DOI:10.14753/SE.2020.2425

SEMMELWEIS EGYETEM

DOKTORI ISKOLA

Ph.D. értekezések

2425.

SARNYAI FARKAS

Patobiokémia

című program

Programvezető: Dr. Mandl József, professzor emeritus

Témavezető: Dr. Csala Miklós, egyetemi tanár

DOI:10.14753/SE.2020.2425

Investigation of the metabolism and deleterious or protective effects of two main dietary trans fatty acids in rat insulinoma cells

PhD thesis

Farkas Sarnyai

Molecular Medicine Doctoral School

Semmelweis University

Supervisor: Dr. Miklós Csala, MD, D.Sc.

Official reviewers: Dr. Katalin Monostory, Ph.D.

Dr. Beáta Törőcsik, MD, Ph.D.

Final Examination Committee

Head: Dr. László Tretter, MD, D.Sc.

Members: Dr. Erzsébet Komorowicz, MD, Ph.D.

Dr. Veronika Deák, Ph.D.

Budapest

2020

DOI:10.14753/SE.2020.2425

Table of Contents

DOI:10.14753/SE.2020.2425

List of Abbreviations

acyl-CoA	acyl-coenzyme A
AP-1	activator protein 1
ASK1	apoptosis signal-regulating kinase 1
ATF-4	activating transcription factor 4
ATF-6	activating transcription factor 6
BAK	Bcl-2 homologous antagonist/killer
BAX	Bcl-2 associated X
Bcl-2	B-cell lymphoma 2
BiP/GRP78	binding immunoglobulin protein/glucose-regulated protein 78
cDNA	complementary DNA
CHOP	C/EBP homologous protein
DG	diglyceride
DNA	deoxyribonucleic acid
eIF2α	eukaryotic initiation factor 2α
ER	endoplasmic reticulum
ERAD	ER-associated degradation
FA	fatty acid
FAT/CD36	fatty acid translocase/ cluster of differentiation 36
FFA	free fatty acid
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GC-FID	gas chromatography coupled to flame ionization detector
IKK	inhibitory-κB-kinase
IL-1R	interleukin 1 receptor
IL-1β	interleukin 1β
IL-6R	interleukin 6 receptor
IRE-1α	inositol-requiring enzyme 1α
JNK	c-Jun aminoterminal kinase
LC-MS/MS	Liquid chromatography–mass spectrometry/mass spectrometry
mRNA	messenger ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAFLD	non-alcoholic fatty liver disease

NF-κB	nuclear factor-κB
PDI	protein disulfide isomerase
P-eIF2α	phosphorylated eukaryotic initiation factor 2α
PERK	protein kinase R-like ER kinase
P-JNK	phosphorylated c-Jun aminoterminal kinase
PKC	protein kinase C
PKR	protein kinase R
PLC	phospholipase C
PS	phosphatidylserine
RINm5F	rat insulinoma cell line
RNase	ribonuclease
ROS	reactive oxygen species
S1P	site-1 protease
S2P	site-2 protease
SAPK	stress activated protein kinase
Ser-Thr	serine-threonine
sXBP-1	spliced X box-binding protein-1
TFA	trans-fatty acid
TG	triglyceride
TLR-4	Toll-like receptor-4
TNF-α	tumor necrosis factor-α
TXNIP	thioredoxin interacting protein
UPR	unfolded protein response
uXBP-1	unspliced X box-binding protein-1
VLDL	very low density lipoprotein
XBP-1	X box-binding protein-1

DOI:10.14753/SE.2020.2425

1. Introduction

1.1. Obesity and diabetes

Non-esterified or free fatty acids (FFA) are excellent nutrients for most aerobic cells of the organism. They are produced in the adipocytes by the hydrolysis of storage triglycerides in starvation, and secreted in the blood plasma where they are mostly associated to albumin [1]. The fatty acid (FA) molecules entering the cytosol are readily conjugated with Coenzyme A. This reaction serves both activation and an entrapment of FAs in the cell [2]. Fatty acyl-coenzyme A (acyl-CoA) is a central intermediate in lipid metabolism, as it can be transported into the mitochondrial matrix or peroxisome for β-oxidation, it can be elongated and/or desaturated in the endoplasmic reticulum (ER) membrane, and it can be inserted into complex lipids, such as di- and triglycerides (DGs and TGs), phosphoglycerolipids, ceramides and other sphingolipids or cholesteryl esters.

Generally, a permanent increase in the FFA levels can be linked to obesity, one of the most concerning problems of the modern world, which is largely caused by the so called “western-type” diet, as well as the lack of exercise. Metabolic and hormonal changes in a sustained overnutrition favor adipocyte hypertrophy in the human body [3]. The FAs originated from food are absorbed in the intestines as resynthesized complex lipids, mostly triglycerides packed in chylomicrons. They can reach from the intestinal lymphatics through the systemic blood circulation directly to the fat tissue. Amino acids and monosaccharides from digested food reach the liver through the portal system, where a large portion of them is converted to FAs and incorporated in TGs. These TGs are also transported to the fat tissue packed in very low density lipoprotein (VLDL), through the systemic circulation. The esterified FAs transported in lipoproteins, either chylomicrons or VLDL can enter the adipocytes, after getting liberated with the help of lipoprotein lipase [4]. The excess nutrients, which are accumulated because of an overfeeding for an extended period of time, are a heavy burden on the adipose tissue. Due to the limited number of adipocytes, excessive fat deposition causes adipocyte hypertrophy, which may lead to a local inflammation. The amount and activity of macrophages and lymphocytes increase, which is caused by the secretion of adipokines (e. g. tumor necrosis factor-α; TNF-α) and leukotrienes from the swollen cells. Stimulated inflammatory macrophages secrete cytokines, such as TNF-α and interleukin-1β (IL-1β), which disturb the local insulin signaling, and thus enhance degradation of TGs in the cells [5]. The inflammation-

driven TG mobilization combined with TG deposition maintained by the oversupply of nutrients, result in an accelerated TG turnover, which in turn increases the FFA release of the tissue. Ultimately, the local inflammation in the adipose tissue is spread by elevated plasma FFA and cytokine levels to the whole body. The excessive and sustained supply of FFA not only disrupts the function of the most important metabolic tissues (liver, muscle), but damages the β-cells too. It causes insulin resistance, which leads to higher blood sugar levels and an intensified insulin demand but it also hinders the compensatory insulin synthesis, and it can even decrease insulin levels through reducing the β-cell mass [6]. This process called lipotoxicity is widely regarded as a key link between overweight and type 2 diabetes [5]. The complex intracellular mechanism of lipotoxicity has been intensely investigated in various cell cultures, including diverse insulinoma cell lines, by using high concentrations of palmitate (16:0) and oleate (18:1 cis-Δ^9), i.e., the major endogenous saturated and mono-unsaturated FAs, respectively.

1.2. Molecular background of the toxicity of fatty acids

Elevated plasma FFA levels can disturb the function of the cells since they are both signal and nutrient molecules. They can exert their toxic effect on the cell in several ways. Connecting to the Toll-like receptor-4 (TLR-4), interleukin receptors (IL-1R, IL-6R) and tumor necrosis factor receptor, all of which can be found on the outer surface of the plasma membrane, they can initiate signaling pathways related to inflammation [7]. These pathways meet at the activation of two major serine-threonine (Ser-Thr) kinases, i.e., the inhibitory-κB-kinase (IKK) complex and the c-Jun aminoterminal kinase (JNK). Both of these kinases contribute to the inflammatory cell response on the level of the genes, through nuclear factor-κB (NF-κB) and activator protein 1 (AP-1) transcription factors [5]. They contribute to the development of insulin resistance and the activation of JNK can also induce apoptosis. It is important to emphasize, that JNK phosphorylation is not specific to inflammation, rather it is a general stress activated protein kinase (a.k.a. SAPK) [5, 8]. The cell does not regulate, or only to a limited amount, the uptake of FAs, so the FFA consumption is proportional in a wide range to the supply. The entry of FAs into the cell can happen in two ways, with passive diffusion through the double layered plasma membrane and with facilitated membrane protein transport [9]. Among several FA transporters, the most investigated is the FA translocase (FAT/CD36), which is part

of the type B scavenger receptor family. This protein works also as a receptor, whose stimulation turns on the phospholipase C (PLC) signaling pathway [10]. Extended stimuli can keep a constant level of the different activated protein kinase C (PKC) isoforms, and it can also lead to Ca^{2+} depletion in the ER [11]. The consumed FAs are converted to acyl-CoA by acyl-CoA synthetase and enter different catabolic and anabolic pathways.

