they were effective even at concentrations 20x lower than the concentration of saturated FAs used for induction of apoptosis, i.e. 1 mM. Moreover, the concentration of 0.2 mM and higher, i.e. 5x lower than cell death-inducing concentration of saturated FAs, was even able to completely revert the cell loss induced by saturated FAs and enabled cell growth (see Fig. 1., p. 79). Similar observations were made in other β-cells lines and isolated islets as already mentioned (see 5.3.3.3, p. 28). Importantly, the inhitory potential of unsaturated FAs against the saturated FA-induced cell death was demonstrated in other cell types as well [248, 259, 260].

These results are very important due to their physiological relevance as FAs in the plasma never exist in the form of a single species but always in combination that reflects at least partially the composititon of various FAs species in diet. This implies that understanding of molecular mechanisms governing the survival of β-cells in the

environment containing both saturated and unsaturated FA species would be of great physiological significance. Moreover, such knowledge could be also used for targeted regulation of β-cell survival.

Taken together, we found that saturated FAs are able to induce apoptosis in NES2Y cells in contrast to *cis* unsaturated FAs that are not harmful and are even able to inhibit the cell death-inducing effect of unsaturated FAs. *Trans* FAs are not directly cytotoxic but exert more deleterious effect on cell growth than their *cis* counterparts.

7.3.2 Basic mechanisms of saturated fatty acid-induced apoptosis and its inhibiton by unsaturated fatty acids

7.3.2.1 Activation of caspase-3

To elucidate the mechanisms of apoptosis induction by saturated FAs in NES2Y cells, we assessed caspase-3 activity as its activation belongs to the basic hallmarks of apoptosis. As experimental approach, we used colorimetric assay based on direct measurement of caspase-3 activity in cell lysates *via* cleavage of chromogenic caspase-3 specific substrate. Surprisingly, we did not detect any significant increase in caspase-3 activity after PA and SA treatment (see Fig. 5., p. 70). As this was rather unexpected finding, we confirmed it by independent methods, flow cytometry employing fluorescent conjugate of caspase-3 inhibitor and Western blotting, employing specific caspase-3 antibody (see Fig. 2., p. 79 and Fig. 3., p. 80).

Caspase-3 activation was detected after PA treatment in several β-cell lines and primary animal β-cells by colorimetric and fluorimetric assays [140-142, 167, 261, 262] (see also 5.3.4.1, p. 29). In contrast, the data concerning human β-cells are very spare. Only Maedler et al. [132] mentioned in their paper the specific detection of cleaved caspase-3 in PA-treated human islets but any information documenting the extent of its activation is missing. Thus in fact, to our best knowledge, no data demonstrating specific involvement of caspase-3 in saturated FA-induced apoptosis in human β-cells are available so far. Our findings may therefore suggest that there are perhaps differences in the role of caspase-3 activation in FA-induced apoptosis between rodent and human β-cells. Another possibility is that NES2Y cells may possess functionally defective caspase-3.

However, the assays based on measuring the caspase activity by specific substrate cleavage are not absolutely specific for the caspase tested, as various

substrates previously reported to be specific for a given caspase were later shown to be cleaved also by other caspases, albeit with lower efficacy [263]. This raises doubts about validity and relevance of a huge amount of data obtained in the apoptotic field of research and implicates the need for employment of more specific methods when serious conclusion are to be drawn. This raises objections to the published data concerning caspase-3 activation in β-cells as well and, of course, also to all our data concerning caspase activation obtained by both colorimetric and flow cytometric assays as they also rely on the use of "specific" caspase substrates or inhibitors. Nevertheless, we verified our data obtained by these methods also by Western blotting, employing specific antibodies against individual full length caspase forms (data not published) and against their cleaved forms, which is still the most specific and reliable method available.

Based on all our results concerning caspase-3, we concluded that its activation is not essential for saturated FA-induced apoptosis. However, the role of caspase-3 may be substituted by activation of executioner caspase-6 and -7 that we demonstrated to be activated by SA treatment in NES2Y cells (see Fig. 3., p. 80).

7.3.2.2 Activation of mitochondrial pathway of apoptosis and caspase-9

Based on our demonstration of caspase-9 activation by SA treatment (see Fig. 2., p. 79 and Fig. 3., p. 80), we were interested whether this is a result of activation of mitochondrial apoptotic pathway, as already suggested in literature [115, 132, 156, 225]. To solve this question, we assessed potential changes in the mitochondrial membrane potential ($\Delta\Psi_m$) and reactive oxygen species level (ROS) that are usually associated with mitochondrial pathway of apoptosis and the cytochrome c release which is the basic hallmark of activation of this pathway. Employing flow cytometric approaches, we found only slight, nonsingnificant changes in $\Delta\Psi_m$ and ROS level (see Fig. 4., p. 81), and, most importantly, we did not find a massive cytochrome c release that would indicate activation of mitochondrial pathway of apoptosis (see Fig. 4., p. 81). As this was very fundamental information for our next experiments, we confirmed it by confocal microscopy (see Fig. 5., p. 82).

Our finding was in contradiction to some extent with already published data by Maestre et al. [156] who found increased ROS production and reduced $\Delta\Psi_m$ after PA

treatment, however, the magnitude of changes they detected was rather modest. Another factor contributing to the discrepancy of the results may be our use of chemically-defined media allowing the growth of the cells during the experiment (see also 7.3.1, p. 47) in contrast to Maestre et al. [156] who used serum-depleted media that serve as an additional stress factor in the experiments as admitted by the investigators themselves. Such effect of serum-free media is documented in the paper being just discussed by a marked change in ROS level and $\Delta\Psi_m$ in comparison to cultivation under standard serum-containing conditions. In addition, serum-free media was shown to cause a certain level of ER stress in β-cells as well [165]. In contrast, we did not observe any negative effect of our chemicaly-defined media on the cell viability and ER stress-associated parameters.

As we did not find signs of significant activation of mitochondrial pathway, the question concerning the molecular mechanism of caspase-9 activation in SA-treated β-cells has not been answered. However, caspase-9 activation can occur independently of cytochrome-c release and apoptosome formation as already reported for various cell types and apoptotic stimuli [264-266] and, recently, also for streptozotocin and c-Myc induced apoptosis in β-cells [267].

To conclude, our data indicate that SA-induced apoptosis in NES2Y cells can occur without prominent changes in mitochondrial functions and without cytochrome c release.

7.3.2.3 Activation of receptor pathway of apoptosis and caspase-8

The detected activation of caspase-8 pointed to the possible involvement of receptor-pathway of apoptosis induction (see 5.2.1.2, p. 17) in SA-induced β-cell apoptosis. Because of the possible role of Fas/FasL interaction in pathogenesis of T2DM [172, 268], we analyzed whether FA-treatment results in change of either Fas or FasL expression.

Both molecules were expressed in NES2Y cells under control conditions. Fas expression in control cells was caused, very likely, by the persistent hyperglycemic environment [268] resulting from the use of RPMI 1640-based culture media where the standard glucose concentration is 11 mM. Of note, because of the common use of the RPMI 1640 media for cell cultures, not only ours but also the overwhelming majority of all other experimental results published in the world is in fact affected by the

hyperglycemic setting of the experiments. Nevertheless, some other commonly used media are even worse from this point of view, e.g. the standard formulation of the Dulbecco´s Modified Eagle´s Medium (DMEM) contains 25 mM glucose, contrasting dramatically with the normal glycemic level of 5.5 mM in the human body and thus expected as optimal for human cell cultivation.

We found no significant changes in Fas and FasL expression after SA treatment (see Fig. 6., p. 83), indicating that these two molecules are unlikely to play an important role in SA-induced apoptosis. This is consistent with the finding that β-cell specific deletion of Fas receptor is not able to protect mice against high fat diet-induced T2DM [269]. Nevertheless, we could not exclude the possibility that caspase-8 activation results from activation of some other death receptor on the β-cell surface, nor the involvement of other regulatory circuits at the level of caspase-8 activation, e.g. *via* regulation of expression of FLIP (FLICE-inhibitory protein) that has already been demonstrated to be downregulated by high glucose level in β-cells [270]. In addition, the potential of PA to induce expression of TRAIL receptor DR5 and activate caspase-8 in human hepatoma cells *in vitro* was shown recently [271].

Rather unexpectedly, caspase-8 activation may also result from direct cleavage by caspase-7 and thus bypass the receptor pathway of apoptosis induction [272]. Caspase-7 is activated by SA in NES2Y cells, however, whether it is responsible for the observed caspase-8 activation remains to be clarified.

Summarizing, caspase-8 activation is involved in SA-induced apoptosis in NES2Y cells. Moreover, its activation by saturated FAs appears to be a common mechanism of their action in various cell types [250, 273], however, the experimental evidence is very spare and irrespective of cell type studied, the mechanisms leading to caspase-8 activation by saturated FAs remains elusive.

7.3.2.4 Role of caspase-2 in saturated fatty acid-induced apoptosis

We were the first who demonstrated caspase-2 activation by saturated FAs in β-cells (see Fig. 7. on p. 70, Fig. 2. on p. 79 and Fig. 3 on p. 80). So far, only one other study exists, besides our published data, that deals with caspase-2 in this context but only the level of its expression in cytosol and mitochondria, not its activation, was evaluated there [156]. However, the possible importance of caspase-2 for apoptosis induction in pancreatic β-cells was indicated earlier by the demonstration that caspase-2

mediates apoptosis induced by GTP-depletion in HIT-T15 β-cell line [274]. Caspase-2 activation has also been demonstrated in human islets during pancreas procurement and storage before its use for islet isolation and associated with lower islet viability [275]. In contrast, another study found that caspase-2 is not involved in better survival of pseudoislets formed from β-cells of MIN6 line under two different cultivation conditions [276]. However, to the best of our knowledge, this is the complete list of papers that deal with caspase-2 in relation to pancreatic β-cells and Langerhans islets besides our own contributions.

Caspase-2 was already shown to function as the key initiator caspase in many experimental systems employing various stimuli, e.g. [69, 277, 278]. So we hypothesized that it could serve as an initiator caspase in saturated FA-induced apoptosis of pancreatic β-cells as well. However, we did not find any significant effect of targeted silencing of caspase-2 expression on the viability of NES2Y cells after SA application (see Fig. 5., p. 116), indicating no key role of caspase-2 in SA-induced cell death of NES2Y cells. There was also no apparent delay in activation of other caspases (see Fig. 6., p. 117) which implied that caspase-2 is not involved in activation of other caspases in SA-treated NES2Y cells.

To elucidate whether the caspase-2 activation occurs *via* PIDDosome formation (see 5.2.1.5, p. 20), we assessed the expression level of PIDD protein, the regulatory component of this signaling complex formation, after FA treatment. We did not detect any change which indicated that caspase-2 is very probably activated *via* a PIDDosome-independent mechanism. Such mechanisms have been already demonstrated to exist in some other experimental models where apoptosis was associated with caspase-2 activation [279-284]. For example, DISC formation (see 5.2.1.2, p. 17) was shown to mediate caspase-2 activation, besides its indisputable role in activation of caspase-8/10 [279, 280, 283], however, active caspase-2 activation must not necessarily participate in further propagation of apoptotic signal [280]. Such behaviour of caspase-2 would be also consistent with our data - clear activation of both caspase-2 and caspase-8, but no effect of caspase-2 silencing on the apoptotic signaling *per se*.

Caspase-2 was also reported to be activated by caspase-7 [272] which could occur in NES2Y cells as caspase-7 is activated by SA in these cells (see Fig. 3, p. 80). However, to rule out the involvement of PIDDosome in caspase-2 activation definitely,

additional studies employing different approaches, e.g. siRNA-mediated PIDD silencing, are necessary.

To summarize, we showed that caspase-2 is activated by saturated FAs in NES2Y β-cells, very likely by a PIDD-independent mechanism. However, caspase-2 is not an apical caspase in the caspase activation cascade and is dispensable for saturated FA-induced apoptosis.

7.3.2.5 Role of p53

Fas receptor and PIDD belong to a large group of p53 regulated proteins (see also 5.2.1.4, p. 19) [67, 285]. Their expression, as well as the expression of another well known transcriptional target of p53 protein, the $p21^{WAF1/CIP1}$ protein [286], was not upregulated by SA (see Fig. 6., p. 83). This implied that SA-induced apoptosis may be independent of p53 activation.

Concerning the literature, the evidence documenting the effect of FAs on p53 activation in β-cells is very poor. The activation of p53 by both PA and OA during cell-death induction was demonstrated in animal β-cell lines [287, 288]. Another study found p53 mRNA expression significantly, but only modestly, increased in PA-treated MIN6 cells [289]. This seems not to correlate with our finding, however, p53 was shown not to be activated in mouse islet β-cells by γ-irradiation which is a well-known p53-activating signal. This raises the question about the general requirement of p53 for β-cell apoptosis [290]. So it is clear that further experiments are needed to solve this issue completely.

7.3.2.6 Inhibitory effect of unsaturated fatty acids

Based on the interesting results concerning the inhibitory effect of unsaturated FAs on the cell death-inducing effect of saturated FAs, we studied the molecular mechanisms underlying this effect of unsaturated FA in parallel with molecular mechanisms of saturated FA-induced apoptosis. We believed that this approach could reveal the step(s) in the apoptotic signaling pathways activated by saturated FAs, where the inhibitory effects of unsaturated FAs takes place.

Very importantly, all the caspases we found activated by SA (caspase-2, -6, -7, -8 and -9) were not activated when OA was applied together with SA. This was consistent with data from others [130, 145] and indicated that the inhibitory intervention

of unsaturated FAs into saturated FAs-induced cell death signaling occurs upstream of caspase activation.

Our results also revealed that the inhibitory intervention of OA into SA-induced signaling is not exerted *via* regulation of p53 activation, PIDDosome and Fas/FasL interaction (see Fig. 6., p. 83) nor regulation of mitochondrial pathway of apoptosis activation (see Fig. 4 on p. 81 and Fig. 5. on p. 82). However, importantly, we found that OA is able to inhibit SA-induced upregulation of several ER stress markers (see 5.4.1, p. 34), i.e. BiP, CHOP and XBP1s (see Fig. 6., p. 83). This indicated that the inhibitory effect of unsaturated FAs is exerted upstream or at the level of ER stress induction (see also our unpublished data, p. 119).

Interestingly, the available experimental data point to the fact that inhibitory effect of unsaturated FAs occurs very rapidly and is able to block the apoptotic signaling even if its execution has already been started to some extent at the molecular level [130, 144]. Unrestrictedly to β-cells, unsaturated FAs are also effective against wider spectrum of apoptosis-inducing molecules (but not all) with different modes of action [291, 292]. This would support the hypothesis that their inhibitory effect is exerted *via* triggering of some signaling pathway(s) that can intervene at some rather distal step to the cell death signaling initiated by saturated FAs or other proapoptotic stimuli [85, 129].

Several signaling pathways have been already shown to be differently activated/regulated by saturated and unsaturated FAs and proposed as possible mediators of the inhibitory effect. The molecules under consideration include several G protein-coupled receptors (GPR), e.g. GPR119 and GPR120 [293], protein kinase B [294] and protein phosphatase 1, involved in regulation of ER stress signaling [165]. However, the role of GPR119 in cytoprotection was questioned recently [295].

7.3.3 Regulation of ER stress by fatty acids

7.3.3.1 Activation of individual ER stress pathways

We found that several ER stress markers, i.e. BiP, CHOP and XBP1s (see 5.4.1, p. 34) are upregulated by SA treatment (see Fig. 6., p. 83). This indicated that SA activates at least some branches of ER stress signaling and stimulated us to study the ER stress signaling in detail.

The time-course analysis of SA effects on individual ER stress pathway activation (see 5.4.1, p. 34) revealed that SA activates all three ER stress pathways (i.e. IRE1α, PERK and ATF6 pathway) within 3 h after application (see Fig. 1. on p. 112, Fig. 2. on p. 113 and Fig. 3. on p. 114). The effect of SA on PERK pathway activation and eIF2α phosphorylation in NES2Y cells is in agreement with data obtained in PA-induced β-cell lines of animal origin [131, 135, 139, 158, 159, 164, 165, 226].

Furthemore, SA treatment activated IRE1α pathway in NES2Y cells as demonstrated by XBP1 splicing and JNK activation (see Fig. 1. on p. 112 and Fig. 2. on p. 113). This finding is consistent with experimental data of some investigators [131, 139, 157, 159, 226], whereas not with data from others [135, 136, 165]. This may reflect the level of ER stress achieved in experiments as this pathway appears to be activated only under more severe ER stress [135].

SA treatment also caused ATF6 pathway activation in NES2Y cells (see Fig. 3., p. 114), similarly to PA treatment in MIN6 cells [139]. In contrast to others [131, 157, 159] who used reporter constructs and luciferase assays for demonstration of ATF6 transcriptional activity, we used confocal microscopy as an alternative method for direct detection of ATF6 translocation into the nucleus.