Oxidative degradation of FAs takes place in the mitochondria and peroxisomes. The FA oversupply causes an increased oxidation, which inevitably amplifies the generation of the reactive oxygen species (ROS) and the resulting oxidative stress is an additional effective activator of IKK and JNK [5]. Besides this, the shift in the redox equilibrium impairs the function of the ER. There is no other effective process of FA degradation apart from β-oxidation, which can decrease the increased acyl-CoA supply, therefore in case of lipotoxicity, when β-oxidation is saturated, the acyl-CoA dependent lipid synthesis also intensifies. To store the FAs, the optimal anabolic pathway is the TG synthesis [4]. Unfortunately, this process cannot be considered as physiological in case of most cell types, thus its capacity is often limited. The synthetized TG is neutral, hydrophobic, which needs to be stored in the cytoplasm in the form of lipid droplets. This ectopic lipid deposition is an essential part of lipotoxicity and formerly it was considered as the main reason for cellular damage. Nevertheless, it is possible that the formation of lipid droplets is the least harmful way to relieve the cells from the noxious acyl-CoA oversupply. Therefore, the steatosis can be considered as a self-defense strategy of the cells, rather than a mechanism of lipotoxicity [4]. FAs can also be used in the synthesis of a wide variety of membrane lipids, but these cannot be accumulated by the cells.

1.2.1. Diglycerides

DGs have a glycerol backbone and two of its hydroxyl groups are connected with two fatty acyl groups through ester bonds. These molecules can function as precursors in the synthesis of TGs and other complex lipids (e.g. phosphatidylserine (PS), galactolipids). DGs are important intermediates of phospholipid metabolism, so alterations in their structure can have a major effect on the structure and fluidity of the membrane lipids as well [4]. DGs, as major lipid species have signaling functions since their accumulation results in the activation of the PKC isoforms [12] and the role of some PKC isoforms in the formation of insulin resistance has been already proved. Last but not

least, in case of elevated FA levels, DGs also have a negative effect on the insulin secretion of β-cells [13].

1.2.2. Ceramides

The forced stimulation of the synthesis of sphingolipids in lipotoxicity is particularly interesting and results in the accumulation of ceramides. These molecules are amides of a sphingosine molecule and a FA chain. They compose the hydrophobic central part of many different sphingolipids and also have an important role as signal molecules. Ceramides affect cell growth, proliferation and the aging of cells, and they also influence the induction of apoptosis [14-17]. The role of ceramides in lipotoxicity, and within that, their role specifically in β-cell death is demonstrated and discussed in several studies [18, 19]. Many publications also suggested they have a role in the development in insulin related diseases, such as non-alcoholic fatty liver disease (NAFLD), insulin resistance and in β-cell failure, thus they are important in type 2 diabetes [20-23]. It was also proved that the cytotoxic effect of two major inflammatory cytokines (TNF-α and IL-1β) is at least partly mediated through an increased ceramide production [24, 25]. Fatty acyl-CoA surplus intensifies the intracellular *de novo* synthesis of ceramides, which are intermediates in the formation of membrane sphingolipids. The sphingolipids, by their nature, cannot be accumulated in the cells and the product of their hydrolytic degradation is sphingosine. During the salvage pathway, this compound is acylated to form ceramides again. Fatty acyl-CoA enters into the ceramide synthesis at more than one point, so it is evident that the oversupply of fatty acyl-CoA can increase the ceramides levels in the cells. The adverse effect of ceramides is caused partly due to their role in the induction of ER stress and partly due to their direct stimulatory influence on apoptosis [18, 19]. As it has been shown, the mechanism of lipotoxicity is converging to the dysfunction of the ER, so it is not surprising that the ER stress is the central factor of lipotoxicity and its associated adverse conditions.

1.3. The endoplasmic reticulum stress

The ER is an important center of protein synthesis and maturation and, cooperating with the Golgi apparatus, takes part in the sorting of mature proteins. The ER also has a role in the adaptation of the cells to environmental changes, so it is essential in keeping the homeostasis of the cells. The protein maturation process in the ER is helped

by a regulatory mechanism known as the unfolded protein response (UPR) [26]. The UPR is activated when the amount of misfolded proteins raises, which can be caused by several reasons. These are, for example, synthesis of mutant proteins or disturbance in the protein processing, but the UPR can also be activated by changes in the Ca^{2+} levels, by glucose or energy deficit, hypoxia, high acyl-CoA and ceramide concentrations or by different toxins [5, 27]. The UPR is initiated by three ER stress sensor proteins and these are the protein kinase R (PKR)-like ER kinase (PERK), the inositol-requiring enzyme 1α (IRE-1α) and the activating transcription factor 6 (ATF-6) [26]. All three of the mentioned proteins have ER-luminal domains, which enables them to detect the amount of misfolded proteins, directly or indirectly. In case the amount of unfolded proteins reaches critical levels, they activate different intracellular signaling pathways. The dimerization or oligomerization of IRE-1α or PERK is inhibited by an ER chaperon, the binding immunoglobulin protein (BiP or GRP78), in normal cells. In case of stress, the termination of inhibition happens, because the BiP protein binds to the misfolded proteins with greater affinity [28]. In addition, the misfolded proteins also bind to the luminal domain of IRE-1α and PERK as activators [29, 30]. In any case, the primary goal of the cell is to restore the protein synthesis and maturation processes and to survive [31]. The pathways of the ER stress response are tightly coupled to each other, and the hallmarks of the complex process can be considered as either early or late ER stress markers. Early ER stress markers are those which appear almost immediately after the stress forms, for example modification of X box-binding protein-1 (XBP-1) mRNA and translation of XBP-1, as well as the phosphorylation of eukaryotic initiation factor 2α (eIF2α) and JNK, although, as it has been mentioned above, the latter cannot be considered to be ER stress specific. Late ER stress markers are the ones, whose development requires longer stimulation, and thus occur in a prolonged stress such as the induction BiP and protein disulfide isomerase (PDI) chaperons (Fig. 1).

DOI:10.14753/SE.2020.2425

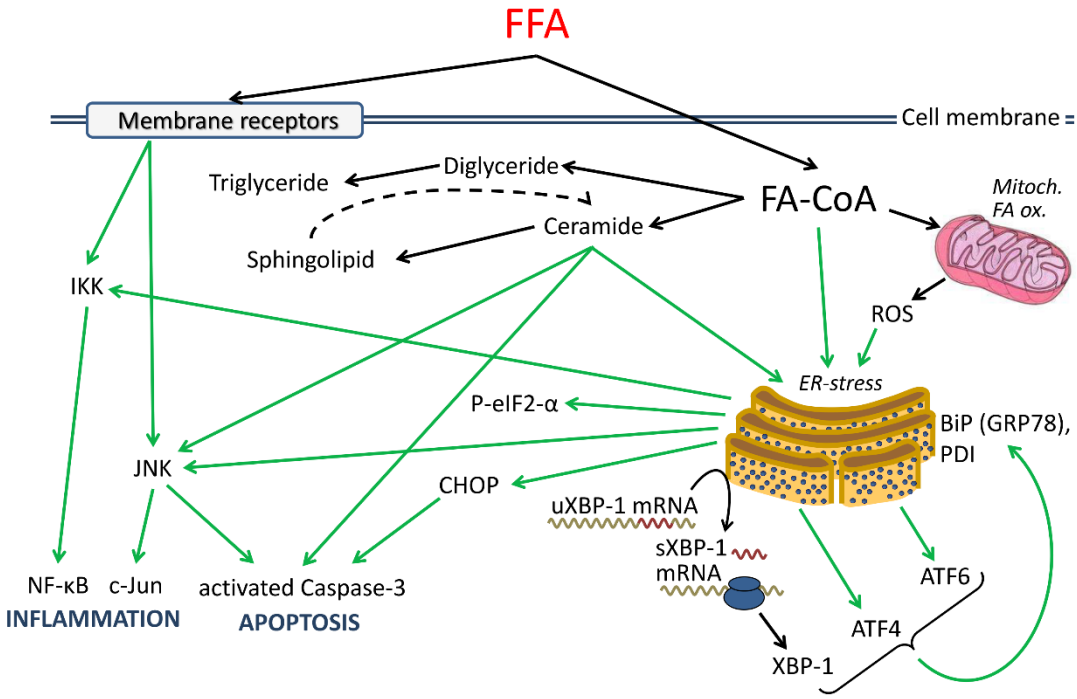

Figure 1. Cellular effects of free fatty acids. FFAs bind to the cell surface receptors or get metabolized through binding to CoA, after entering into the cells. The membrane receptors stimulate pathways, which activate IKK and JNK kinases and this leads to inflammation. The elevated level of fatty acyl-CoA increases the amount of DGs, TGs and ceramides. Because of the enhanced mitochondrial FA oxidation, the amount of ROS increases. The increased fatty acyl-CoA concentration induces ER stress directly and indirectly, via ceramides and ROS as well. During ER stress, different transcription factors, like XBP-1, ATF-4 and ATF-6 and chaperons, like BiP and PDI are activated and/or induced. The ceramides have an effect directly and via ER stress on the phosphorylation of eIF2α and JNK, and also activate the CHOP transcription factor. These changes can lead to activation of the effector caspase-3 and start the apoptosis.

The ER stress, which is caused by an increased secretory demand to compensate for insulin resistance is further aggravated by a FA induced ER dysfunction in β-cells [32, 33]. IRE-1α triggers several signaling routes, which provoke a combination of pro-survival and pro-apoptotic responses. The RNase domain of IRE-1α cuts a short sequence from the XBP-1 mRNA, thus yielding a so called spliced mRNA (sXBP-1 mRNA), and giving rise to the XBP-1 transcription factor, which in turn induces various ER chaperones and proteins of ER-associated degradation (ERAD) [34]. IRE-1α also causes activation of JNK, a central stress kinase that promotes apoptotic cell death [35]. The kinase domain of PERK phosphorylates the α subunit of eIF2 protein, which leads to the inhibition of protein translation by blocking the initiation step [36, 37]. As a result of the decreased protein synthesis, the pressure on the maturation processes in the ER lessens,

and there is more time for the cell to correct the misfolded proteins. Another result of the eIF2α phosphorylation is the increase in the expression of activating transcription factor 4 (ATF-4) [38]. ATF-4 promotes the transcription of genes which are responsible for restoring the homeostasis, and on the other hand, it increases the transcription of the proapoptotic C/EBP homologous protein (CHOP) [39]. In the presence of misfolded proteins, ATF-6 is translocated to the Golgi-apparatus and is cleaved by Site-1 and Site-2 proteases (S1P and S2P). The N-terminal of the cleaved ATF-6 with the help of XBP-1 protein takes part in the restoration of the size and permeability of the ER changed by the stress [40]. During ER stress the induction of both chaperons, the BiP and PDI, are linked to ATF-6. The cleaved ATF-6 enters the nucleus, where it induces the transcription of UPR target genes, such as BiP and PDI, increasing the amount of these chaperons [41, 42]. BiP regulates the signalization steps during the UPR, and it can also inhibit the activation of the caspase cascade, functioning as an antiapoptotic protein [43]. In the process of protein folding, PDI helps in the formation of disulfide bonds, which are needed for the correct protein folding.

In case the mechanisms of the UPR cannot restore the homeostasis, the constant activation of the signalization pathways leads to chronic ER stress. In such cases, the UPR is transformed to an alternative signalization complex, which is called terminal UPR and it actively promotes apoptosis. All three of the pathways activated during the UPR contain a proapoptotic elements, such as B-cell lymphoma 2 (Bcl-2), thioredoxin interacting protein (TXNIP), apoptosis signal-regulating kinase 1 (ASK1), and JNK, which promotes the development of programed cell death, when the progress of the ER stress is irreversible [44-46].

1.4. Apoptosis

As we have seen, apoptosis can be induced by a chronic and/or severe ER stress. It has been established that a persistently high amount of FAs leads to the development of lipotoxicity, including the induction of ER stress [4, 34]. The apoptosis developed because of sustained lipotoxicity is a certain type of programmed cell death, and it is called lipoapoptosis. The apoptotic cells interrupt their connection to their environment, do not receive signals and inhibit their gene expression. Their DNA, nucleus and organelles are fragmented. The cell is shrinking and at the end, it gets fragmented to

apoptotic bodies. The cells undergoing apoptosis use so called “eat me” signals to indicate, for other cells in their surroundings, that they are readily available for phagocytosis. In this process, the accumulation of phosphatidyl serine on the surface of the plasma membrane plays a central role. This also creates an opportunity to detect apoptosis in experimental conditions. The commercially available, fluorescent Annexin binds with great affinity to the phosphatidyl serine rich region of the membrane [47]. These fluorescent regions can be detected by different methods, offering a variety of possibilities for the detection of apoptotic cells even in early stage of the process. Apoptosis is the terminal program of the cell, which is executed mostly by caspases [48]. These proteases cleave the so called death substrates in the right recognition site and the cleavage of hundreds of these death substrates is responsible for the caspase dependent pathway of apoptosis. The most important effector caspase is Caspase-3, which is activated by limited proteolysis, and hence detection of its cleaved fragments is commonly used to estimate the intensity of apoptosis [49].

Among the different pathways of apoptosis, the mitochondrial pathway, which is based on the protein efflux from the intermembrane region of the organelle, exceeds the others. One portion of these proteins entering the cytosol induces the caspase dependent pathway, while the other portion reaches the nucleus, where it fragments and deconstructs the DNA and the chromatin structure in a caspase independent manner. The increase in permeability of the mitochondrial outer membrane is caused by the channels created by members of the Bcl-2 multidomain proapoptotic protein family [50]. These, such as Bcl-2 homologous antagonist/killer (BAK) and Bcl-2 associated X (BAX), are necessary for the execution of a classic and typical apoptosis. In the last decade, we managed to get to know the details of the mechanism of apoptosis generated by ER stress and the UPR. Among these of outstanding importance is JNK activation by IRE-1α, since the JNK by phosphorylating the Bcl-2 proteins increases the permeability of the mitochondria [51]. Induction of the expression of proapoptotic CHOP protein is managed by more than one element of the UPR. These mechanisms contribute greatly to the development of the ER stress mediated apoptosis, because CHOP not only induces the expression of some of the proapoptotic genes (death receptor 5 and Bcl-2 like protein 11), but also inhibits the expression of antiapoptotic genes (Bcl-2) [52]. The Ca^{2+} efflux, which is part of the ER

stress, also contributes to the mitochondrial dysfunction, which forces the cells in the direction of apoptosis [11].

1.5. Toxicity of saturated and unsaturated fatty acids

The induction of ER stress contributes to cell death and reduces β-cell mass through apoptosis [53, 54]. This phenomenon is further exasperated by the accumulation of ceramide intermediates [18, 19].

A greater toxicity of the saturated palmitate compared to cis-unsaturated oleate has been demonstrated in several studies [55, 56]. In contrast, very little is known about the toxicity of dietary trans-fatty acids (TFAs) and even those findings contradict each other. Although double bonds of trans configuration in FAs are not formed by human enzymes, most people ingest considerable amounts of TFAs. Formation of a trans-11 double bond by isomerization is the first step of microbial unsaturated FA biohydrogenation in the digestive system of ruminant animals [57], and this makes tallow, beef, milk, and other dairy products natural sources of TFAs, mainly vaccenate (18:1 trans-Δ^{11}). TFAs are also produced artificially via industrial hydrogenation of vegetable oils; however, the predominant TFA in margarine is elaidate (18:1 trans-Δ^{9}), a positional isomer of vaccenate and a geometric isomer of oleate [58]. TFAs have been shown to exert various deleterious health effects *in vivo*, and chronic TFA intake has been found to be associated with the risk of type 2 diabetes [59]. Some studies have shown a difference between the harmfulness of the natural vaccenate and the industrial elaidate [60], and some studies have suggested that limited amounts of TFAs improve life expectances [61, 62]. Although the results of *in vivo* studies on the health impacts of trans fat consumption are often contradictive, and very little data is available on the metabolic fate and cellular toxicity of TFAs, these food components are widely considered as harmful, and current legislations aim to eliminate them from food products in several countries [63].

Not only has it been repeatedly demonstrated that saturated FAs are more harmful than the unsaturated ones, the ability of oleate to reduce the toxicity of palmitate has also been reported, and it has also become a widely investigated phenomenon. Administration of oleate and palmitate at the same time was shown to be less toxic than palmitate alone in various cell types [64, 65]. It also has been observed that activation of the UPR triggered by palmitate can be prevented or at least diminished with the co-administration

of oleate [66]. These observations raised a very interesting question, whether or not the TFAs also alleviate the palmitate induced toxicity like oleate does.