In conclusion, we found potent activation of all three proposed ER stress signaling pathways by SA. Notably, our results obtained with human β-cell line NES2Y seem to correlate better in respect to ER stress signaling with primary β-cells of rat as well as human origin [136, 159] than with rodent β-cell lines [157, 159, 165].

7.3.3.2 Role of JNK in ER stress

JNK is considered as a molecule that may link excessive ER stress with apoptosis induction. In contrast to Cunha et al. [159], we found no inhibition or delay of caspase activation and CHOP induction after specific inhibition of JNK activity by SP600125 (see Fig. 4., p. 115). Thus, our data disqualify JNK as the mediator of SA-induced apoptosis in NES2Y cells irrespective of whether ER stress signaling is involved in this apoptotic signaling as well. Of note, our data also do not suggest any prosurvival role of JNK that was also described [296, 297].

However, surprisingly, inhibition of JNK activity decreased the BiP upregulation induced by SA (see Fig. 4., p. 115). This implies that JNK is involved somehow in ER stress regulation. This inhibitory effect might be mediated *via* decreased level of

phosphorylated c-Jun caused by JNK inhibition. Phosphorylated form of c-Jun can participate in formation of AP-1 complex that can activate BiP expression besides the predominant activation by XBP1 and ATF6 [298, 299]. However, this explanation is not in agreement with absence of effect of JNK inhibition on the expression of CHOP (see Fig. 4., p. 115) which is also regulated *via* AP-1 complex [300]. Moreover, it was shown previously that AP-1 complexes formed after PA treatment in INS-1 cells do not contain c-Jun, but Jun-B [300], another member of the Jun family of transcription factors whose activation is independent on JNK [301]. Thus the explanation of this particular effect of JNK inhibition is elusive.

7.3.3.3 Role of caspase-2 in ER stress

We found that caspase-2 silencing also reduced the SA-induced upregulation of BiP expression (see Fig. 7, p. 118). There is no simple explanation for this effect either. However, caspase-2 expression was shown to be transcriptionally regulated by the sterol-regulatory element binding protein 2 (SREBP2) and sterol-regulatory element binding protein 1c (SREBP1c), major activators of cholesterol and fatty acid synthesis genes [302, 303] and, importantly, caspase-2 silencing caused decrease in cellular cholesterol and triacylglycerol levels [302]. Thus it appears that caspase-2 is involved by some so far poorly characterized pathway in cellular lipid homeostasis which may in turn regulate the level of ER stress in cells. Interestingly, caspase-2-deficient mice display reduced body fat content when compared with age-matched wild-type mice [304], which supports its suggested role in regulation of lipid homeostasis. Further research of this so far poorly characterized role of caspase-2 might also shed some light on the effect on BiP expression we observed in our study.

In conclusion, although we did not prove an important role for caspase-2 in SA-induced apoptosis, its involvement in non-apoptotic signaling *via* so far unknown molecular mechanism is very likely. Interestingly, a role for caspase-2 and also JNK in diseases associated with some form of lipid metabolism disorder is emerging, e.g. association of both caspase-2 and JNK activation in liver with the progression of non-alcoholic fatty liver disease was shown recently [305].

7.3.4 Concluding remarks

Taking into account the available experimental evidence concerning FAs from a various cell types, it appears that the more deleterious effect of saturated FAs in comparison with unsaturated FAs is their inherent feature [291, 306-309]. The molecular mechanisms underlying the harmfull effect of saturated FAs in various cell types seem to be similar as well and their understanding could thus be of high significance not only in the field of T2DM but also in other areas of medicine and human health care.

Unfortunately, despite the intensive research in our and many laboratories worldwide, a lot of questions still remain unanswered. Especially, the molecular mechanisms of apoptosis induced by saturated FAs and the identity of the apical caspase(s) or protease(s) in this process are still not well characterized. Additionally, it is unclear how excess of saturated FAs induce the ER stress in β-cells. Furthermore, the basic cause of the different effect of saturated and unsaturated FAs on β-cells is also unknown.

Based on recent experimental data [310], a new hypothesis has emerged that suggests that assembly of specific lipid rafts with different signaling potential could be the general principle underlying the different effects of saturated and unsaturated FAs in cells. This concept would be applicable to various signaling pathways starting at membrane-bound molecules, including ER stress sensors IRE1α, PERK and ATF6 and would be consistent with our and other data concerning the effect of saturated and unsaturated FAs in β-cells. However, further studies are necessary to validate this hypothesis.

Taken together, the mechanisms by which saturated and unsaturated FAs regulate the cell fate are hot topics with potentialy very profitable impact not only in β-cell and diabetes area of research. Although the amount of data is still growing, an unifying concept explaining their different effects is still missing. Nevertheless, we believe that our results did provide some new insights and may be the basis for further experimental studies. Also, we believe that our research contributed specifically to the know-how in the research field of diabetology in the Czech Republic.

8 PAPERS

8.1 Development of an alternative method for the isolation of Langerhans islets from mice

8.1.1 PAPER 1

Kopska T., **Fürstova V.**, Kovar J.:

Modified method for isolation of Langerhans islets from mice.

Transplantation Proceedings 40(10): 3611-4, 2008

ISLETS

Modified Method for Isolation of Langerhans Islets From Mice

T. Kopska, V. Fürstova, and J. Kovar

ABSTRACT

Successful isolation of Langerhans islets is a crucial prerequisite for their experimental or possible clinical use such as transplantation. Centrifugation in a Ficoll gradient is a common step used for separation of Langerhans islets from exocrine tissue. However, islets have been reported to be negatively affected by employing Ficoll gradients. Therefore, the aim of this study was to modify the isolation procedure by excluding Ficoll gradient centrifugation to obtain a similar or better yield of viable, functional islets. In our modification of the isolation procedure, the separation of islets from exocrine tissue was based on their sedimentation rate combined with their differential ability to attach to the surface of culture dishes for suspension cells. The resulting purity of islets facilitated their handpicking from the suspension. The mean yield was 900 viable, insulin-producing islets per mouse, which was comparable to or even higher than the yield in commonly used protocols. Our modification of the isolation method may be useful when centrifugation in Ficoll gradient is undesirable due to potential toxicity.

ALTHOUGH LANGERHANS ISLETS have been routinely isolated for various purposes for decades, some steps in the isolation procedure have shown a negative effect on islet yield, viability, and function. Enzymatic digestion of pancreas is the first crucial step affecting islet yield and viability. It should be rapid; collagenase must reach every part of the digested pancreas more or less synchronously. This effect can be achieved either by intraductal injection of the collagenase solution[1–3] or by incubation of minced pieces of pancreas in the solution.[4] The next crucial isolation step is Langerhans islets separation from exocrine tissue, which is usually accomplished by centrifugation in a Ficoll density gradient.[1–3,5] However, recent studies have shown that Ficoll affects islet yield,[6,7] viability, and function.[5,8–10] Ficoll toxicity has been also confirmed for mouse islets.[5] Furthermore, in vitro studies have shown that Ficoll has the capacity to induce interleukin-1β release from human peripheral blood mononuclear cells.[11] The isolation procedure is usually finalized by time-consuming handpicking of individual islets from the exocrine tissue rests.

Langerhans islets can be successfully cultured for prolonged times.[12–14] Langerhans islets are usually cultured in

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dishes for suspension cultures where they do not attach.[15] However, it has not been tested whether this feature of Langerhans islets may also be used for efficient separation from exocrine tissue during the isolation procedure and thus to substitute for centrifugation in a Ficoll gradient for the isolation protocol.

We developed a modified method for isolation of Langerhans islets, avoiding centrifugation in a Ficoll gradient. In contrast to other methods, Langerhans islets are separated from the exocrine tissue on the basis of their differential ability to attach to the surface of culture dishes for suspension cells. When combining this approach with elimination of single cells and debris by sedimentation, we obtained a purity of islets that enabled their easy handpicking for experiments. Our modification yielded viable, insulin producing islets at higher or at least comparable numbers to other isolation protocols.[2,3,16]

MATERIALS AND METHODS

Media

The basic medium was Dulbecco's modified Eagle's medium containing only 5.5 mmol/L glucose (Sigma-Aldrich, St Louis, Mo, USA) and 17.8 mmol/L $NaHCO_3$ (Sevafarma, Prague, Czech Republic) with the addition of extra 15 mmol/L HEPES (Sigma-Aldrich) and 50 μmol/L mercaptoethanol (Gibco-Invitrogen, Carlsbad, Calif, USA). Culture medium represented the basic medium supplemented with 10% fetal bovine serum (Gibco-Invitrogen).

Isolation of Islets

The pancreas excised from female mice of ICR or BalbC strains, at 8 to 16 weeks old immediately after cervical dislocation was transferred to cold basic medium. The pancreas was minced with scissors under sterile conditions. The pieces transferred to a 15 mL centrifugation tube containing 10 mL of cold basic medium without mercaptoethanol were allowed to settle by gravity to the bottom of the tube for 10 minutes on ice. The medium was then carefully removed before addition of 0.5 mL of collagenase P solution (2 mg/mL in phosphate-buffered saline, Roche, Mannheim, Germany). Digestion was performed in a water bath at 37°C under continuous shaking as described previously.[17] After 12 to 15 minutes, the digestion was stopped by diluting the collagenase P with 12 mL of cold culture medium.

The digested material filtered through a sterile mesh (500-nm pores) was transferred to a 15 mL centrifugation tube filled with culture medium to allow settling by gravity to the bottom of the tube for 7 minutes. The islets sediment was resuspended in 15 mL of fresh culture medium for another 7 minutes. Afterward, the sediment resuspended in 18 mL of fresh culture medium was divided into three dishes for suspension culture (Ø 6 cm; Sarstedt, Numbrecht, Germany) and incubated for 24 hours at 37°C in 5% CO_2. After the incubation we carefully collected, the supernatant which contained islets and residual nonislet cells that did not attach to the bottom of the culture dish. The collected supernatant was gently pipetted several times through a 5-mL glass pipette to dismantle islets from surrounding exocrine cells. The supernatant was allowed to settle in a 15-mL centrifugation tube filled with culture media for 7 minutes. The sediment was again resuspended in medium for another 24-hour incubation at 37°C in 5% CO_2. Then islets and residual unattached exocrine cells were collected. Islets were pipetted several times through a 5-mL glass pipette and separated from individual cells and debris by allowing them to settle in 15 mL culture medium for 7 minutes. Afterward, islets were handpicked from the sediment using a pipette.

Assessment of Cell Viability, Number, and Attachment

Viability of islet cells was assessed by trypan blue staining. The islets that contained only sporadic trypan blue-positive cells (<5%) were considered viable.

The number of cells per islet was assessed as the number of nuclei on slide preparations. After the isolation, islets were fixed in 3.7% formaldehyde solution in phosphate-buffered saline. Fixed islets were transferred into a drop of mounting medium containing 0.001 mmol/L 4′, 6-diamidino-2-phenyindole (DAPI) on a microscopic slide, gently pressed by a cover slip to flatten them and counted under a fluorescence microscope.

The capability of islet cells to attach was assessed by employing incubation on cover slips. Islets seeded onto cover slips in a drop of culture medium at a density of 5 to 10 islets per cover slip (1 cm^2) were allowed to spread at 37°C in 5% CO_2 for 48 hours. The number of islet cells attached to the cover slip after the incubation was estimated by counting the number of nuclei after the fixation and DAPI staining as described above. Attached cells were considered to be elements capable of forming a monolayer culture.

Assessment of Insulin Production

The number of insulin-producing islets was estimated by counting the number of dithizone-positive islets, which was performed as described previously.[4,18] The ability of islet cells to produce insulin was assessed after their attachment. Islets seeded into 24-well culture plate were incubated for 48 hours at 37°C in 5% CO_2. After the incubation, culture medium was changed and insulin secretion determined during the next 24-hour incubation employing. Mouse Insulin Elisa EIA-3440 (DRG Diagnostics, Marburg, Germany).

RESULTS

We observed the differential ability of Langerhans islets versus exocrine cells to attach to the surface of culture dishes for suspension cells. On this basis, we developed a modified protocol for isolation of Langerhans islets. We performed 20 independent isolations of Langerhans islets from 50 mouse pancreas by the method described above.

We optimized the time required for efficient sedimentation of islets from digested and filtered material. Most islets (and also bigger pieces of exocrine tissue) settled to the bottom during 7 minutes under the described conditions. During incubation of this settled material in dishes for suspension culture, the major part of the exocrine tissue attached to the bottom of the culture dish (Fig 1A). However, a small portion of exocrine tissue remained as individual cells or small clusters in suspension together with the islets. Some exocrine cells also clustered around the islets (Fig 1B). These exocrine cells were partially dismantled from islets employing gentle pipetting; single cells and debris were eliminated from the islet suspension by subsequent sedimentation (Fig 1C). Most remaining nonislet cells attached to the bottom of the culture dish during the second 24-hour incubation. After the last sedimentation, we

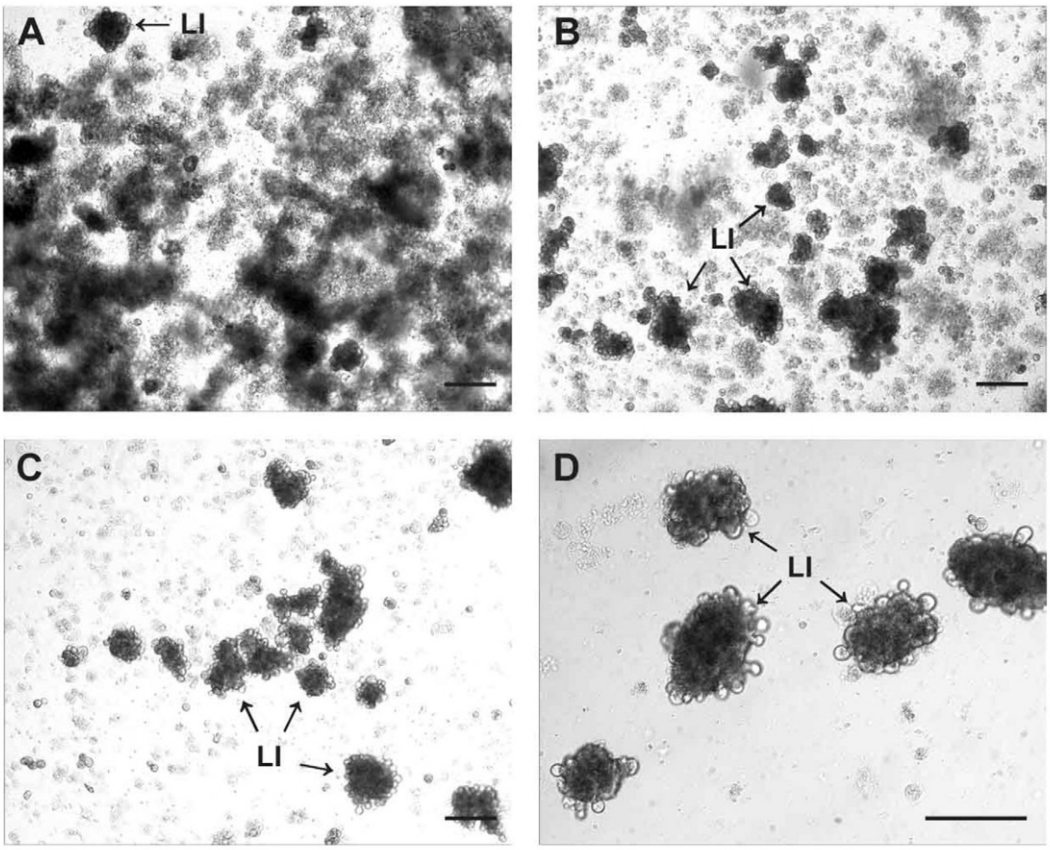

Fig 1. The separation of exocrine and endocrine cells during the procedure of isolation of Langerhans islets (LI). **(A)** After 24-hour incubation of islets and exocrine cells in the dishes for suspension culture, the cells of exocrine tissue are attached as large clusters to the bottom of the culture dish. **(B)** However, some exocrine cells stay in the suspension together with the islets. **(C)** Subsequent sedimentation and a second 24-hour incubation lead to further reduction in the number of exocrine cells. **(D)** After the second incubation followed by sedimentation, we obtain a relatively pure islet suspension. The bar represents 100 μm.

obtained an islet suspension of purity suitable for easy handpicking of islets (Fig 1D). The mean yield of isolated islets was 895 ± 105 (ranging from 800 to 1000) insulin-producing, viable Langerhans islets per mice. The differences in islet yield, using three independent batches of collagenase, were negligible.

To further characterize the islets isolated by our modified method, we assessed the size of the islets. Islets isolated by our modified method contained on average 350 ± 115 cells. The range of islet size was 90 to 1000 cells per islet. The majority of islets (80%) contained 100 to 450 cells, approximately 50% contained 200 to 400 cells.