DOI:10.14753/SE.2020.2425

2. Objectives

High FA levels cause ER stress and apoptosis in β-cells, largely due to ceramide and diglyceride accumulation. The deleterious effect of the saturated palmitate (16:0) is much more pronounced than that of cis-unsaturated oleate (18:1 cis-Δ^9). It is also described in the literature that the simultaneous addition of oleate can mitigate the effects of palmitate. Despite their importance, very little information is available on the effects of dietary TFAs, such as elaidate (18:1 trans-Δ^9) and vaccenate (18:1 trans-Δ^{11}) and their health effects are even more obscure due to the contradictory data. Although it is true that humans are not able to synthetize FAs with double bonds in trans configuration, we consume plenty of TFAs in the 21st century. Based on these facts, the aim of our investigation was:

- to compare the toxicity of the two major dietary TFAs (i.e., elaidate and vaccenate) with that of the best studied endogenous saturated and cis-unsaturated FAs (i.e., palmitate and oleate) in RINm5F cells,

- to determine the change in FA profile and in the ceramide and diglyceride levels after palmitate, oleate, elaidate and vaccenate treatment,

- to test the potential mitigating effect of elaidate and vaccenate on palmitate-induced toxicity,

- to investigate how oleate, elaidate and vaccenate influence palmitate-induced alterations in the FA profile and the intracellular accumulation of ceramides and diglycerides.

We carried out our experiments on RINm5F rat insulinoma cell line since it is widely used to investigate β-cell apoptosis, including lipoapoptosis, and the experimental parameters were already successfully adjusted in our laboratory during the investigation of the protective effect of metformin against palmitate induced ER stress and cell death [34]. We estimated the cell viability and the intensity of apoptosis. The accountable ER stress and phosphorylation of stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK) were also observed. In addition, the amounts of the most relevant FAs were determined in the cells, and the intracellular levels of various ceramides and diglycerides were investigated.

3. Results

3.1. Effects of palmitate and various unsaturated fatty acids on RINm5F cell viability

The toxicity of saturated palmitate has been investigated in RINm5F cells earlier in our laboratory [34, 67]. In the present study, RINm5F cell survival was estimated after 24-hour long treatments with different FAs and their combinations at 250 or 500 μM concentration separately by using MTT assay. In accordance with our and others' previous results, palmitate was found to decrease the cell viability in a dose dependent manner. The number of viable cells dropped to about 40% and below 20% of the control value after 250 and 500 μM palmitate treatment, respectively (Fig. 2). Cell viability in samples treated with oleate was much higher, as expected based on previous observations. The comparison between the three observed unsaturated FAs did not show any difference. Neither the cis- nor trans-unsaturated FAs reduced the viability significantly at 250 μM concentration, nor did the damage caused by them reach the levels caused by palmitate at 500 μM (Fig. 2). In combination treatment with oleate, the damage caused by palmitate was lessened significantly in accordance with several earlier observations. Importantly, in case of the trans-unsaturated elaidate and vaccenate co-treatment, significant viability improvements could also be observed too. At 250 μM FA concentration, the cis-unsaturated oleate improved the viability of palmitate-treated cells by 40%, while the trans-unsaturated FAs lessened the toxicity of palmitate by at least 20%. At higher, 500 μM concentration the oleate co-treatment resulted in a 30% viability improvement, and the amount of viable cells also increased by 20% upon TFA co-treatment. It is also remarkable that no obvious difference was observed between the protective effect of the two trans-unsaturated FAs (Fig. 2).

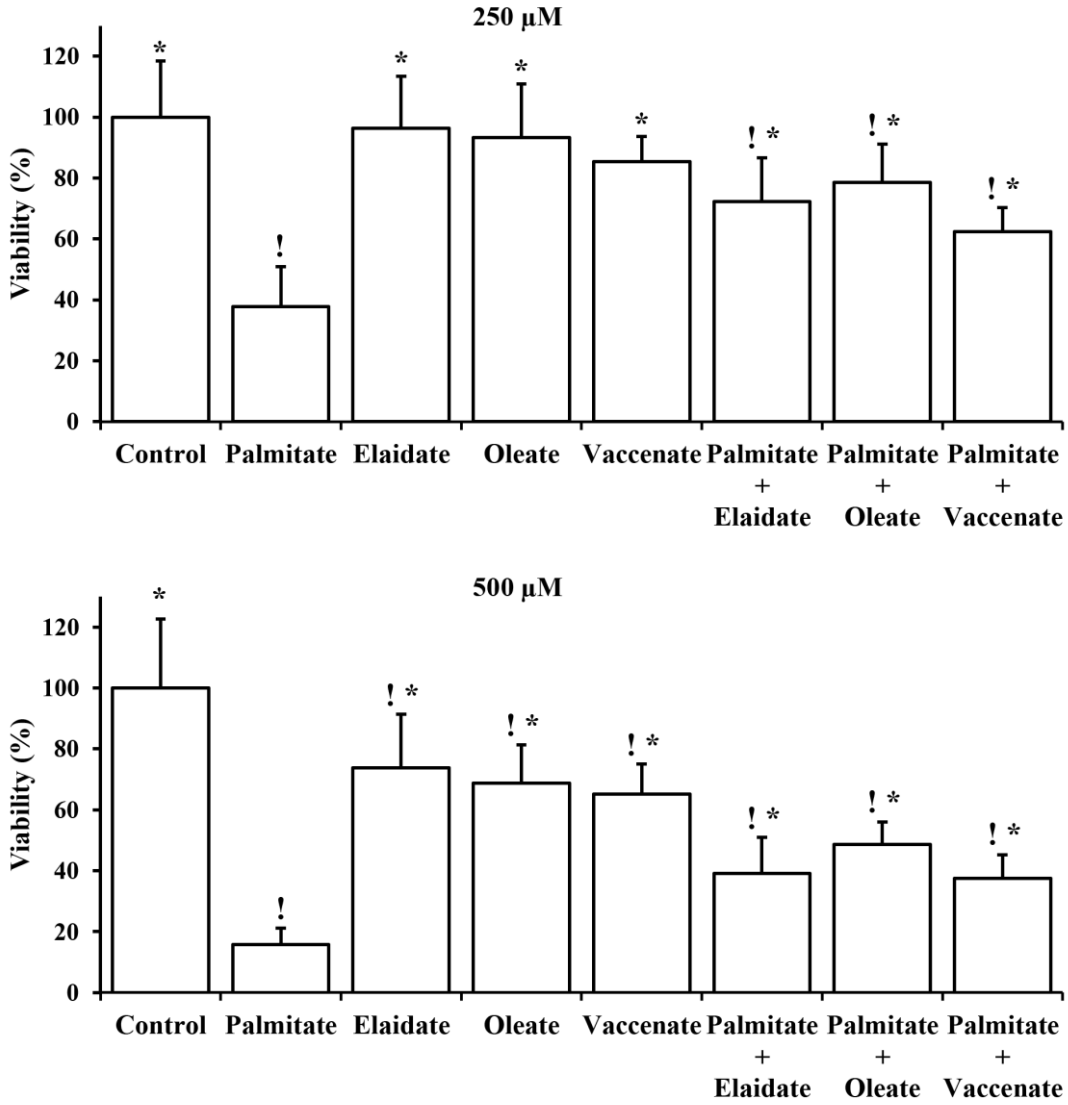

Figure 2. Cell Viability. RINm5F insulinoma cells were treated with BSA (control cells) or BSA-conjugated palmitate, elaidate, oleate or vaccenate alone, or with palmitate and one of the unsaturated FAs at two concentrations (250 or 500 µM) at 70-80% confluence and incubated for 24-hours. Cell viability was measured by Colorimetric (MTT) Kit for Cell Survival and Proliferation (Millipore) and expressed as the percentage of Control. Data are shown as mean values ± S.D. of six experiments; statistically significant differences: $^{!}$ P <0.05, v.s. BSA-treated control; *P <0.05, v.s. palmitate treated cells.

3.2. Fatty acid-induced apoptosis

Apoptosis is one of the major factors in β-cell lipotoxicity. To reveal the contribution of apoptotic cell death in the change of cell viability, apoptotic index was determined. After an 8-hour long FA treatment, the number of apoptotic cells among 100 total cells was counted with fluorescence microscopy. Palmitate treatment caused a definite 5-fold increase in apoptotic index (Fig. 3). The three unsaturated FAs alone

caused a small and statistically not significant elevation in the apoptotic index when compared to the control level. A distinct change can be observed upon unsaturated FA co-treatments compared to palmitate-treated cells, as the simultaneously added unsaturated FAs lessened the amount of apoptotic cells significantly. The amount of the apoptotic cells in these co-treated samples does not show statistically relevant difference from the control (Fig. 3).

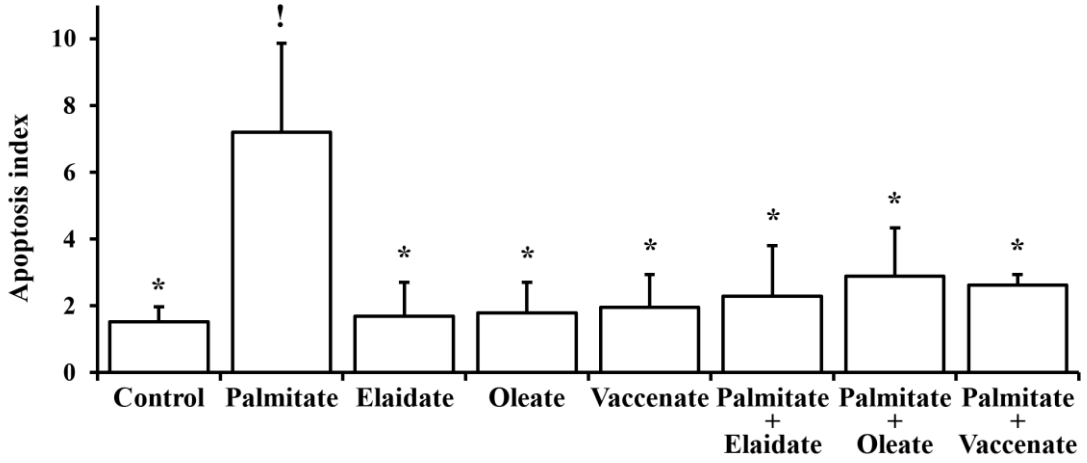

Figure 3. Apoptosis index. The cells were treated with BSA (control cells) or BSA-conjugated palmitate, elaidate, oleate or vaccenate alone or in combination with palmitate and one of the unsaturated FAs at 500 μM concentration for 8-hours at 70-80% confluence, in 96-well plates. Apoptosis index was calculated as the relative number of apoptotic cells and expressed as percentage of the total cell number. Apoptotic cells were detected and counted by annexin and propidium iodide staining and fluorescence microscopy. Typical microscopic images obtained in three independent experiments and data are shown in the diagram as mean values ± S.D. of three experiments; $^{!}$ $P < 0.05$, v.s. BSA-treated control; *$P < 0.05$, v.s. palmitate-treated cells.

By investigating the caspase-3 activation levels after the different FA treatments, further proofs can be found about the effects of different FAs on apoptosis. As expected based on the results shown above, the amount of cleaved caspase-3 was found to increase at least 10-fold in palmitate-treated cells compared to control levels (Fig. 4). The single treatment with one of the unsaturated FAs revealed a slightly higher level of caspase-3 than the control cells. The difference was most noticeable, but still not significant, in case of vaccenate (Fig. 4). The cells co-treated with palmitate and one of the unsaturated FAs contained lower amount of the cleaved caspase-3 enzymes compared to palmitate alone treatment. Oleate caused a remarkable, 55% decrease in the cleaved caspase-3 levels compared to palmitate (Fig. 4). While in the case of elaidate a still significant 40%

decrease can be observed, the co-treatment with vaccenate caused a 25% decrease in the enzyme levels, which was deemed non-significant by the statistical analysis (Fig. 4).

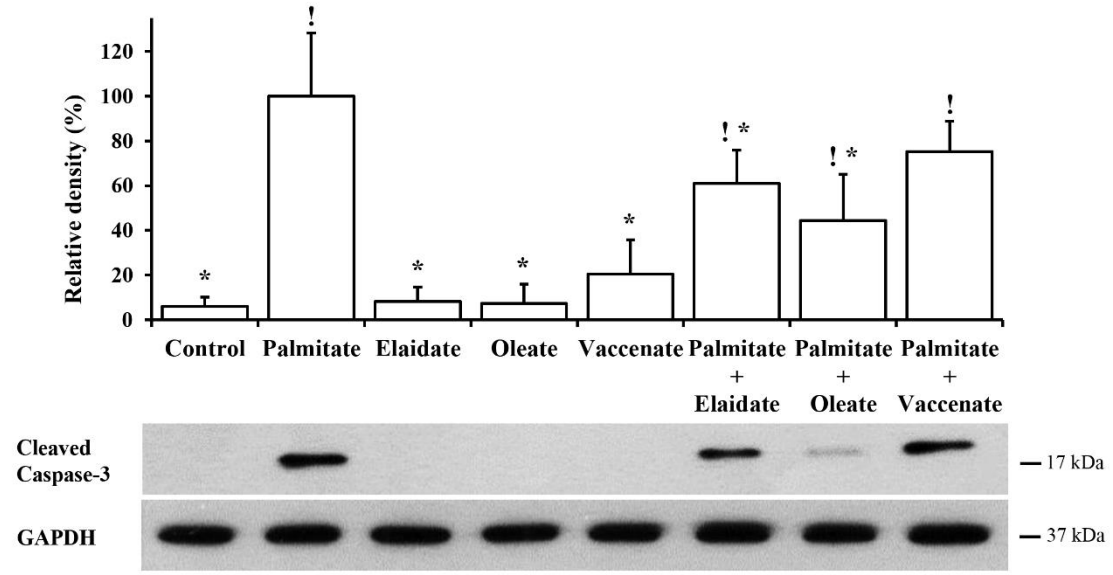

Figure 4. Cleaved Caspase-3 levels. The cells were treated with BSA (control cells) or BSA-conjugated palmitate, elaidate, oleate or vaccenate alone or in combination with palmitate and one of the unsaturated FAs at 500 μM concentration for 8-hours at 70-80% confluence, in 6-well plates. Cleaved Caspase-3 was detected by western blot in cell lysates. The results were quantified by densitometry, normalized to GAPDH as a constitutive reference protein and are shown as relative band densities in the percentage of palmitate values. The western blot image shows typical results of three independent experiments with two parallels. Data are shown in the diagram as mean values ± S.D. of three experiments; ! $P < 0.05$, v.s. BSA-treated control; *$P < 0.05$, v.s. palmitate-treated cells.

3.3. Induction of ER stress

Lipotoxicity is a complex process, which involves numerous signaling pathways, some of which belong to the cellular mechanism of ER stress. The earliest momentum of the UPR involves the excision of 26 nucleotides from the so-called unspliced version of XBP-1 (uXBP-1) mRNA, a process catalyzed by the IRE-1 ER stress receptor. The resulting spliced (sXBP-1) mRNA codes for a transcription factor and it is also a specific marker of the ER stress and the UPR. For better visualization, the IRE-1 mediated splicing was detected by endonuclease cleavage. After palmitate treatment, an enhanced splicing was observed, resulting in a more than 5-fold increase in the sXBP-1/uXBP-1 ratio, in turn, the cis- and trans-unsaturated FAs only caused a minor and non-significant elevation

of the ratio compared to control (Fig. 5A). The addition of oleate next to palmitate caused the ratio to diminish by almost 50% compared to palmitate alone. The TFA co-treatment also resulted in a moderation of sXBP-1/uXBP-1 ratio, where elaidate proved to be the less effective, resulting in a significant 30% decrease, while vaccenate caused an almost 40% lessening in the ratio compared to palmitate (Fig. 5A).