We further assessed the ability of islet cells to form an attached culture of proliferating cells. Not all islet cells were capable of attaching to the surface of standard culture dishes to form a monolayer culture. On average, only 115 cells per islet attached. After the formation of an attached culture, the islet cells still produced and secreted insulin, approximately 30 ng insulin per 10^5 cells within 24 hours.

DISCUSSION

We developed a modified method to isolate Langerhans islets that provided a high yield of viable, insulin-producing islets with relatively low expense of time for manipulation and materials. Islets of Langerhans are usually isolated employing the separation of islets from the exocrine tissue by centrifugation in a Ficoll density gradient. However, it has been shown that methods avoiding Ficoll gradient purification lead to higher yield,[6,8,9] and better-quality of

islets.[5,7,8,10] These methods use either other chemical gradients[6–9] or mechanical means.[5] Generally, avoidance of chemical and mechanical stress (eg, centrifugation) should lead to better islet quality. We introduced two alternative steps for Langerhans islet separation from exocrine cells; both steps contributed to a higher yield of islets.

First, we used sedimentation to eliminate debris and individual cells. As the second step, we incubated the entire settled material, including exocrine cells, in dishes for suspension culture. During this incubation, most exocrine cells attached to the bottom of the culture dish, while islets stayed in suspension to be easily collected. Moreover, the amount of exocrine cells was gradually reduced by subsequently repeating these two procedures. Finally, the purity of isolated islets in suspension facilitated handpicking of islets for use.

Since the quality and quantity of islets are affected by the quality of collagenase,[19,20] we tested three independent batches of collagenase to prove that high yield is not merely a result of an enzyme batch that was exceptionally good. In our protocol, we also minced the pancreas into small pieces before the digestion. This approach is not used as often as intraductal injection of collagenase solution,[2] although it has been described previously.[4] We suppose that this alteration did not affect islet yield or viability to a great degree.

The reported yields of islets in commonly used protocols range from 200 to 700 islets per one mouse pancreas,[2,3,16] whereas using our modified method, the mean yield was about 900 viable, insulin-producing islets per mouse. This improvement may help to overcome the problem of low islet yield that particularly limits the use of islets for transplantation.[21]

It has been already proven that islets isolated without the use of Ficoll show higher quality[5,8,10] and are more suitable for both experimental and transplantation use.[5,10] We did not perform an experimental transplantation with islets isolated by our modified method, but it has been shown that islets purified by filtration[5] were more potent to restore normoglycemia than those isolated by Ficoll gradients. Islets isolated by our modification were viable, producing insulin; therefore, they should be suitable for experiments as well as for experimental transplantation.

Taken together, we have developed a modified method for Langerhans islet isolation. The method is based on the different ability of Langerhans islets versus exocrine cells to attach to the surface of culture dishes for suspension cells. Thus, the method avoids the use of Ficoll or any other chemical or mechanical stress. We suppose that this method may be useful for experimental cultivation of islets or islet cells as well as for experimental transplantation, because it is easy to perform and effective as to the time and costs. Moreover, it provides a high yield of viable, functional islets. It can become suitable particularly for experiments where Ficoll centrifugation is considered undesirable, particularly due to its potential toxicity, and when one considers the consumption of time and costs.

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8.2 Effect of fatty acids on apoptosis and ER stress in β-cells

8.2.1 PAPER 2

Fürstova V., Kopska T., James R.F., 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.

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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

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Abstract

We tested the effects of various types of fatty acids, differing in the degree of saturation and in the cis/trans configuration of the double bond, on the growth and viability of NES2Y cells (a human pancreatic β-cell line). We found that during a 48-hour incubation period, saturated fatty acids, i.e. palmitic and stearic acids, at a physiologically relevant concentration of 1 mM and higher concentrations induced death of the β-cells while their counterpart unsaturated fatty acids, i.e. palmitoleic and oleic acids, did not induce cell death at concentrations up to 3 mM. We also found that unsaturated elaidic acid with a trans double bond exerted significant inhibition of growth of the β-cells at a concentration approximately ten times lower, i.e. 0.1 mM vs. 1 mM, than counterpart oleic acid with a cis double bond. This is the first direct evidence that a trans unsaturated fatty acid is significantly more effective in inhibiting β-cell growth than a counterpart cis unsaturated fatty acid. Furthermore, we newly demonstrated that β-cell death induced by saturated fatty acids is related to significant increase of caspase-2 activity (2 to 5-fold increase) but not to caspase-3 activation. The growth-inhibiting effect of saturated fatty acids at concentrations lower than death-inducing concentrations correlates with certain increase of caspase-2 activity.

Keywords: Saturated fatty acids; Unsaturated fatty acids; Trans fatty acids; Cell growth; Cell death induction; Caspase-2; Pancreatic β-cells; NES2Y cells

Introduction

Recently it was clearly demonstrated that a reduction of β-cell mass by apoptosis contributes to the development of type 2 diabetes (Sakuraba et al., 2002; Butler et al., 2003). The generally accepted candidate triggers of β-cell death are fatty acids and glucose because elevated levels of free fatty acids and hyperglycemia are common features of the disease.

The apoptosis-inducing potential of fatty acids has previously been described for isolated human and animal β-cells in vitro as well as pancreatic β-cells lines (Maedler et al., 2001, 2003; Lupi et al., 2002; El Assaad et al., 2003; Kharroubi et al., 2004; Welters et al., 2004; Higa et al., 2006; Karaskov et al., 2006). It seems that the toxicity of fatty acids depends particularly on the degree of their saturation. Most papers (Maedler et al., 2001, 2003; Eitel et al., 2002; Welters et al., 2004, 2006; Higa et al., 2006), but not all (Maestre et al., 2003; Kharroubi et al., 2004; Karaskov et al., 2006), suggest that saturated fatty acids exert a proapoptotic effect whereas unsaturated fatty acids do not cause cell death. There are even some indications that unsaturated fatty acid treatment can counteract cell death induced by saturated fatty acids (Maedler et al., 2001, 2003; Eitel et al., 2002; Welters et al., 2004), serum removal and exposure to proinflammatory cytokines (Welters et al., 2004).

The molecular mechanisms of the cell death-inducing effect of fatty acids in pancreatic β-cells are not well understood. However, there is growing evidence that β-cell death induced by fatty acids is caspase dependent. Apoptosis induced by fatty acids in human and animal β-cells can be reduced by pancaspase inhibitor z-VAD (Lupi et al., 2002; Welters et al.,

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2006) and cell death induced by palmitate in rat β-cells BRIN-BD11 was accompanied by the activation of caspases as detected by FITC-VAD-FMK binding (Welters et al., 2004).

Unfortunately, the detection of cell death/apoptosis is very often based only on the detection of DNA degradation (e.g. TUNEL assay) and information on the involvement of specific caspases is missing. Nevertheless, according to available data caspase-3 seems to be the prominent caspase involved in the execution of fatty acid-induced cell death. Activation of this key executioner caspase after fatty acid-treatment was detected both in animal β-cell lines (Eitel et al., 2003; El-Assaad et al., 2003; Rakatzi et al., 2004) and in isolated human islets (Maedler et al., 2003). According to Hirota et al. (2006), caspase-6 could be also involved in cell death induced by fatty acids. Caspase-6 was found to be activated upstream of caspase-3 in a mouse β-cell line (βTC1) treated with palmitate. However, the role of other caspases in fatty acid-induced apoptosis has not been studied in detail so far.

In this study, we compared the effects of several types of individual fatty acids, differing in their degree of saturation and the cis/trans configuration of the double bond, on the growth and viability of the human β-cell line NES2Y. We found that saturated fatty acids at physiologically relevant concentrations induced cell death in contrast to unsaturated fatty acids. The trans fatty acid elaidic acid inhibited cell growth at a ten times lower concentration than its cis counterpart oleic acid. Furthermore, we found that caspase-2 but not caspase-3 was activated when saturated fatty acids induced cell death in NES2Y cells.

Materials and methods

Materials

Human apo-transferrin (Sigma-Aldrich, St. Louis, MO, USA) was rendered iron-saturated as described previously (Kovar and Franek, 1989). Fatty-acid free bovine serum albumin (BSA) and fatty acids (palmitic, stearic, palmitoleic, oleic, elaidic and linoleic acid) were also obtained from Sigma-Aldrich.

Cells and culture conditions

The human pancreatic β-cell line NES2Y is a proliferating insulin-secreting cell line with insulin promotor unresponsive to glucose. This line was derived from a patient with persistent hyperinsulinemic hypoglycemia of infancy (Macfarlane et al., 1997). The doubling time of NES2Y cells in vitro is approximately 24 h. The cells lack functional ATP-sensitive potassium channel and they also have a defect in the insulin gene-regulatory transcription factor PDX1 (Macfarlane et al., 2000). Cells were routinely maintained in an RPMI 1640 based culture medium supplemented with 10% heat-inactivated fetal bovine serum (Musilkova and Kovar, 2001) at 37 °C in a humidified atmosphere of 5% CO_2 in air. In experiments, a defined serum-free medium was used. This serum-free medium was RPMI 1640 containing extra L-glutamine (300 μg/ml), sodium pyruvate (110 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), HEPES (15 mM), ethanolamine (20 μM), 2-mercaptoethanol (50 μM), ascorbic acid (20 μM), hydrocortisone (5 nM), iron-saturated human transferrin (5 μg/ml) and eleven trace elements as described previously (Kovar and Franek, 1989; Koc et al., 2006). In addition, the serum-free medium contained fatty-acid free BSA for binding tested fatty acids, i.e. 2% BSA for 0.03–1 mM fatty acids and 5% BSA for 3 mM fatty acids. Serum-free medium with the BSA alone and without bound fatty acid was used as a control medium. Media with 1 mM fatty acid/2% BSA and with 3 mM fatty acid/5% BSA were prepared as stock media as described below. Media containing 0.03–0.3 mM fatty acid/2% BSA were prepared by diluting the stock medium (1 mM fatty acid/2% BSA) with the control medium.

Preparation of stock media with fatty acids

Solutions of individual fatty acids (0.3 M) in ethanol were prepared. Solutions with palmitic, stearic and elaidic acid were warmed up to 45 °C to complete dissolving. These ethanol solutions were mixed with the serum-free medium containing 10% fatty acid-free BSA (pH 7.4) employing intensive shaking for 6 h at 37 °C in order to prepare 10 mM fatty acid/10% BSA solutions in the serum-free medium. After sterilization by filtration, actual concentrations of individual fatty acids were determined employing the Nefa C kit (Wako, Neuss, Germany). Prepared solutions were then adjusted to get stock media containing 1 mM fatty acid/2% BSA or 3 mM fatty acid/5% BSA. The stock media were stored at −80 °C. Fatty acid/BSA molar ratios used were lower than the ratios known to exceed the binding capacity of BSA (Cnop et al., 2001).

Cell growth and survival experiments

Cells maintained in culture medium were harvested and then seeded at 5×10^3 cells/100 μl of the culture medium into wells of a 96-well plastic plate. Cells were allowed to attach during a 24-hour incubation period and afterwards the culture medium was replaced by media containing individual fatty acids at tested concentrations or control media. Cell growth and survival were evaluated after 48 h and 96 h of incubation. The number of living cells was determined by hemocytometer counting after staining with trypan blue.

Measurement of caspase-2 and caspase-3 activities

A commercial colorimetric Caspase-3 Assay Kit (Sigma-Aldrich, St. Louis, MO, USA) and solutions from a Caspase-3 Colorimetric Kit (Biosource, Camarillo, CA, USA) combined with the chromogenic caspase-2 substrate and caspase-2 inhibitor (Alexis Biochemicals, Lausen, Switzerland) were used for assessment of caspase-2 and caspase-3 activity. After 24 h or 72 h of incubation in media containing tested fatty acids and in control media, the cells (approximately 10×10^6 cells per sample) were harvested by low-speed centrifugation and lysed. The total protein concentration was determined by the BCA™

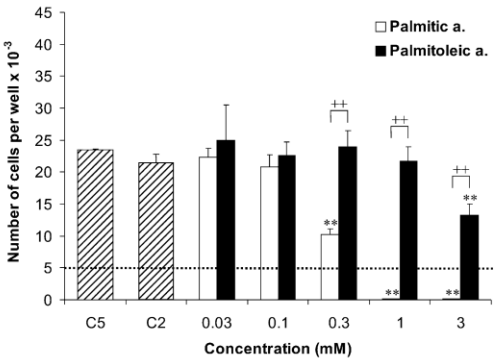

Fig. 1. Comparison of the effect of palmitic acid and palmitoleic acid (0.03–3 mM) on the growth and survival of NES2Y cells. Cells incubated without fatty acid but with 2% (C2) or 5% (C5) fatty-acid free bovine serum albumin (BSA) represented control cells for 0.03–1 mM fatty acids bound to 2% fatty-acid free BSA or for 3 mM fatty acids bound to 5% fatty-acid free BSA, respectively. The cells were seeded at 5×10^3 cells/100 µl of culture medium in the well. The number of cells of the inoculum is shown as a dotted line. After 24-hour incubation period allowing the cells to attach, the culture medium was replaced by media containing fatty acids or control media. The number of living cells was determined after following 48 h of incubation (see Materials and methods). Each column represents the mean of 4 separate cultures ± SEM. ** $P<0.01$ when comparing the effect of individual fatty acids with relevant control. $^{++}$ $P<0.01$ when comparing the effect of both fatty acids.

Protein Assay Kit (Pierce, Rockford, IL, USA). Assays were performed in 96-well plates and 100 µg of total protein per sample was analyzed. Samples were incubated with the specific chromogenic substrates for 4 h at 37 °C. The activity of caspases was determined as the sample absorbance measured at 405 nm using a Sunrise ELISA Reader (Tecan, Maennedorf, Switzerland). Appropriate positive and negative controls of the assays were used.

Statistical analysis

Data are presented as mean ± SEM of 4 (numbers of cells) or 2 (caspase activity) experimental values obtained in one representative experiment of several independent experiments performed. Statistical significance of differences, when comparing obtained data with relevant control, was determined by unpaired Student's *t*-test for two groups. $P<0.05$ was considered statistically significant.

Results

Effect of fatty acids on growth and survival

The effect of individual fatty acids on the growth and survival of NES2Y cells was tested in the physiologically relevant concentration range of 0.03–3 mM. Saturated palmitic acid (16 carbons) and stearic acid (18 carbons), unsaturated palmitoleic acid (16 carbons, one cis double bond) and oleic acid (18 carbons, one cis double bond), elaidic acid (18 carbons, one trans double bond) and linoleic acid (18 carbons, two cis double bonds) were tested. The selection of fatty acids for testing was based on their abundance in human blood. Fatty acids were tested at the highest concentration of 3 mM due to technical limits because 5% fatty-acid free bovine serum albumin (BSA) in medium is required as a carrier for complete binding of 3 mM fatty acids (see Materials and methods) and higher concentrations of BSA were found to be toxic for NES2Y cells (data not shown). However, control cells incubated with 5% BSA and without fatty acid displayed slower growth than control cells incubated with 2% BSA after 96 h of incubation (Figs. 3 and 4). After 48 h of incubation, no significant difference in growth was detected (Figs. 1 and 2).

Saturated palmitic acid induced cell death of nearly all NES2Y cells at a concentration of 1 mM and higher concentrations during 48 h of incubation. The D_{50} of palmitic acid (the dose of fatty acid producing 50% of living cells in comparison with the control) after 48 h of incubation was approximately 0.3 mM. In contrast, its unsaturated counterpart, palmitoleic acid, did not induce death of NES2Y cells at concentrations up to 3 mM. The D_{50} of palmitoleic acid was approximately 3 mM, i.e. ten times higher (Fig. 1).

Saturated stearic acid also showed 100% death-inducing effect at a concentration of 1 mM and higher concentrations during 48 h of incubation with a D_{50} of approximately 0.2 mM. Its unsaturated counterpart, oleic acid (like palmitoleic acid in Fig. 1), did not induce the death at concentrations up to 3 mM. The D_{50} of oleic acid was higher than 3 mM. In fact oleic acid at concentrations below 3 mM even somewhat stimulated growth of the cells during the 48-hour incubation period (Fig. 2).

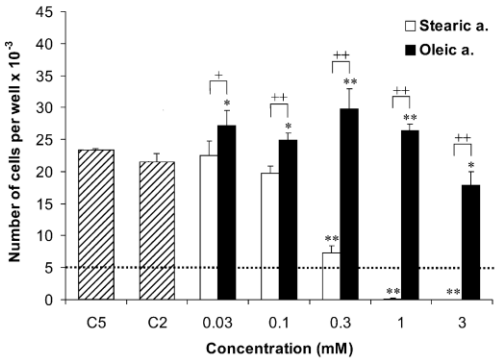

Fig. 2. Comparison of the effect of stearic acid and oleic acid (0.03–3 mM) on the growth and survival of NES2Y cells. Cells incubated without fatty acid but with 2% (C2) or 5% (C5) fatty-acid free bovine serum albumin (BSA) represented control cells for 0.03–1 mM fatty acids bound to 2% fatty-acid free BSA or for 3 mM fatty acids bound to 5% fatty-acid free BSA, respectively. The cells were seeded at 5×10^3 cells/100 µl of culture medium in the well. The number of cells of the inoculum is shown as a dotted line. After 24-hour incubation period allowing the cells to attach, the culture medium was replaced by media containing fatty acids or control media. The number of living cells was determined after following 48 h of incubation (see Materials and methods). Each column represents the mean of 4 separate cultures ± SEM. * $P<0.05$, ** $P<0.01$ when comparing the effect of individual fatty acids with relevant control. $^{+}$ $P<0.05$, $^{++}$ $P<0.01$ when comparing the effect of both fatty acids.