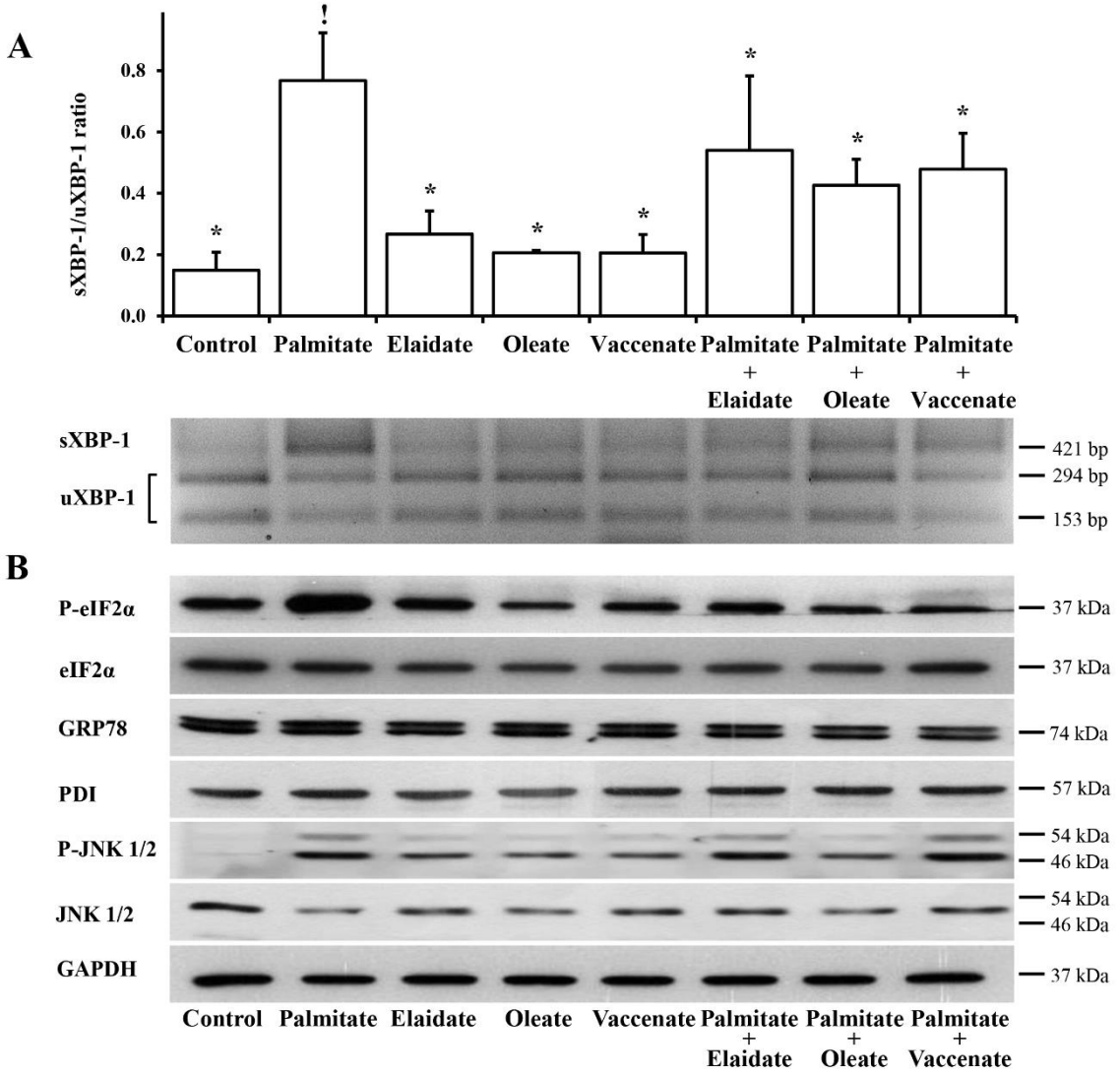

Figure 5. Endoplasmic reticulum stress markers. The cells were treated with BSA (control cells) or BSA-conjugated palmitate, elaidate, oleate or vaccenate alone or in combination with palmitate and one of the unsaturated FAs at 500 μM concentration for 8-hours at 70-80% confluence, in 6-well plates. (**A**) After treatment total RNA was prepared and a 447 and a 421 bp long sequence was amplified by RT-PCR from sXBP-1 and uXBP-1 mRNA, respectively. For better visualization the two versions were further digested by PstI restriction endonuclease, which leaves the spliced cDNA uncut (421), while cleaves the unspliced cDNA to two fragments (153 and 294 bp). The digested samples cDNAs were separated by 2% agarose gel electrophoreses, the band densities were quantified by densitometry, and sXBP-1/uXBP-1 density ratios were calculated. A

typical gel image obtained in one of three independent experiments is presented. (**B**) From the samples phosphorylated eIF2α (P-eIF2α), 78 kDa glucose-regulated protein (GRP78/BiP), protein disulfide isomerase (PDI), phosphorylated SAPK/JNK isoforms (P-JNK 1/2) protein levels were detected by western blot. The western blot images show typical results of three independent experiments. The results were quantified by densitometry, normalized to overall GAPDH levels and are presented as relative band densities in the percentage of palmitate-treated samples. Data are shown as mean values ± S.D. of three experiments; ! P <0.05, v.s. BSA-treated control; *P <0.05, v.s. palmitate-treated cells.

The other major signalization pathway of the ER stress is closely associated to PERK. Thus, as a PERK-mediated ER stress marker, the eIF2α phosphorylation was assessed, by comparing the levels of phosphorylated and overall eIF2- α levels. Palmitate caused a significant increase in the P-eIF2α levels compared to control cells, while the single treatments with unsaturated FAs were deemed insignificant (Fig. 5B). After the co-treatment with unsaturated FAs, a remarkable decrease could be observed in case of the cis-unsaturated oleate as it diminished the phosphorylated eIF2α amount by almost 70%. The trans-unsaturated vaccenate was almost as potent as oleate, while the trans-elaidate treatment resulted in a 25% symbolic, but non-significant decrease in the P-eIF2α levels (Fig. 5B). As the stress conditions remain for a longer period of time, the expression of ER chaperons grows as an important adaptive element of the UPR. The chaperon development comes with a delay as generally the transcriptional regulations do. As long-term ER stress markers, two main ER chaperons, the BiP and PDI were investigated by western blotting. In both cases, the 8-hour long FA treatment did not result in any elevation in the amount of the proteins mentioned above (Fig. 5B).

The JNK phosphorylation is connected to lipotoxicity because, as a major stress kinase among others, it is sensitive to oxidative and ER stress and it is also activated by inflammation and ceramide accumulation. The enzyme is activated by phosphorylation and helps in the development of insulin resistance and apoptosis. The isoforms of the activated JNK were detected by immunoblotting and as expected, the palmitate raised the phosphorylation of the JNK five-fold compared to control samples, while the three unsaturated FAs caused a much smaller increase in JNK phosphorylation (Fig. 5B). The co-treatment with the cis-unsaturated oleate remarkably decreased the amount of the activated JNK and the level was slightly, but not significantly higher than the control. Interestingly, however, the TFAs did not change the JNK phosphorylation significantly; nevertheless, elaidate caused a 40% decrease compared to palmitate treatment, but it was

proven to be non-significant by the statistical analysis, while vaccenate did not cause any notable changes (Fig. 5B).

3.4. Changes in the fatty acid profile of the cells

During a 24-hour long treatment, the incorporation of the FAs in question were observed in different times. After the extracellular FAs were removed, the FA profile of adherent cells was constructed with saponification and esterification of complex lipids followed by a gas chromatography coupled to flame ionization detector (GC-FID) measurement. The investigation proved that all of the FAs were incorporated into the cells efficiently, though with different time courses. Palmitate and oleate reached their maximum levels after 8 and 16-hours of incubation (Figs. 6 and 7).

In case of the single treatments, the trans-unsaturated elaidate and vaccenate topped at 24-hour (Fig. 6). The amount of palmitate and elaidate increased by about 150 μg/mg protein, that of oleate and vaccenate increased by about 100 μg/mg protein above the control levels. Since the cells contained substantial amounts (nearly 50 μg/mg protein) of palmitate and oleate originally, the maximum level of palmitate was the highest (above 200 μg/mg protein) and that of vaccenate was the lowest (below 100 μg/mg protein) among the 4 FAs added (Fig. 6).

Although the same concentration of palmitate was applied in single and co-treatments, intracellular amount of palmitate in co-treatments did not reach at any given time the values observed after palmitate alone treatment, and remained at around 65 μg/mg protein in all combinations (Fig. 7).

As a result of desaturation and elongation, an elevation can be observed in stearate and cis-palmitoleate (16:1 cis-Δ^9) levels after palmitate treatment. Interestingly, the addition of both TFAs caused an obvious accumulation of its degradation intermediate (16:1 trans-Δ^7 or 16:1 trans-Δ^9 in case of elaidate or vaccenate, respectively), which was in a striking contrast to oleate, as the concentration of 16:1 cis-Δ^7 FA did not increase in oleate-treated cells (Fig. 6). The increase of degradation intermediates can be detected in case of the TFA co-treatment as well (Fig. 7).