Both oleic acid (unsaturated fatty acid with a cis double bond) and elaidic acid (trans double bond counterpart of oleic acid) were unable to induce death of NES2Y cells at concentrations up to 3 mM during 96 h of incubation. However, elaidic acid was more efficient in inhibiting growth of the cells than oleic acid. The D_{50} of elaidic acid was approximately 0.3 mM while the D_{50} of oleic acid was approximately 1 mM. Furthermore, the lowest detected concentration of elaidic acid with a significant inhibitory effect was 0.1 mM but in the case of oleic acid it was 1 mM (Fig. 3).

Linoleic acid (unsaturated fatty acid with two double bonds) was also more efficient than oleic acid (one double bond) during 96 h of incubation. The D_{50} of linoleic acid was approximately 0.5 mM and the D_{50} of oleic acid was 1 mM. Linoleic acid even induced death of some of the cells at a concentration of 3 mM (Fig. 4).

We also tested a possibility that fatty acid effects were potentiated by glucose. We did not detect any significant enhancement of the effect of fatty acids by increased level of glucose, i.e. from 11 mM (normal level in used RPMI 1640 medium) to 33 mM. However, the increased level of glucose itself seemed to have certain effect resulting in a slight decrease of the number of NES2Y cells (data not shown).

The effect of fatty acids on caspase-3 and caspase-2 activities

In order to further characterize the cell death induction and growth inhibition of NES2Y cells by the fatty acids, the activation of the key executioner caspase-3 was tested. We chose to test caspase activities after 24-h incubation with a death-inducing concentration of the saturated fatty acids because after 48-h incubation nearly all cells were dead. As to the unsaturated fatty acids, we chose to test caspase activities after 72-h incubation with their growth-inhibiting concentration because the most pronounced effect on the cell number, when comparing with control cells, was achieved after 96-h incubation (see previous chapter of Results).

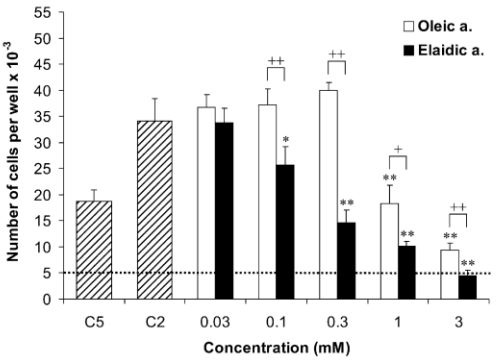

Fig. 3. Comparison of the effect of oleic acid and elaidic acid (0.03–3 mM) on the growth and survival of NES2Y cells. Cells incubated without fatty acid but with 2% (C2) or 5% (C5) fatty-acid free bovine serum albumin (BSA) represented control cells for 0.03–1 mM fatty acids bound to 2% fatty-acid free BSA or for 3 mM fatty acids bound to 5% fatty-acid free BSA, respectively. The cells were seeded at 5×10^3 cells/100 µl of culture medium in the well. The number of cells of the inoculum is shown as a dotted line. After 24-hour incubation period allowing the cells to attach, the culture medium was replaced by media containing fatty acids or control media. 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 ± SEM. * $P<0.05$, ** $P<0.01$ when comparing the effect of individual fatty acids with relevant control. $^{+}$ $P<0.05$, $^{++}$ $P<0.01$ when comparing the effect of both fatty acids.

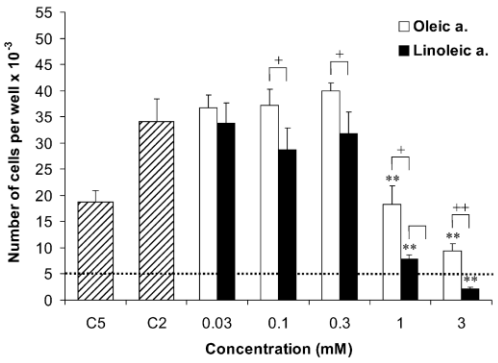

Fig. 4. Comparison of the effect of oleic acid and linoleic acid (0.03–3 mM) on the growth and survival of NES2Y cells. Cells incubated without fatty acid but with 2% (C2) or 5% (C5) fatty-acid free bovine serum albumin (BSA) represented control cells for 0.03–1 mM fatty acids bound to 2% fatty-acid free BSA or for 3 mM fatty acids bound to 5% fatty-acid free BSA, respectively. The cells were seeded at 5×10^3 cells/100 µl of culture medium in the well. The number of cells of the inoculum is shown as a dotted line. After 24-hour incubation period allowing the cells to attach, the culture medium was replaced by media containing fatty acids or control media. 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 ± SEM. ** $P<0.01$ when comparing the effect of individual fatty acids with relevant control. $^{+}$ $P<0.05$, $^{++}$ $P<0.01$ when comparing the effect of both fatty acids.

Using a colorimetric assay (see Materials and methods) we showed that after 24 h of incubation with a death-inducing concentration (1 mM) of the saturated fatty acids (palmitic and stearic acid) the activity of caspase-3 did not increase significantly (Fig. 5). Similarly, after 72 h of incubation with a growth-inhibiting concentration (3 mM) of the unsaturated fatty acids (palmitoleic, oleic, elaidic and linoleic acid) the activity of caspase-3 did not increase (Fig. 6). We verified that the assay being used (see Materials and methods) was working by testing it on an established system employing 38C13 cells after treatment with doxorubicin. The activity of caspase-3 increased several times in this system. Furthermore, we also tested the activity of caspase-3 in NES2Y cells after the treatment with fatty acids by an independent method. Employing flow cytometry after staining with a specific monoclonal antibody against activated caspase-3 (Active Caspase-3 PE Mab Apoptosis Kit, BD Biosciences Pharmingen, San Diego, CA, USA), we obtained similar results (data not shown) as we obtained with the colorimetric assay.

In contrast, incubation (24 h) of NES2Y cells with a death-inducing concentration (1 mM) of palmitic and stearic acids led

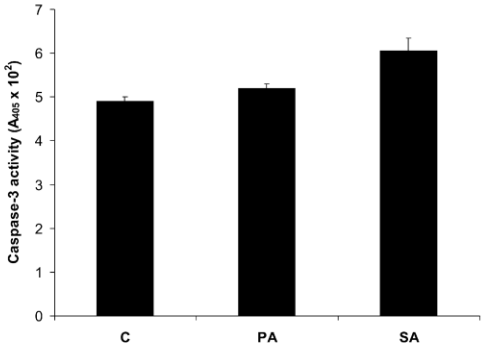

Fig. 5. Effect of palmitic acid (PA) and stearic acid (SA) at a concentration of 1 mM on caspase-3 activity in NES2Y cells. Cells incubated without fatty acid but with 2% fatty-acid free bovine serum albumin (BSA) represented control (C) cells. After 24 h of incubation in media containing fatty acids or in control medium, the activity of caspase-3 was measured as the absorbance of the cleaved product of a caspase-3 chromogenic substrate at 405 nm, employing a commercial colorimetric kit (see Materials and methods). Each column represents the mean of two experimental values ± SEM. No significant increase of caspase-3 activity was detected here and therefore appropriate functioning of the used assay was verified (see Results).

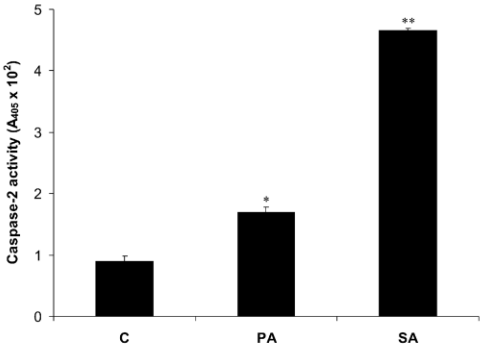

Fig. 7. Effect of palmitic acid (PA) and stearic acid (SA) at a concentration of 1 mM on caspase-2 activity in NES2Y cells. Cells incubated without fatty acid but with 2% fatty-acid free bovine serum albumin (BSA) represented control (C) cells. After 24 h of incubation in media containing fatty acids or in control medium, the activity of caspase-2 was measured as the absorbance of the cleaved product of a caspase-2 chromogenic substrate at 405 nm, employing a commercial colorimetric kit (see Materials and methods). Each column represents the mean of two experimental values ± SEM. * $P<0.05$, ** $P<0.01$ when comparing with control.

to significant increase (approximately 2 to 5-fold) of caspase-2 activity (Fig. 7). However, incubation (72 h) of the cells with a growth-inhibiting concentration (3 mM) of the unsaturated fatty acids did not lead to an increase of caspase-2 activity and in the case of palmitoleic and oleic acids, the activity of caspase-2 even decreased (Fig. 8).

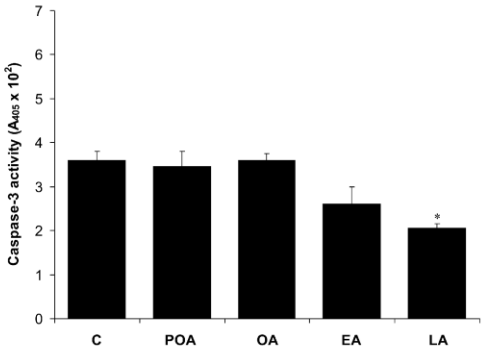

Fig. 6. Effect of palmitoleic acid (POA), oleic acid (OA), elaidic acid (EA) and linoleic acid (LA) at a concentration of 3 mM on caspase-3 activity in NES2Y cells. Cells incubated without fatty acid but with 5% fatty-acid free bovine serum albumin (BSA) represented control (C) cells. After 72 h of incubation in media containing fatty acids or in control medium, the activity of caspase-3 was measured as the absorbance of the cleaved product of a caspase-3 chromogenic substrate at 405 nm, employing a commercial colorimetric kit (see Materials and methods). Each column represents the mean of two experimental values ± SEM. * $P<0.05$ when comparing with control. No significant increase of caspase-3 activity was detected here and therefore appropriate functioning of the used assay was verified (see Results).

We also tested the effect of saturated fatty acids on caspase-2 activity at concentrations lower than death-inducing concentration. As demonstrated for stearic acid (Fig. 9), we found that incubation of NES2Y cells with a growth-inhibiting concentration (0.3 mM) of saturated fatty acid resulted in significant increase (more than 2-fold) of caspase-2 activity. Increasing activity of caspase-2 after 24-h incubation with increasing concentration (0.1–1 mM) of the fatty acid corresponded to decreasing number of cells after 48-h incubation. It supports a suggestion that the effect of saturated fatty acids on growth

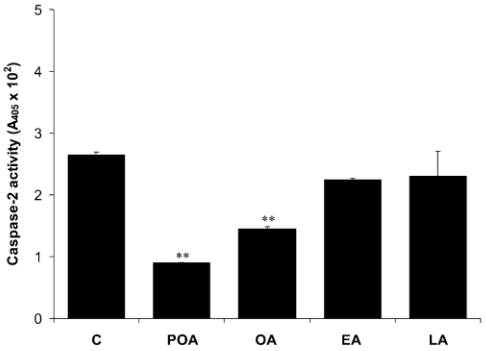

Fig. 8. Effect of palmitoleic acid (POA), oleic acid (OA), elaidic acid (EA) and linoleic acid (LA) at a concentration of 3 mM on caspase-2 activity in NES2Y cells. Cells incubated without fatty acid but with 5% fatty-acid free bovine serum albumin (BSA) represented control (C) cells. After 72 h of incubation in media containing fatty acids or in control medium, the activity of caspase-2 was measured as the absorbance of the cleaved product of a caspase-2 chromogenic substrate at 405 nm, employing a commercial colorimetric kit (see Materials and methods). Each column represents the mean of two experimental values ± SEM. ** $P<0.01$ when comparing with control.

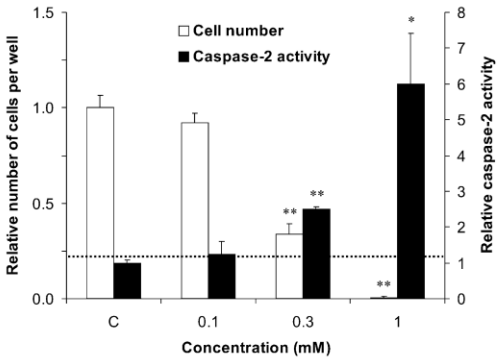

Fig. 9. Comparison of the effect of stearic acid (0.1–1 mM) on the growth and survival and on caspase-2 activity of NES2Y cell. Cells incubated without fatty acid but with 2% fatty-acid free bovine serum albumin (BSA) represented control (C) cells. The cells were seeded at 5×10^3 cells/100 μl of culture medium in the well. The number of cells of the inoculum is shown as a dotted line. After 24-hour incubation period allowing the cells to attach, the culture medium was replaced by media containing fatty acids or control medium. The number of living cells was determined after following 48 h of incubation (see Materials and methods) and it is expressed as a relative number when comparing with control cell number ($21.4\times10^3\pm1.4\times10^3$). The activity of caspase-2 was measured after 24 h of incubation as the absorbance of the cleaved product of a caspase-2 chromogenic substrate at 405 nm, employing commercial colorimetric kit (see Materials and methods) and it is expressed as a relative activity when comparing with control caspase-2 activity ($2.00\times10^{-3}\pm0.14\times10^{-3}$). Each column represents the mean of four separate cultures (number of cells) or two experimental values (activity of caspase-2) ± SEM. * $P<0.05$, ** $P<0.01$ when comparing with relevant control.

of the cells is due to increased death induction rather than decreased proliferation activity.

Discussion

In this study we have compared the effect of various fatty acids on the growth and viability of the human β-cell line NES2Y employing chemically defined serum-free culture media (see Materials and methods) allowing us to test the effect of individual fatty acids without interactions with other fatty acids and also allowing precise control of fatty acid concentrations. The effect of fatty acids was tested within the range of concentrations physiologically available in vivo (Lagerstedt et al., 2001).

Our previous experiments employing flow cytometry showed that tested saturated and unsaturated fatty acids at relevant concentrations do not exert significant effect on cell cycle profile of NES2Y cells (unpublished data). This means that proliferation activity of the cells per se is not significantly affected. Furthermore, it implies that detected effect of tested fatty acids on growth of the cells results from changed frequency of death induction rather than from changed proliferation activity. This suggestion is also supported by our finding concerning the correlation between increasing caspase-2 activity after incubation with increasing concentration of saturated fatty acid and decreasing number of cells after the incubation (see Fig. 9).

We found that saturated fatty acids, i.e. palmitic and stearic acids, at a concentration of 1 mM and higher concentrations induced cell death of β-cells. On the other hand, counterpart unsaturated fatty acids, i.e. palmitoleic and oleic acids, did not induce the death of β-cells at concentrations up to 3 mM. These findings correspond with previous data concerning the cytotoxic effects of saturated fatty acids on pancreatic β-cells in contrast to unsaturated fatty acids (Maedler et al., 2001, 2003; Eitel et al., 2002; Welters et al., 2004, 2006; Higa et al., 2006). However, unsaturated fatty acids do not appear to be completely ineffective in β-cells. Our data indicate that unsaturated fatty acids are approximately 10-fold less effective in inhibiting growth than their counterpart saturated fatty acids. Surprisingly, oleic acid at lower concentrations (0.03–1 mM) even slightly supported growth of the cells. This finding also corresponds with some previous data of Maedler et al. (2003). It seems that unsaturated fatty acids influence growth of the cells at relevant concentrations via changed frequency of death induction rather than via changed proliferation activity (see above). However, in the case of unsaturated fatty acids growth inhibition is not related to caspase-2 activation as in the case of saturated fatty acids.

Our data could have an important physiological relevance, particularly to the pathological events associated with the induction of type 2 diabetes, because the concentrations of fatty acids which affect cell growth or induce cell death in human pancreatic β-cells under our experimental conditions are equivalent to those found in human blood (Lagerstedt et al., 2001). Thus the growth-affecting and cell death-inducing potential of fatty acids may be important in the long-term homeostasis of β-cells in vivo.

For a long time it has been speculated that trans fatty acids can affect human health in several ways (reviewed in Stender and Dyerberg, 2004). It was shown that high trans fat consumption represents a significant risk factor for coronary heart disease (Sun et al., 2007) and several animal and epidemiological studies have indicated that there might be an association between trans fatty acid intake by food and the incidence of type 2 diabetes (reviewed in Odegaard and Pereira, 2006). The influence of trans fatty acids on insulin release and other β-cell functions has also been studied. In the β-cell line INS-1, trans isomers induced higher fatty acid oxidation than cis isomers (Alstrup et al., 2004). In isolated mouse islets, trans fatty acids appeared more effective in the potentiation of insulin secretion and glucose oxidation than their cis counterparts (Alstrup et al., 1999). Despite a number of studies concerning trans fatty acid consumption and their effects on metabolic parameters of pancreatic β-cells, differences in the effect of cis and trans fatty acids on the growth and viability of pancreatic β-cells have yet to be fully elucidated.

In our studies we selected elaidic acid as a representative trans fatty acid because it is the predominant monounsaturated trans fatty acid formed in the process of industrial oil hardening and is therefore the major component of trans fatty acids present in food (Stender and Dyerberg, 2004). We found that the lowest concentration of elaidic acid exerting significant inhibition of growth of the β-cells was 0.1 mM in contrast to the lowest

concentration of oleic acid which was 1 mM. As far as we know, this is the first direct evidence demonstrating that the effect of an individual unsaturated fatty acid on the growth of β-cells depends upon the configuration of the double bond. The fact that a trans unsaturated fatty acid is significantly more effective in inhibiting β-cell growth than its cis counterpart could be explained by different physical properties of trans and cis unsaturated fatty acids. The melting point of trans unsaturated fatty acids is more similar to the melting point of saturated fatty acids than that of cis unsaturated fatty acids. Such a difference in melting point of incorporated fatty acids can lead to changed membrane fluidity affecting membrane functions including signal transduction (Kondoh et al., 2007). As a consequence it can result in different cell responses to various signals and stimuli.

The molecular mechanisms involved in β-cell death induction by fatty acids are not still fully understood. According to our present knowledge, endoplasmatic reticulum (ER) stress as well as mitochondrial pathway are supposed to be involved here (Cnop et al., 2005; Choi et al., 2007). Mitochondrial pathway is realized via cytochrome c release from mitochondria and subsequent activation of caspase-9 and finally the activation of key executioner caspase-3. Surprisingly, cell death induction by saturated fatty acids in our experiments with the NES2Y cells was associated with only a marginal increase of caspase-3 activity which was not significant. Thus it seems, at least in our experimental system, that cell death induced by saturated fatty acids in β-cells is not mediated via caspase-3 activation and thus is not probably realized via mitochondrial pathway. Another possibility is that specifically NES2Y cells are unable to employ mitochondrial pathway and/or caspase-3 activation in the case of cell death induced by saturated fatty acids. Some detected increase in caspase-3 activity after cell death induced by saturated fatty acids (see Fig. 5), as also mentioned by several authors (Eitel et al., 2003; El-Assaad et al., 2003; Maedler et al., 2003; Rakatzi et al., 2004; Hirota et al., 2006), could only represent a side effect of cell death induction via a different main pathway. This pathway could involve the activation of caspase-2.

We detected a significant (2 to 5-fold) increase in caspase-2 activity after cell death was induced by saturated fatty acids (see Fig. 7). We believe that this is the first time when β-cell death induced by saturated fatty acids has been shown to be related also to caspase-2 activation. Caspase-2 was shown to mediate apoptosis induced by GTP-depletion in HIT-T15 cells (β-cell line) and its activation preceded both caspase-3 and caspase-9 activation (Huo et al., 2002). These findings implicate the existence of caspase-2 mediated cell death pathway(s) also in β-cells. Otherwise, our knowledge concerning the role of caspase-2 in cell death induction is still incomplete. Caspase-2 is supposed to be activated in so called PIDDosome complex and to function upstream of mitochondrial permeabilization (Troy and Shelanski, 2003; Zhivotovsky and Orrenius, 2005).

Taking into account our knowledge concerning described mechanisms involved in cell death induction and particularly our present knowledge concerning caspase-2 function, it is difficult to connect somehow caspase-2 activation (see Fig. 7) and expected involvement of ER stress in cell death induction by saturated fatty acids (Cnop et al., 2005). Molecules like CHOP and caspase-12 (only in mice) seem to be involved in cell death induced by ER stress but the mechanisms remain to be fully understood (Araki et al., 2003; Cnop et al., 2005; Xu et al., 2005; Lipson et al., 2006). One explanation of our findings could be that the activation of caspase-2 represents a new pathway, related or unrelated to ER stress, of β-cell death induction by saturated fatty acids.

The reduction of caspase-2 activity after treatment with some unsaturated fatty acids, i.e. palmitoleic and oleic acids, (see Fig. 8) remains obscure. It could be related to previously presented suggestions that unsaturated fatty acids can protect β-cells against cell death induction (Maedler et al., 2001, 2003; Eitel et al., 2002; Welters et al., 2004).

Taken together, we can conclude that saturated fatty acids at physiologically relevant concentrations, in contrast to unsaturated fatty acids, induce cell death in the human β-cell line NES2Y and that this could reflect events occurring in vivo. We have provided the first direct evidence that a trans unsaturated fatty acid is significantly more effective in inhibiting β-cell growth than its counterpart cis unsaturated fatty acid. We have newly demonstrated that β-cell death induced by saturated fatty acids is related to caspase-2 activation but not to caspase-3 activation. Furthermore, the inhibitory effect of saturated fatty acids on β-cell growth results from increased death induction, probably via caspase-2 activation, rather than from decreased proliferation activity. This may have important implications for the development of therapeutics that could treat or delay the onset of type 2 diabetes.

Acknowledgments

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8.2.2 PAPER 3

Němcová-Fürstová V., James R.F., Kovář J.:

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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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 coincubation 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 coincubation 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.

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

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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 socalled 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 ($5x10^5$ 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% CO_2 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 $5x10^3$ cells/100 µl of the culture medium into wells of a 96-well plastic plate. Cells were allowed to attach during a 24-h incubation period (approximatelly 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 $3x10^5$ 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 $5x10^5$ 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 [$DiOC_6(3)$] 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 µM 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 $5x10^5$ 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 µM 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 $2x10^6$ 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 1h 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 2×10^5 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 μM 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 μl 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 μl 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 coveslips were transfered 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 μM staurosporine for 24 hours.

Western blot analysis

Cells (approximately $20x10^6$ 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 μl RIPA lysis buffer (Sigma-Aldrich, St. Louis, MO, USA) containing 1.5 μl Protease Inhibitor Coctail (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 μl) containing 40 μg of proteins were mixed with 10 μl of sample loading buffer (0.125 mM Tris-HCl, pH 6.8, 10% glycerol, 4% SDS, 250 mM DTT, 0.004% bromphenol 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 μm nitrocellulose transfer membrane (Protran BA83, Schleicher-Schuell, Dassel, Grermany) for 2 h at 0.25 A using a Mini-Protean 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 chemi-luminescence 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 $1x10^6$ 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,

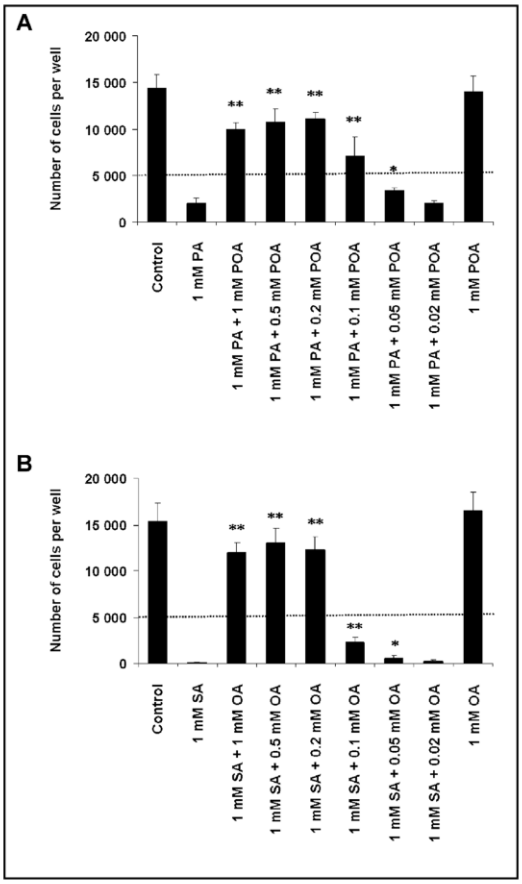

Fig. 1. Concentration-dependent effect of (A) palmitoleic acid (POA) and (B) oleic acid on the cell death-inducing effect of (A) palmitic acid (PA) and (B) stearic acid (SA) in NES2Y cells. The cells were seeded at $5x10^3$ cells/100 µl of culture medium in the well. The number of cells of the inoculum 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 ± 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.

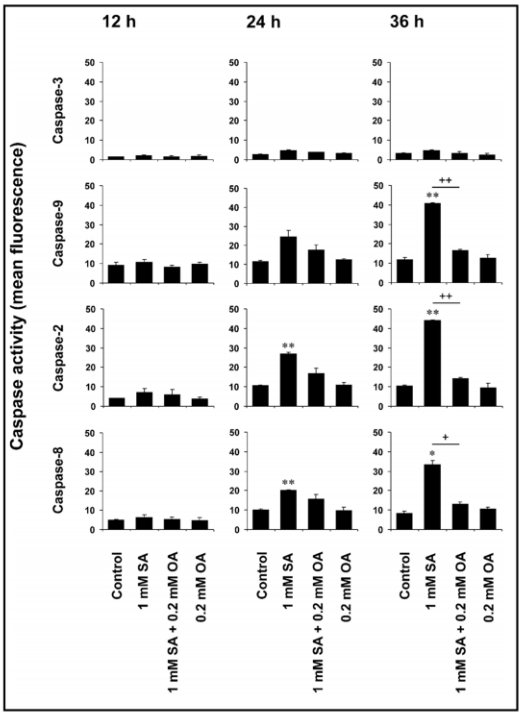

Fig. 2. Effect of 1 mM stearic acid (SA), 1 mM stearic acid applied together with 0.2 mM oleic acid (OA), and 0.2 mM oleic acid on the activity of caspase-3, -9, -2 and -8 in NES2Y cells. Cells incubated without fatty acids represented control cells. After 12, 24 and 36 h of incubation in media containing 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 ± 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 unsignificant 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).

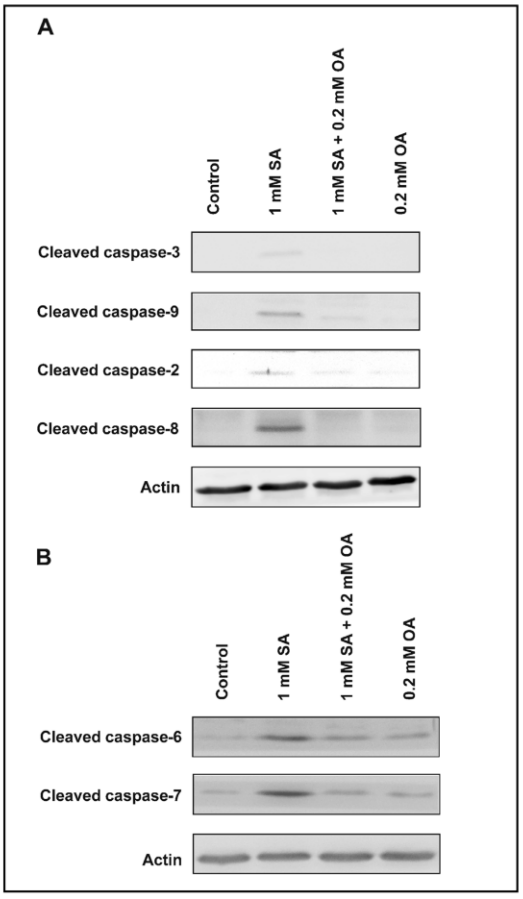

Fig. 3. Effect of 1 mM stearic acid (SA), 1 mM stearic acid applied together with 0.2 mM oleic acid (OA), and 0.2 mM oleic acid on the expression of (A) cleaved caspase-3, -9, 2, -8, and (B) cleaved caspase-6, -7. Cells incubated without fatty acids represented control cells. After 24 h of incubation, 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.

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 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).

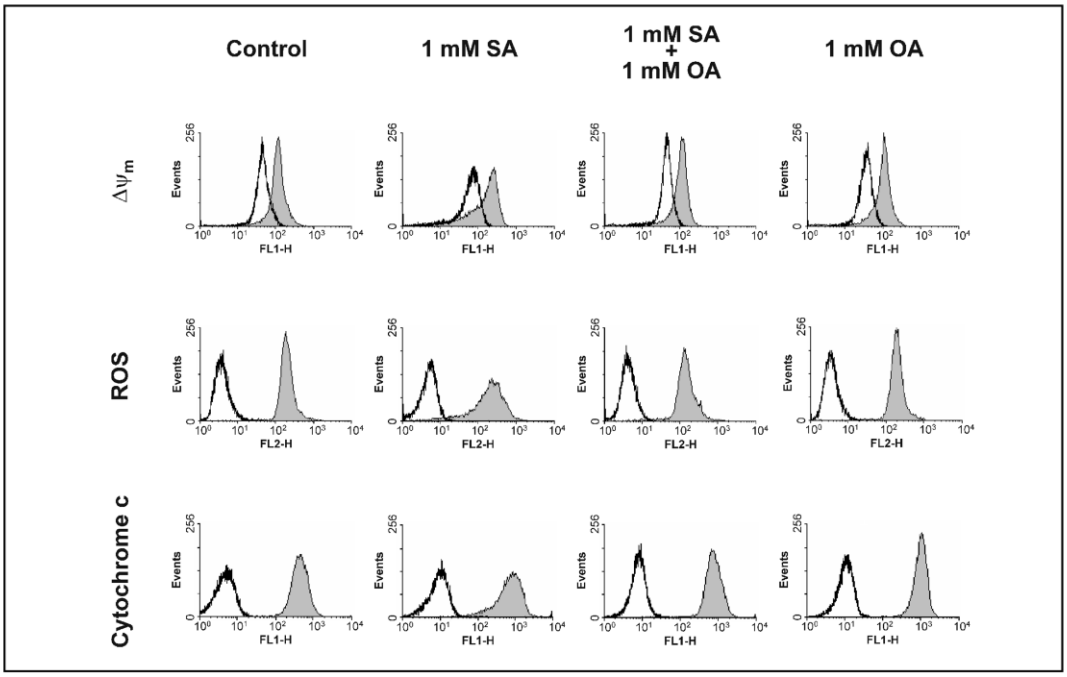

Fig. 4. Effect of 1 mM stearic acid (SA), 1 mM stearic acid applied together with 0.2 mM oleic acid (OA), and 0.2 mM oleic acid on the mitochondrial membrane potential ($\Delta\psi_m$), reactive oxygen species (ROS) level and the cytochrome c release from mitochondria in NES2Y cells. Cells incubated without fatty acids represented control cells. After 24 h of incubation, the mitochondrial membrane potential was assesed by flow cytometric analysis of cells after staining with 20 nM [$DiOC_6(3)$] (see "Materials and Methods"). The collapse of $\Delta\psi_m$ caused by the treatment of cells with 100 μM 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 μM 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 cytochtome 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

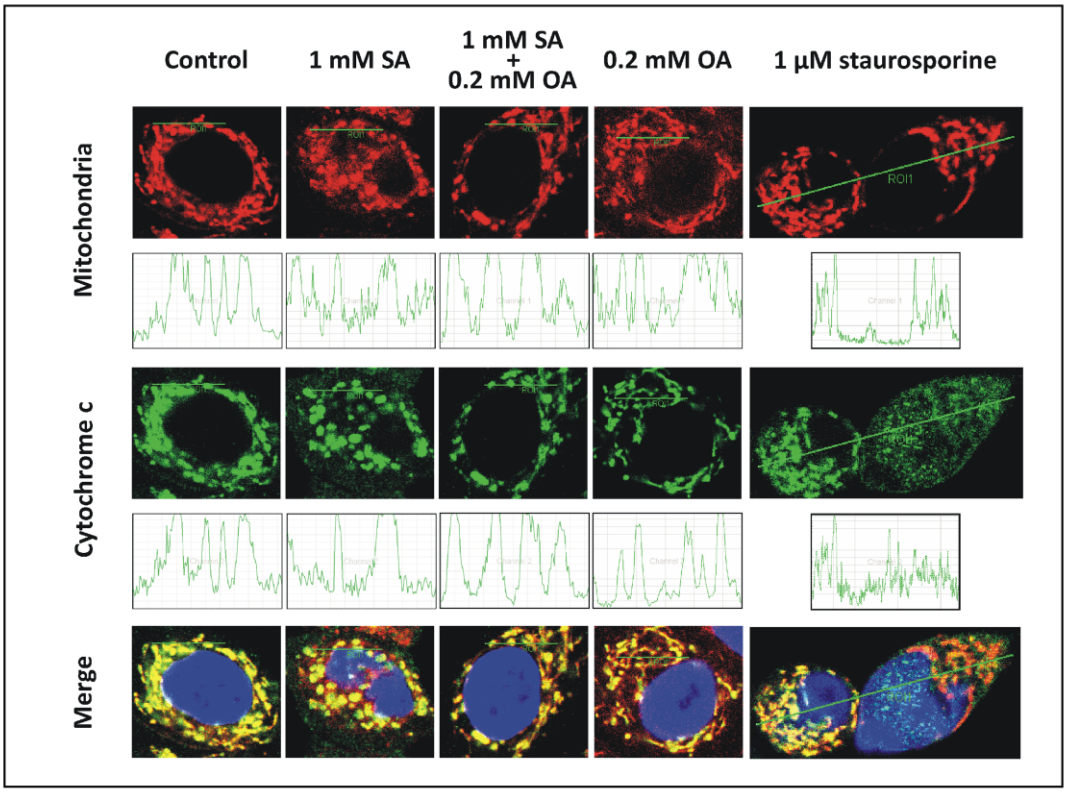

Fig. 5. Effect of 1 mM stearic acid (SA), 1 mM stearic acid applied together with 0.2 mM oleic acid (OA), and 0.2 mM oleic acid alone on cytochrome c release from mitochondria in NES2Y cells. Cells incubated without fatty acids represented control cells. After 24 h of incubation, mitochondria (red), cytochrome c (green) and nuclei (blue) within the cells were stained and representative images were aquired 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 („ROI1", 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 µM 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 (approximatelly 100 kDa) was not found in NES2Y cells, we did detect a cleaved form of PIDD (approximatelly 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