DOI:10.14753/SE.2020.2425

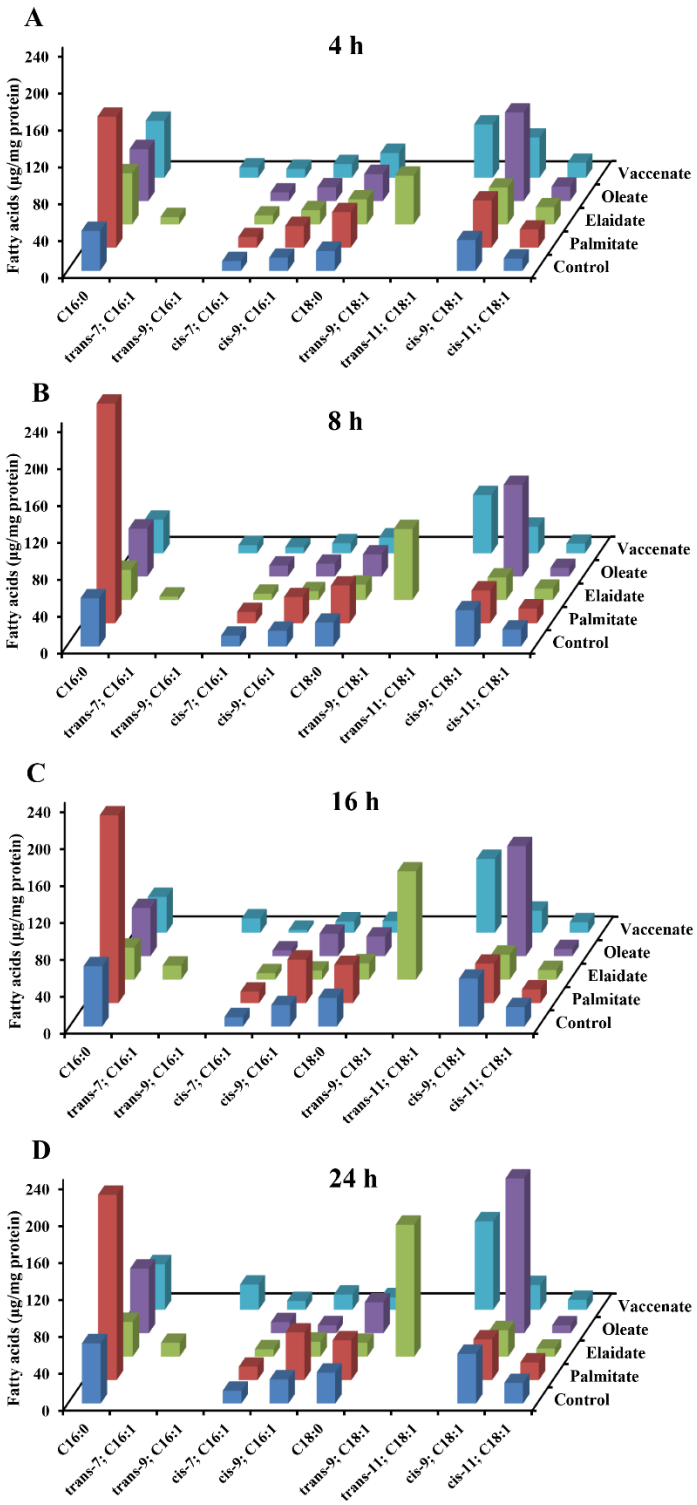

Figure 6. Fatty acid profile of RINm5F cells after single treatments. The cells were treated with palmitate, elaidate, oleate or vaccenate (250 µM) at 70-80% confluence, in 6-well plates. Cell samples were withdrawn at 4, 8, 16 and 24 h times of incubation, and the amount of 10 different saturated and mono-unsaturated FAs was measured by GC-FID after saponification and methylation. Data were analyzed of three independent experiments with two parallels and normalized to the total protein content of the samples.

DOI:10.14753/SE.2020.2425

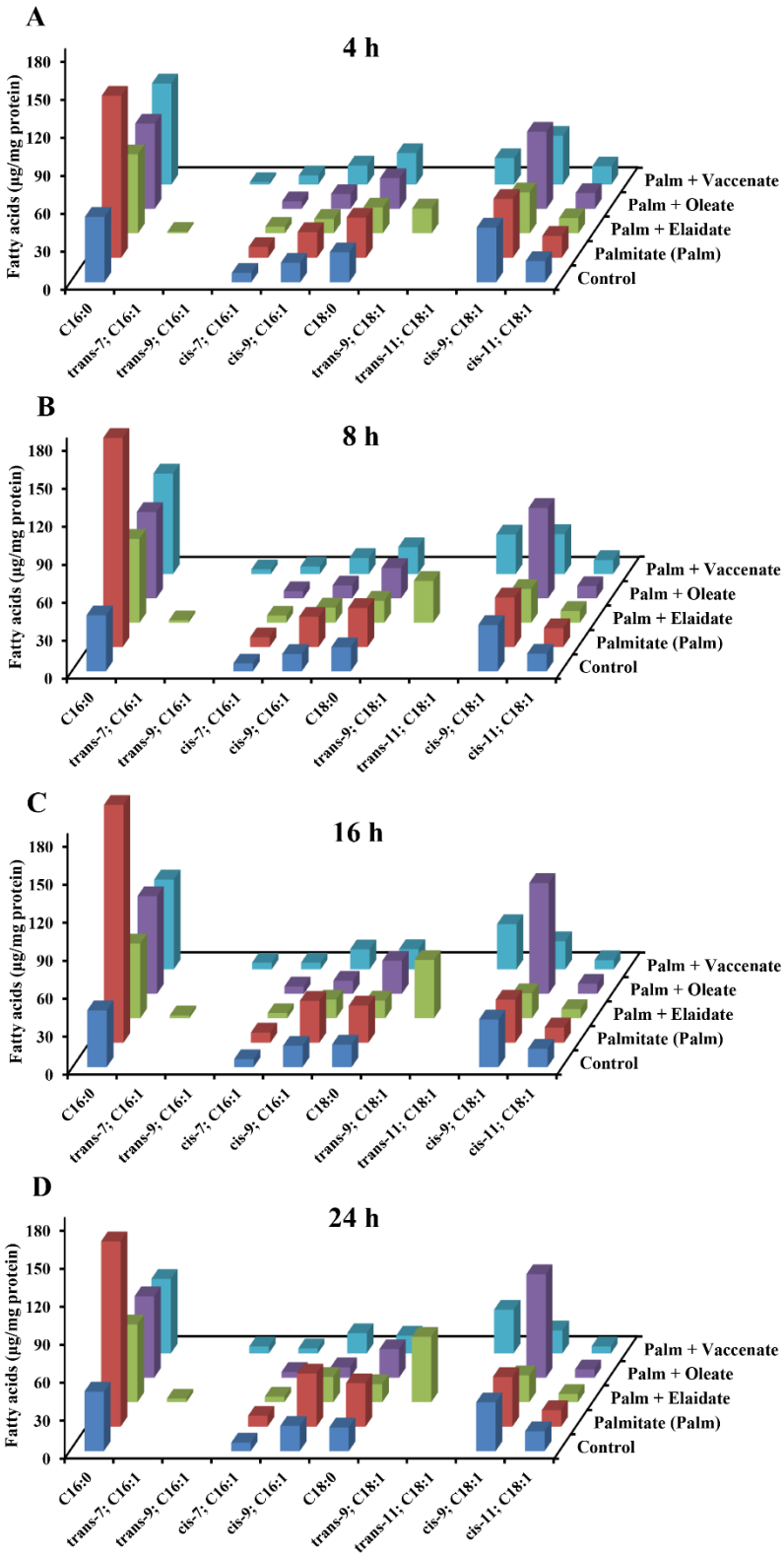

Figure 7. Fatty acid profile of RINm5F cells after co-treatments. The cells were treated with palmitate or in combination with palmitate and elaidate, oleate or vaccenate (250 µM) at 70-80% confluence, in 6-well plates. Cell samples were withdrawn at 4, 8, 16 and 24-hour times of incubation, and the amount of 10 different saturated and mono-unsaturated FAs was measured by GC-FID after saponification and methylation. Data

were analyzed of three independent experiments with two parallels and normalized to the total protein content of the samples.

3.5. Diglyceride levels

The excess acyl-CoA can cause an increased ER stress and apoptotic tendency, which is detrimental, especially for β-cells. Triglyceride synthesis is an efficient way for the cells to eliminate the acyl-CoA surplus, but an uneven saturated and unsaturated FA supply can result in a DG build-up. The LC-MS/MS measurement revealed an increase in the amount of the most important DG types containing 16 and 18 long carbon chains from saturated and mono-unsaturated FAs. In case of the palmitate treatment, the most outstanding elevation can be observed after 8-hour long treatment, where the levels of 1,2-dipalmitoyl-glycerol (16:0/16:0), 1-palmitoyl-2-stearoyl-glycerol (16:0/18:0), 1,2-distearoyl-glycerol (18:0/18:0), 1-palmitoyl-2-palmitoleoyl-glycerol (16:0/16:1) were increased by at least 45-fold, 18-fold, 8-fold and 5-fold, respectively. A gentle elevation can be observed in the concentration of 1-palmitoyl-2-oleoyl-glycerol (16:0/18:1), while the levels of 1-stearoyl-2-oleoyl-glycerol (18:0/18:1) and 1,2-dioleoyl-glycerol (18:1/18:1) remained practically unaffected. Altogether the palmitate treatment caused a 17-fold increase in the overall DG levels compared the control cells (Figs. 8 and 9).