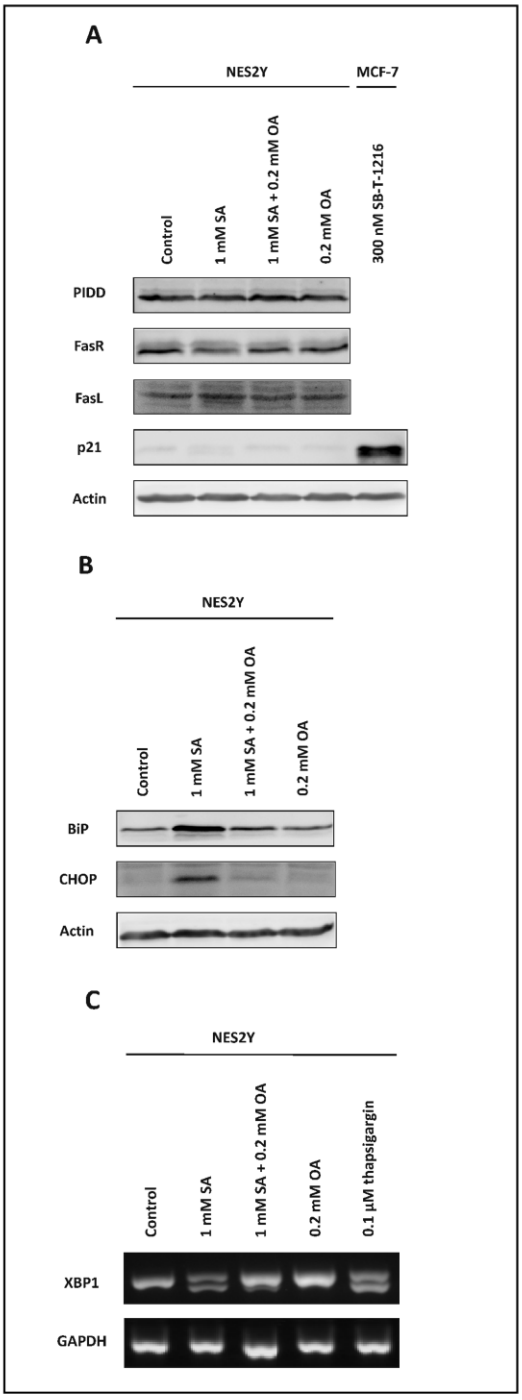

Fig. 6. Effect of 1 mM stearic acid (SA), 1 mM stearic acid applied together with 0.2 mM oleic acid (OA), and 0.2 mM oleic acid on (A) the expression of PIDD, Fas receptor (FasR), Fas ligand (FasL) and $p21^{WAF1/CIP1}$ and (B) the expression of BiP and CHOP and (C) XBP1 mRNA splicing in NES2Y cells. 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 µM 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 acic 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 containig FBS (data not shown) in contrast to data of Maestre et al. [16]. This discrepancy may results 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 phophorylation of eIF2α (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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8.2.3 PAPER 4

Němcová-Fürstová V., Šrámek J., Balušíková K., James R.F., Kovář J.

Caspase-2 and JNK activated by saturated fatty acids are not involved in apoptosis induction but modulate ER stress in human pancreatic β-cells

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Original article

Caspase-2 and JNK activated by saturated fatty acids are not involved in apoptosis induction but modulate ER stress in human pancreatic β-cells

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Abstract

Background: Fatty acid-induced β-cell apoptosis and ER stress contribute to the development of type 2 diabetes, however, the molecular mechanisms involved are not clear.

Aims: In this study we have tested the role of caspase-2 and the proposed ER stress mediator JNK, in saturated fatty acid-induced apoptosis of the human pancreatic β-cell line NES2Y.

Results: We found that stearic acid treatment activated ER stress signaling pathways in NES2Y, i.e. IRE1α, PERK and ATF6 pathways. During stearic acid-induced apoptosis, JNK inhibition did not decrease 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 and caspase-8, -9 and -7 cleavage and CHOP induction, however, it also decreased 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 pancreatic β-cells. However, they appear to be involved in the modulation of ER stress signaling, probably by a phospho-c-Jun independent mechanism.

Introduction

Dramatical increase in the incidence of diabetes mellitus represents one of the principal challenges to human health care and to economy system in parallel in the 21st century [1]. It is becoming evident that the worldwide rise in type 2 diabetes (T2D) correlates with a rise in obesity and an increased level of circulating non-esterified fatty acids which is one of the main factors responsible for the progressive loss of β-cell function can result in triggering apoptotic cell death and a subsequent reduction in β-cell mass [2-4]. There is convincing experimental evidence showing that saturated fatty acid exposure (in contrast to unsaturated fatty acids) is detrimental to pancreatic β-cell survival [5-7] and leads to apoptosis. However, despite intensive research the molecular mechanisms of saturated fatty acid induced apoptosis in β-cells still remain to be elucidated. Saturated fatty acids were also shown to activate endoplasmic reticulum (ER) stress signaling in pancreatic β-cells *in vitro* [7-11] and increased levels of ER stress markers have been demonstrated in β-cells of type 2 diabetes patients [12-14]. Therefore, ER stress is suggested as a likely mechanism that mediates the pro-apoptotic effect of saturated fatty acids in pancreatic β-cells.

Properly tuned ER functioning is crucial for many cellular processes, including synthesis and folding of secretory proteins. As secretory cells, β-cells are especially prone to perturbations in ER function [15]. When the ER folding capacity is compromised, misfolded proteins accumulate and trigger the ER stress signaling pathways known as the "unfolded protein response" (UPR).

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 which results in translation of active transcription factor (XBP1s). Activation of PERK branch of ER stress signaling results in the inhibition of protein translation via phosphorylation of eIF2α and thus in decreasing the demands on ER folding capacity. 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 by decreasing protein translation, increasing the expression of chaperones, such as the prominent ER chaperone BiP (immunoglobulin heavy chain binding-protein), and

inducing the degradation of misfolded proteins [16]. 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). It has been proposed that all ER stress signaling pathways converge in CHOP induction [17].

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 [18]. Fatty acid treatment activates JNK in β-cells [9, 19-21] and it is known that T2D is associated with the activation of the JNK pathway [22], 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 by saturated fatty acids when apoptosis is induced in human pancreatic β-cell line NES2Y. Similarly, ER stress is also induced by saturated fatty acids [11, 23]. Despite being among the first caspases discovered and intensively researched, the role of caspase-2 in apoptotic signal transduction has still not yet been fully elucidated [24]. However, there are several lines of evidence pointing at caspase-2 as a possible transducer of pro-apoptotic ER stress signaling in various cell types [25-28] 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 proposed ER stress mediator JNK, in saturated fatty acid-induced apoptosis of human pancreatic β-cells NES2Y. We have also assessed the role of caspase-2 and JNK in saturated fatty acid-induced ER stress signaling. 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, they do appear to be involved in the modulation of 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-phospho-SAPK/JNK (#4668) 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).

Preparation of stock media with stearic acid

Stock media with stearic acid were prepared as described previously [Furstova et al. 2008]. Briefly, solution of stearic acid in ethanol (0.3 M) was warmed to 45°C to completely dissolve. The solution was then mixed with the serum-free medium (see below) containing 10% fatty acid-free bovine serum albumin (BSA, pH 7.4) and was shaken intensively for 6 h at 37°C in order to prepare 10 mM fatty acid/10% BSA solutions. After sterilization by filtration, the actual concentration of fatty acid was determined using the Nefa C kit (Wako, Neuss, Germany) and the stock solution was stored at -80°C.

Cells and culture conditions

The human pancreatic β-cell line NES2Y [23, 29] 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 [30] supplemented with 10% heat-inactivated fetal bovine serum (Biochrom AG, Berlin, Germany) at 37°C in a humidified atmosphere of 5% CO_2 in air. RPMI 1640 medium contains 11 mM glucose. In experiments, a defined serum-free medium [31] supplemented with stearic acid bound to 2% fatty-acid free bovine serum albumin (BSA) was used as described

previously [23]. The serum-free medium containing 2% BSA alone and without bound fatty acid was used as a control medium. Stock solution containing stearic acid bound to the 10% BSA in the serum-free medium was prepared as described above and was 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 [32].

Western blot analysis

Cells (approximately 1-3x10^5 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 by control medium. After required period of incubation, the cells were harvested by low-speed centrifugation, washed twice with PBS and lysed in RIPA lysis buffer (Sigma-Aldrich, St. Louis, MO, USA) containing Protease Inhibitor Coctail (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 from Pierce (Rockford, IL, USA).

Western blot analysis was performed as described previously [11]. Samples containing 10-20 μg of proteins were separated by SDS-PAGE on 12% polyacrylamide gel (4% polyacrylamide stacking gel) at 30 mA and then blotted onto 0.2 μm nitrocellulose transfer membrane (Protran BA83, Schleicher-Schuell, Dassel, Grermany) for 3 h at 0.25 A using a Mini-Protean 3 apparatus (Bio-Rad, Hercules, CA). The membrane was blocked with 5% BSA 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 probed with the primary antibody in 0.1% Tween-20/TBS containing 1% BSA overnight at 4°C. All primary antibodies were used in 1:1 000 dilution with exception of anti-CHOP antibody that was diluted 1:500. 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 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 by low-speed centrifugation. The splicing of XBP1 mRNA was assessed by RT-PCR as described previously [33]. Reverse transcription from total RNA was performed according to Balusikova et al. [34]. Expression of housekeeping gene GAPDH was determined using primer sequences described previously [34]. 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 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).

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. Fixed cells were washed several times with PBS and permeabilized with 0.1 % Tween in PBS for 10 min. After washing with PBS, the coverslips were blocked with FX Enhancer (Invitrogen-Molecular Probes, Eugene, OR, USA). After washing, cells were incubated with 30 μl of the primary antibody against ATF6 (Abcam, Cambridge, UK) diluted 1:50 in PBS at 4°C overnight. Then, cells were washed with PBS and incubated with 30 μl of the secondary AlexaFluor 488-conjugated goat anti-mouse antibody from Invitrogen (Invitrogen-Molecular Probes, Eugene, OR, 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

coveslips 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 containing JNK inhibitor SP600125 (Enzo Life Sciences, Farmingdale, NY, USA) at desired concentration, or by control medium containing the vehiculum only (DMSO). After 1 h of pretreatment, stearic acid and BSA were added to achieve the required concentration of stearic acid or 2% BSA, respectively. After 18 h and 24 h of incubation the cells were harvested by low-speed centrifugation and lysates were prepared for Western blot analysis as described in "Western blot analysis".

Inhibition 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 to show potential nonspecific effects of the whole transfection procedure. The concentration of siRNA necessary for efficient inhibition of caspase-2 expression was determined from series of preliminary experiments where the effect of various combinations of siRNA and transfection reagent concentrations was tested (data not shown) to provide optimal results. The efficiency of inhibition of caspase-2 expression at the protein level was tested after 72 or 96 h of treatment in each experiment by Western blot analysis of caspase-2 expression.

For caspase-2 silencing, 1.5×10^5 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 concetration of 1 x 10^6 cells/2.5 ml per well. After 24 h allowing cells to attach, the media was changed for classical induction media with or without stearic acid (see e.g. Western blot analysis) but containing also fresh siRNA and transfection reagent at the same concentration as used for the initial inhibition of caspase-2 expression. Both stearic acid-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 application, and at the end of experiment, i. e. 24 h after stearic acid 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

Activation of ER stress signaling by stearic acid

Our previous experiments concerning the effect of stearic acid on NES2Y cells showed that the execution of apoptosis, including caspase-2 activation, starts within 24 h after stearic acid application [11, 23, our unpublished data]. Therefore, all effects were tested until 24 h after application of fatty acid.

We tested for 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 reflected by the 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.

To assess ATF6 translocation to the nucleus, we employed confocal microscopy. 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 3h 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). 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).

Effect of JNK inhibition on caspase activation

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 level of procaspase-2 were not influenced by JNK inhibition during stearic acid treatment (Fig. 4B, only data for 18 h are shown).

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. 5A). 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. 5B). 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. 6).

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. 7, 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. 7, 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. 7, only data for 6 h are shown).

Discussion

In our previous studies, we showed that saturated fatty acids (palmitic acid and, more efficiently, stearic acid) induce caspase-2 activation and apoptosis in NES2Y cells [11, 23]. In these earlier studies we also indicated that there may be a possible role of ER stress in apoptosis induction of pancreatic β-cells by stearic acid [23]. Thus in this study we have gone on to test the role of caspase-2 and the ER stress mediator JNK, in stearic acid-induced apoptosis of NES2Y cells and their role in ER stress signaling.

Experiments were performed in a chemically defined serum-free media allowing precise control of fatty acid concentration. The medium used also allows cells to grow. NES2Y cells respond to saturated and unsaturated fatty acids and also their combination similarly like primary human β-cells [6, 9, 10, 35], as we found in our previous experiments [11, 23]. Thus it may be expected that the responses to saturated fatty acids seen in this study with NES2Y cells are comparable to the effects on human β-cells *in vivo*. Currently, there have only been a limited number of studies investigating the effect of fatty acids on ER stress signaling and apoptosis in β-cells of human origin [9, 10, 35].

In NES2Y cells, we demonstrated activation of all known ER stress pathways (i.e. IRE1α, PERK and ATF6 pathway) by saturated stearic acid at a cell death-inducing concentration (1mM). The activation was clearly detectable as soon as 3 h after the stearic acid treatment and, with exception of eIF2α phosphorylation, the activation of ER stress pathways lasted for 24 h. The effect on PERK pathway activation and the time-course of eIF2α phosphorylation is consistent with data obtained by others in β-cells of animal and human origin [9, 36, 37]. XBP1 splicing and/or JNK activation after saturated fatty acid treatment was also found by other autors [8, 9, 14, 37, 38]. However, other data has suggested that there is no involvement of IRE1α activation in fatty acid-induced ER stress signaling [7, 10, 35]. This discrepancy may reflect the level of ER stress achieved in individual experiments because IRE1α appears to be activated only under more severe ER stress conditions in contrast to the PERK pathway [35]. Finally, we found ATF6 activation by stearic acid treatment in NES2Y cells similarly to others [8, 9, 37]. However, in our studies we have directly assessed ATF6 translocation into the nucleus by confocal microscopy rather than use reporter constructs and luciferase assays.

The treatment of cells with stearic acid led to significant increase in BiP expression in NES2Y cells. Probably because of the necessity of transcriptional activation by other molecules, we did not observed the increase of BiP expression until 12 h of treatment, in contrast to the activation of other tested ER stress-related molecules which were upregulated between 3-6 hours following treatment. Thus BiP seems to be a late marker of ER stress induction in NES2Y cells as has been found in rodent β-cells and human islets. [9, 10, 32]. However, fatty acid-induced ER stress signaling does not always result in an increase in BiP expression as demonstrated by Karaskov et al. [36] and Lai et al. [35]. They were unable to detect an upregulation of BiP expression even in the presence of clear activation of PERK and IRE1α pathways.

The exact mechanisms that connect excessive ER stress with apoptosis induction are still elusive. JNK is considered as one of the most possible mediators. However, in NES2Y cells, inhibition of JNK activity did not lead to significant changes in the level of apoptotic markers (cleaved caspases and PARP). In the study of Cunha et al. with INS-1E cells [9], palmitate, but not oleate, was also found to activate JNK but inhibition of JNK activity resulted in a partial reduction of apoptosis. In another study with INS-1E cells, JNK inhibition was showed to be anti-apoptotic, especially under hyperglycemic conditions [21]. JNK activation by stearate in NES2Y cells is 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 [21, 38]. It is therefore somewhat difficult to directly compare these two conflicting sets of 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 ER stress 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 [19, 21]. JNK can be activated also by MAP kinase kinases MKK4 and MKK7 and thus its activation by saturated fatty acids can result also from the engagement of signaling pathways that are not related to ER stress induction [18]. Our results do not contradict this hypothesis since we observed the full JNK activation after 3 h but splicing of XBP1 reached its maximum at 6 h. To sum up, our data suggest that in NES2Y cells JNK signaling 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 [40]. However, except of specific population of African descendents, the human population does not possess functional caspase-12 [41]. Several lines of evidence indicate that caspase-2 could substitute for caspase-12 in human cells [25-28] but this hypothesis has not yet been tested in pancreatic β-cells. We previously identified that caspase-2 was activated by palmitate and stearate in NES2Y cells [11, 23]. The importance of caspase-2 in β-cell apoptosis may be indicated by the demonstration of the key role played by caspase-2 in GTP-depletion induced β-cell apoptosis [42]. Thus we decided to decipher the role of caspase-2 in fatty acid-induced cell death by directly targeting and blocking its expression using siRNA. The results showed not only that caspase-2 is dispensable for fatty acid-induced apoptosis in NES2Y but also no influence of caspase-2 silencing on the order and extent of activation of other caspases previously shown to be associated with stearate-induced apoptosis [11].

Expression of both BiP and CHOP can be regulated, besides other transcription factors, through an AP-1 dependent mechanism [39, 43, 44]. During stearic acid-induced apoptosis, 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 induction and BiP expression after stearic acid treatment. This indicates that CHOP and BiP expression in NES2Y cells are not regulated directly by c-Jun and activation of JNK pathway. This is consistent with the finding that AP-1 dimers that contribute to CHOP induction by fatty acids in INS-1E β-cells are composed preferentially of c-Fos and jun-B, but not c-Jun [39] and the activity of jun-B is not controlled by JNK-mediated phosphorylation [45]. However, the mechanism mediating the decrease of BiP expression downstream of JNK inhibition and the mechanism responsible for increased c-Jun phosphorylation after caspase-2 silencing remain obscure.

The effect of caspase-2 silencing on BiP expression is unexpected. 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 [46, 47]. Caspase-2

silencing might thus 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. However, a lack of a complete understanding of the involvement of caspase-2 in lipid metabolism hinders speculations concerning the impact of caspase-2 silencing on ER stress signaling.

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 ER stress in NES2Y cells.

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Figure legends

Fig. 1. Effect of 1 mM stearic acid (SA) on the expression of phospho-eIF2α, phospho-JNK, phospho-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.

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.

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, BiP and CHOP and on (B) the cleavage of PARP, caspase-7, caspase-9, caspase-8 and caspase-2 in 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.

Fig. 5. 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. 6. 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.

Fig. 7. Effect of caspase-2 silencing by specific siRNA on the effect of 1 mM stearic acid (SA) on the expression of phospho-c-Jun, CHOP and BiP in NES2Y cells. Cells incubated without stearic acid represented control cells. After 6 h (phospho-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. 1.

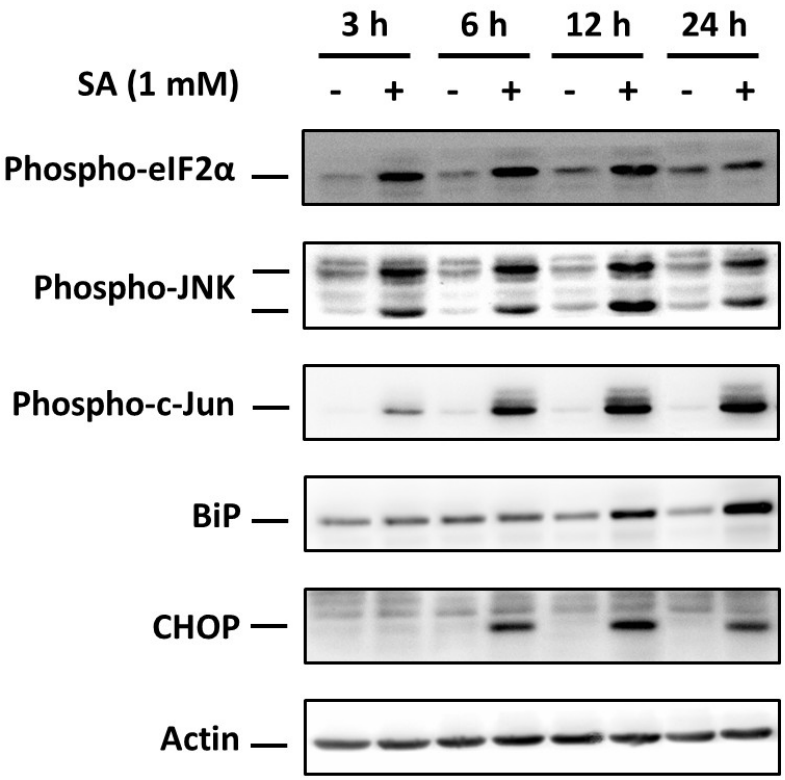

3 h
6 h
12 h
24 h
SA (1 mM)
- + - + - + - +
Phospho-eIF2α
Phospho-JNK
Phospho-c-Jun
BiP
CHOP
Actin

Fig. 2.

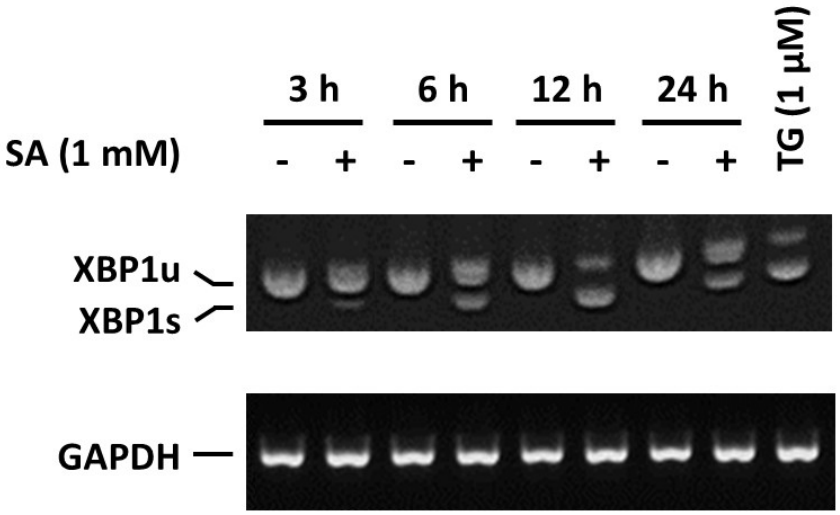


Fig. 3.

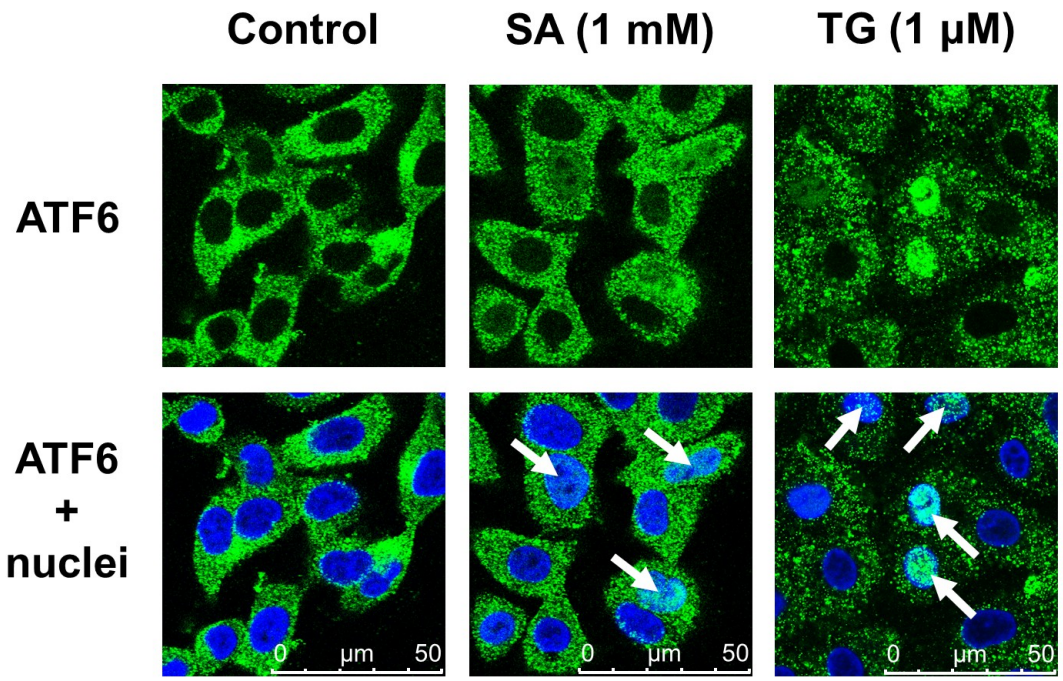


Fig. 4.

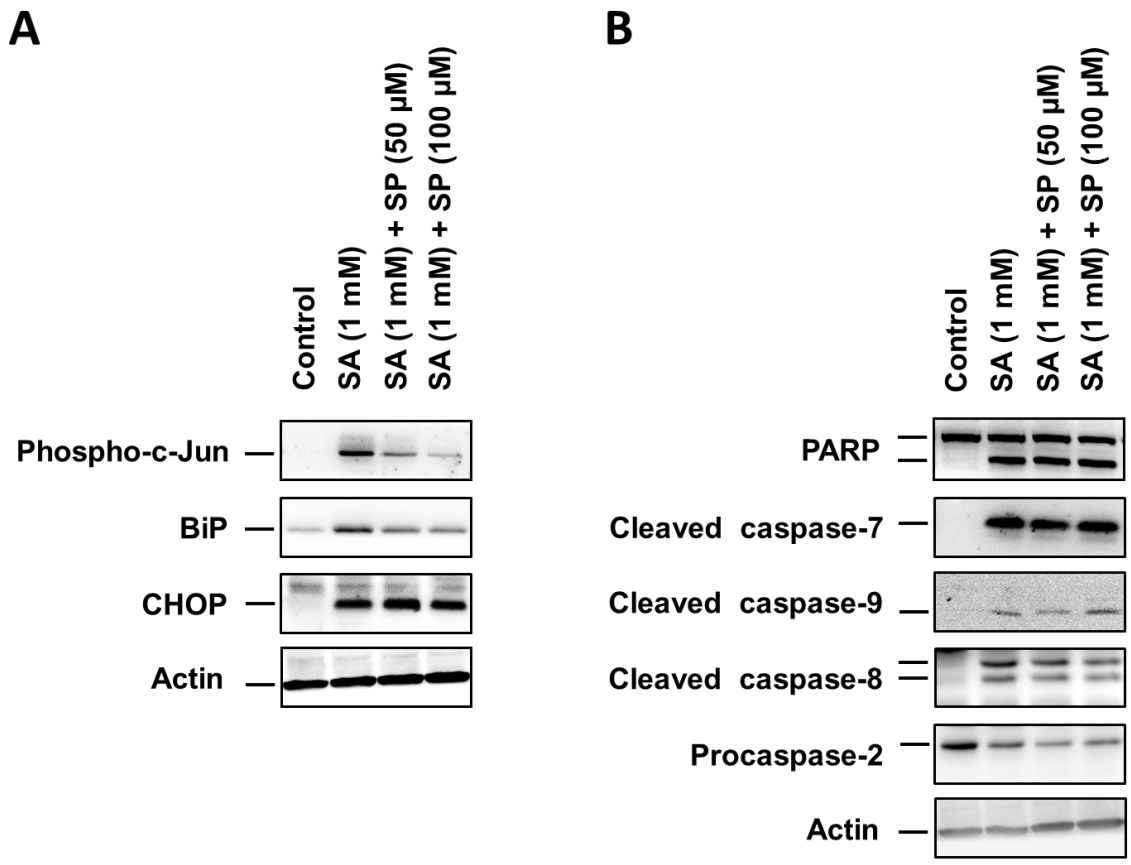

A
Control
SA (1 mM)
SA (1 mM) + SP (50 μM)
SA (1 mM) + SP (100 μM)
Phospho-c-Jun
BiP
CHOP
Actin
B
Control
SA (1 mM)
SA (1 mM) + SP (50 μM)
SA (1 mM) + SP (100 μM)
PARP
Cleaved caspase-7
Cleaved caspase-9
Cleaved caspase-8
Procaspase-2
Actin

Fig. 5.

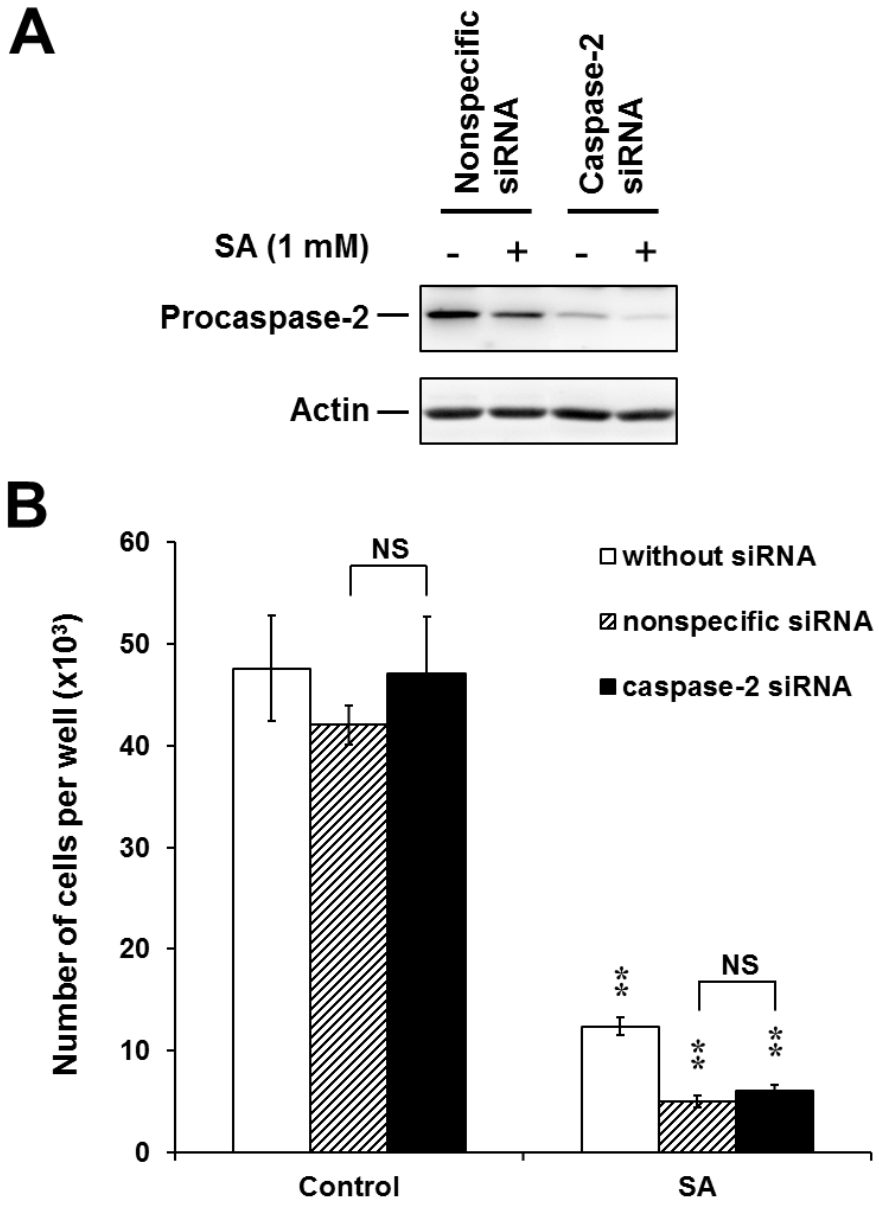

A
Nonspecific siRNA
Caspase-2 siRNA
SA (1 mM)
- + - +
Procaspase-2
Actin
B
Number of cells per well (x10³)
60
50
40
30
20
10
0
NS
without siRNA
nonspecific siRNA
caspase-2 siRNA
**
**
**
NS
Control
SA

Fig. 6.

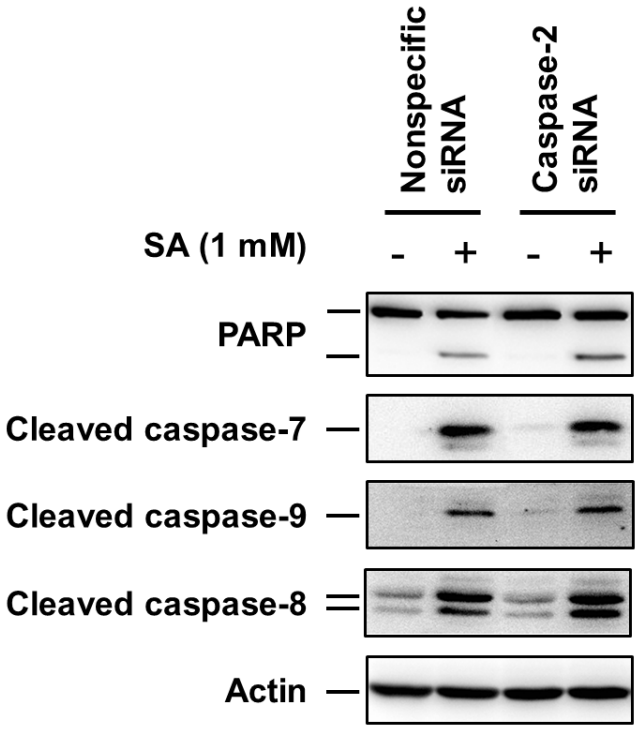
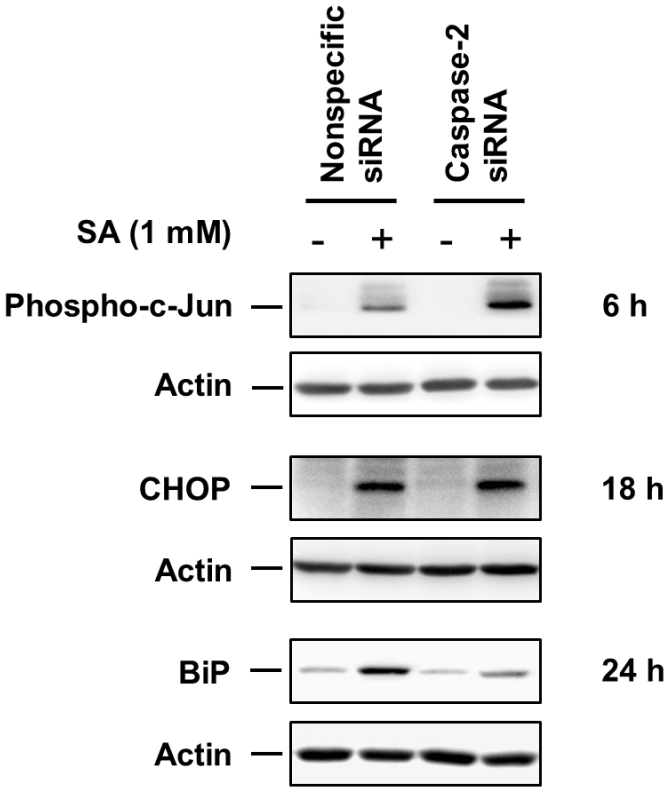

Nonspecific siRNA
Caspase-2 siRNA
SA (1 mM)
- + - +
PARP
Cleaved caspase-7
Cleaved caspase-9
Cleaved caspase-8
Actin

Fig. 7.


Nonspecific siRNA
Caspase-2 siRNA
SA (1 mM)
- + - +
Phospho-c-Jun
Actin
6 h
CHOP
Actin
18 h
BiP
Actin
24 h

9 UNPUBLISHED DATA

Besides the data included in our papers and already discussed, we have available some additional data that concern the effect of oleic acid (OA) on stearic acid (SA)-induced ER stress signaling. These results indicate that OA inhibits the activation of IRE1α and PERK pathway (see Figure 9-1, p. 120). However, surprisingly, OA does not inhibit ATF6 activation and nuclear translocation induced by application of SA but rather potentiates it (see Figure 9-2, p. 121). This is the first time we see a significant difference between the effect of OA *per se* and the effect of OA combined with SA. Nevertheless, to eliminate the possibility that the potentiation of ATF6 activation occurs solely due to increased total FA concentration in media, we induced cells with 1.2 mM SA and 1.2 mM OA. The extent of ATF6 translocation found under these conditions was comparable to the effect of routinely used SA and OA concentrations, i.e. 1 mM SA and 0.2 mM OA (data not shown). This indicated that the potentiation of ATF6 translocation occcurs only when SA and OA are applied in combination.

These data suggest that ATF6 pathway of ER stress signaling might play an important role in mediating the antiapoptotic effect of unsaturated FAs against saturated FAs. The prosurvival potential of the ATF6 protein in β-cells has already been suggested [159, 311], however, further research is needed to elucidate this issue.

So far, there exists only limited number of papers investigating the interplay of saturated and unsaturated FAs in the ER stress signaling in β-cells [159, 164, 165]. However, as to our best knowledge, there is no evidence in the literature concerning the effect of saturated and unsaturated FA applied in combination on ER stress signalling in human β-cells .

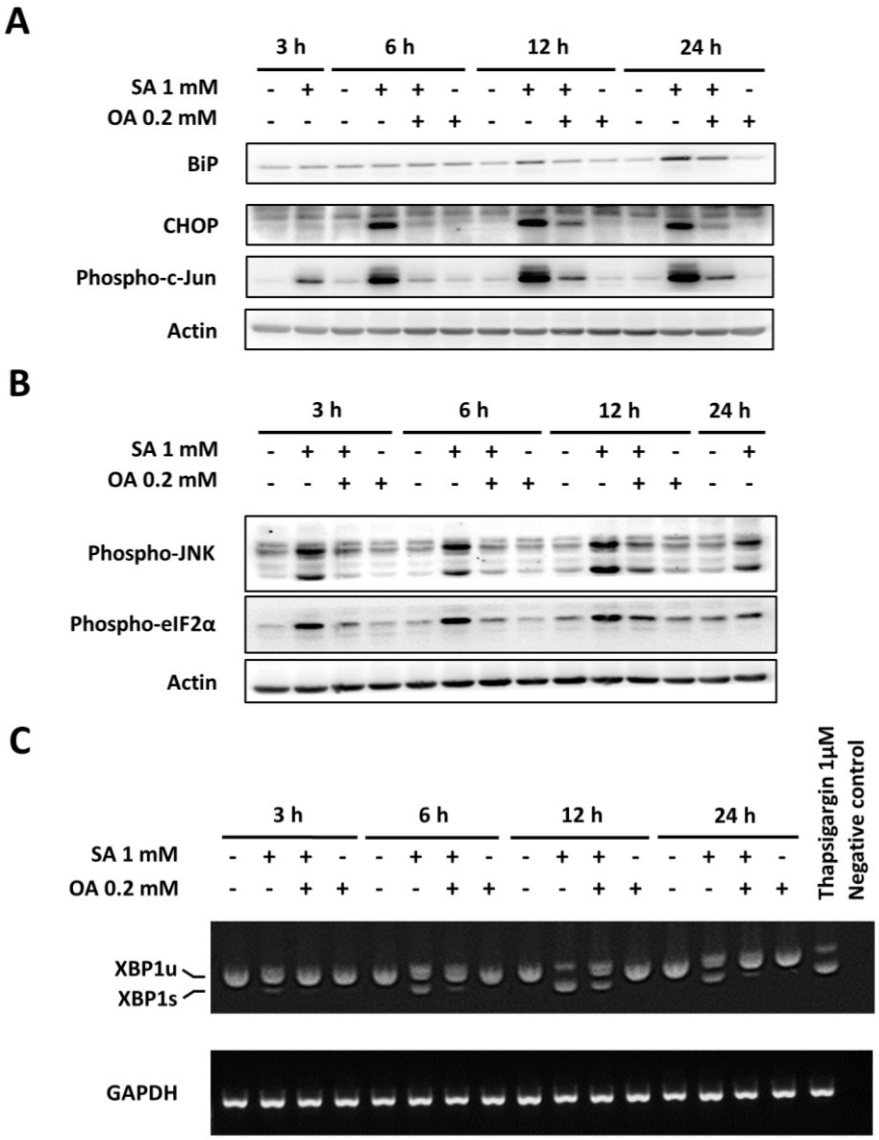


Figure 9-1: Effect of 1 mM stearic acid (SA), 1 mM stearic acid applied together with 0.2 mM oleic acid (OA), and 0.2 mM oleic acid on (A) the expression of BiP, CHOP and phospho-c-Jun and (B) the expression of phospho-JNK and phospho-eIF2α in NES2Y cells. Cells incubated without fatty acids represented control cells. After 3, 6, 12 and 24 h of incubation, the expression of proteins was determined by Western blot analysis employing relevant antibodies. 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. (C) 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 XBP1 mRNA splicing in NES2Y cells. Cells incubated without fatty acids represented control cells. After 3, 6, 12 and 24 h of incubation, the XBP-1 splicing was assessed by RT-PCR using relevant primers. As positive control, NES2Y cells were induced with 1 μM thapsigargin. GAPDH was used as a control gene for RT-PCR. Negative control of PCR reaction is also included. The data shown were obtained in one representative experiment of three independent experiments. The methods used are described in detail in Paper 4 (Němcová-Fürstová et al 2012, submitted), p. 95 and p. 96.

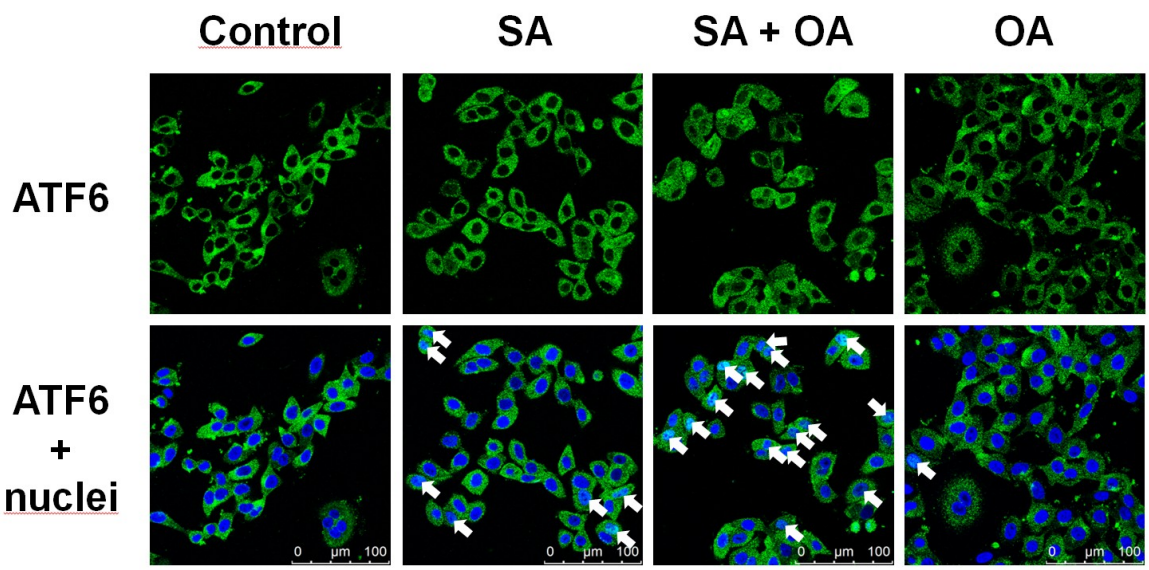


Figure 9-2: Effect of 1 mM stearic acid (SA), 1 mM stearic acid applied together with 0.2 mM oleic acid (OA), and 0.2 mM oleic acid alone on ATF6 translocation into nucleus. Cells incubated without SA represented control cells. After 3 h of incubation, ATF6 (green) and nuclei (blue) within the cells were stained and representative images were aquired with Leica confocal scanning microscope. ATF6 staining and the merge of ATF6 and DAPI signal is shown. Data obtained in one representative experiment of three independent experiments are shown. The methods used are described in Paper 4 (Němcová-Fürstová et al 2012, submitted), p. 96.

10 CONCLUSIONS

To summarize the contributions of this Ph.D. project, we developed an alternative method for isolation of Langerhans islets from mice that avoids the use of Ficoll gradient and its potentially toxic impact on viability and function of isolated islets. The innovativeness of our approach lies in the use of plastic for suspension cells that allows effective separation of exocrine and endocrine tissue during islet isolation.

We also demonstrated the different effects of saturated (e.g. stearic acid, SA) and unsaturated (e.g. oleic acid, OA) fatty acids (FAs) on the growth and viability of human pancreatic β-cells NES2Y. Saturated FA treatment led to apoptosis induction in β-cells, in contrast to unsaturated FA treatment that was even able to block the saturated FA-induced cell death. Searching for molecular mechanisms involved in the observed effects, we found that SA-induced apoptosis is associated with activation of executioner caspase-6 and -7 and initiator caspases-9,-8 and -2. However, it is not accompanied by a significant activation of the key executioner caspase-3. SA-induced apoptosis appeared to occur independently of p53 and mitochondrial pathway of apoptosis activation. Fas/FasL interaction and PIDDosome formation were likely not involved in the observed caspase-8 and caspase-2 activation, respectively. SA caused also rapid activation of IRE1α, PERK and ATF6 pathways of ER stress signaling. OA *per se* did not activate any of the molecules tested. We also demonstrated that caspase-2 and JNK are not key players in β-cell apoptosis induced by saturated FAs but are involved in modulation of ER stress

In addition, our results suggest that the inhibitory effect of OA on SA-induced apoptosis occurs upstream of caspase activation and is not exerted at the level of mitochondrial pathway of apoptosis induction, alteration of Fas, FasL and PIDD expression nor p53 activation since these mechanisms do not play significant role in SA-induced apoptosis. Concerning the effect on ER stress signaling, OA co-application inhibited the activation of IRE1α and PERK pathway of ER stress signaling, but surprisingly, it seemed to potentiate the ATF6 pathway activation. This points at a possible role of the ATF6 branch of ER stress signaling in the inhibitory effect of unsaturated FAs on saturated FA-induced signaling.

Collectivelly, our findings contributed to the knowledge concerning the role of saturated and unsaturated FAs in the regulation of β-cell functions and viability. In wider context, they also contributed to the understanding of mechanisms that play potential role in the pathogenesis of type 2 diabetes. Our results and available data from various other cell types indicate that saturated and unsaturated FAs have the ability to regulate multiple cellular signaling pathways and that their prevailing composition in blood plasma is involved in pathogenesis of various other diseases as well. Thus, FAs seem to be an attractive target for multiple disease prevention and maybe the therapy as well since their level and composition in respect to saturated versus unsaturated FA species can be relatively easily manipulated by the diet.

11 FUTURE PERSPECTIVES

Our experiments brought a lot of information concerning the molecular mechanisms by which saturated fatty acids (FAs) induce apoptosis and endoplasmic reticulum (ER) stress in human pancreatic β-cells NES2Y. However, many questions are still present that we want to address in our further studies.

For example, the identity of the most apical caspase in the caspase activation cascade is not known so far and our results disqualified caspase-2 for this role. So we plan to assess, whether caspase-4, proposed earlier to mediate ER stress-induced apoptosis [213, 214] and neglected by β-cell research so far, is activated by saturated FAs in β-cells.

Available data also point at CHOP as the mediator of ER-induced apoptosis. The experimental evidence concerning its role specifically in saturated FA-induced β-cell apoptosis is very spare. Thus the logical continuation of our research is to assess the impact of the siRNA-mediated CHOP silencing on saturated FA-induced apoptosis. The experiments concerning caspase-4 and CHOP could also show whether ER stress *per se* is involved in apoptosis induced by saturated FAs or whether its induction is only an accompanying effect of FA treatment with no relevance to apoptosis.

Recently, autophagy was also shown to be activated by saturated FAs in β-cells [312-314] and histologically confirmed in islets of type 2 diabetes patients [315]. Our preliminary data indicate that autophagy is regulated differentialy by saturated and unsaturated FAs in NES2Y cells as well. Thus we also plan to assess whether autophagy represents protective or deleterious response to increased levels of saturated FAs in β-cells as both options can principally occur [316, 317]. Targeted modulation of autophagy could open a new way to the regulation of β-cell viability as modulators of autophagy-related molecules, e.g. modulators of mTOR (mammalian target of rapamycin) activity, are already used in clinical practice.

The last but not least, we want to extend the current knowledge concerning the inhibitory mechanisms used by unsaturated FAs to inhibit saturated FA-induced apoptosis. Here, some clues are already available. Our unpublished data show that the activation of ATF6 branch of ER stress signaling is not inhibited but potentiated when SA is administered together with OA. Thus, the silencing of ATF6 expression would

reveal whether activation of this signaling plays some role in the inhibitory effect. In addition, we have some preliminary data in our hands that point to differential regulation of several kinase signaling pathways by saturated and unsaturated FAs that could mediate the inhibitory effect of unsaturated FAs and potentially could be used for improvement of β-cell viability by their targeted modulation.

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13 ANNEXE

Vobořilová J., **Němcová-Fürstová V.**, Neubauerová J., Ojima I., Zanardi I., Gut I., Kovář J.:
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