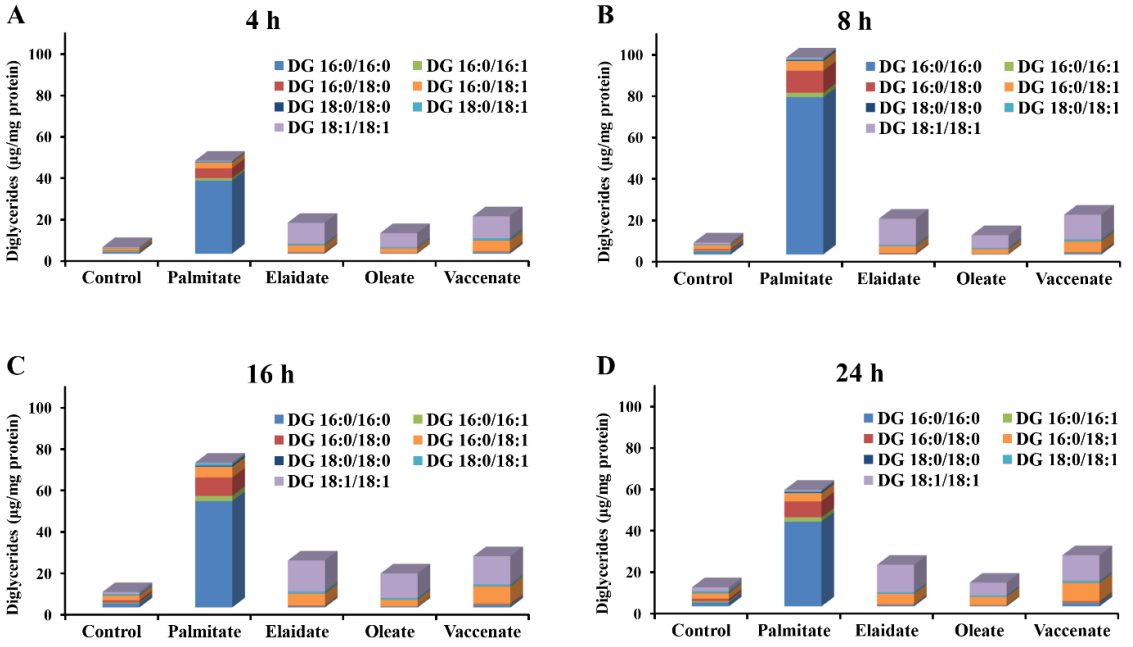

Figure 8. Diglyceride accumulation after single fatty acid treatments. The cells were treated with palmitate, elaidate, oleate or vaccenate (250 µM) at 70-80% confluence, in 6 well plates. Cell samples were withdrawn at 4, 8, 16 and 24 h times of incubation, and

the amount of 7 major diglycerides was measured by LC-MS/MS. Data were normalized to the total protein content of the samples, and are shown as mean values of three independent experiments with two parallels. S.D. was below 10% of the mean values in all cases.

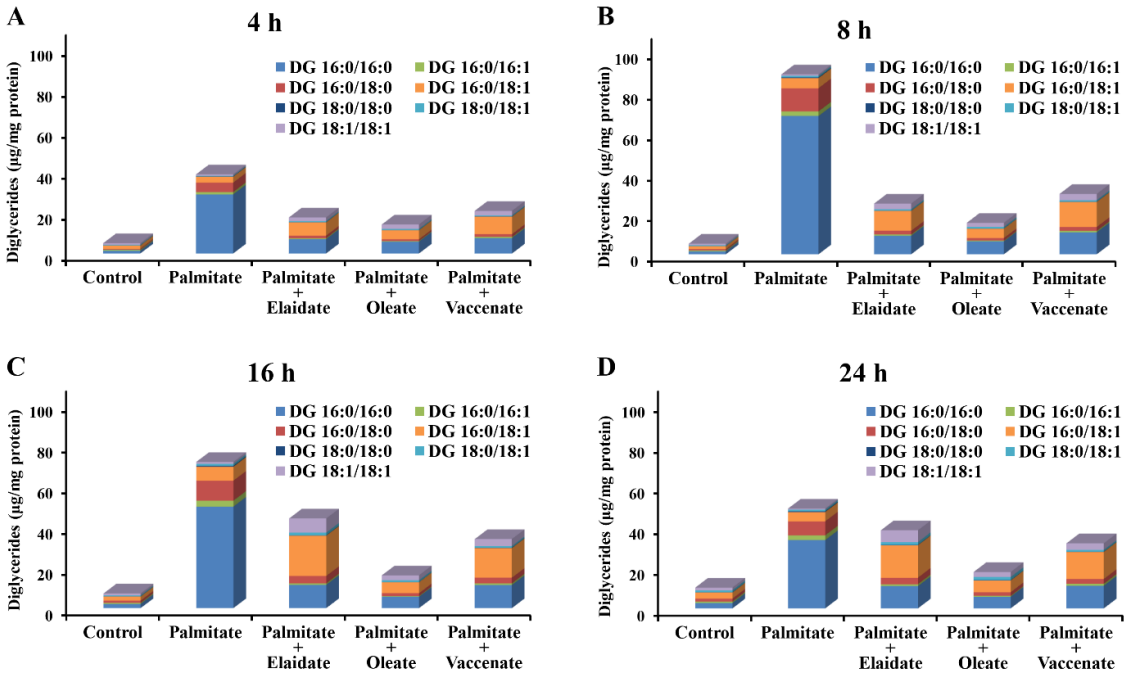

Figure 9. Effect of unsaturated fatty acid co-treatment on diglyceride accumulation. The cells were treated with palmitate or in combination with palmitate and elaidate, oleate or vaccenate (250 µM) at 70-80% confluence, in 6-well plates. Cell samples were withdrawn at 4, 8, 16 and 24 h times of incubation, and the amount of 7 major diglycerides was measured by LC-MS/MS. Data were normalized to the total protein content of the samples, and are shown as mean values of three independent experiments with two parallels. S.D. was below 10% of the mean values in all cases.

The effects of oleate treatment differed greatly from those of palmitate. The increase in overall DG level was the largest at 4 h of oleate treatment, when its amount tripled (9.9 µg/mg protein v.s. 3.2 µg/mg protein in oleate-treated and control, respectively). This change was caused by an 18-fold increase in 1,2-dioleoyl-glycerol (18:1/18:1) and a 2-fold increase in 1-palmitoyl-2-oleoyl-glycerol (16:0/18:1) and 1-stearoyl-2-oleoyl-glycerol (18:0/18:1) content (Fig. 8). It is also notable that the amounts of those four DGs, i.e., 1,2-dipalmitoyl-glycerol (16:0/16:0), 1-palmitoyl-2-stearoyl-glycerol (16:0/18:0), 1,2-distearoyl-glycerol (18:0/18:0) and 1-palmitoyl-2-palmitoleoyl-glycerol (16:0/16:1), which are the most markedly elevated in palmitate-treated cells, were found to be 5-10-fold decreased in oleate-treated cells at all times. More or less the same changes can be observed in the cells after TFA treatment. The overall DG amount stayed far below the level observed after palmitate treatment and was more similar to that

induced by oleate: they were increased 5-fold (14.8 μg/mg protein v.s. 3.2 μg/mg protein) and 6-fold (18.0 μg/mg protein v.s. 3.2 μg/mg protein) in case of elaidate and vaccenate, respectively at 4 h of incubation, and it was mostly due to a 27-28-fold increase in the levels of DGs containing only elaidate or vaccenate (18:1/18:1). The accumulation of 1-palmitoyl-2-elaidyl-glycerol and 1-palmitoyl-2-vaccenyl-glycerol was also more noticeable than that of 1-palmitoyl-2-oleoyl-glycerol (16:0/18:1) (Fig. 8).

An increase in the overall DG content in the palmitate plus oleate co-treatment was also observed, but the elevation was only two-fold, which was considerably lower than that seen after palmitate alone. Another noteworthy difference from single treatments is in the time-course and the pattern of the effect. The overall DG levels were kept stable during the experiment, and the contribution of DG species containing 18 carbon long unsaturated chains were similar to those containing only palmitoyl, palmitoleoyl and/or stearoyl groups (Fig. 9). Briefly, almost all of the DG levels showed a less pronounced increase during the co-treatment, and the biggest difference was observed at the levels of the two most abundant DGs, where a 10-times lower level compared to palmitate treated cells could be seen after 8-hour of combined FA incubation (68.3 μg/mg protein v.s. 6.3 μg/mg protein and 11.5 μg/mg protein v.s. 1,3 μg/mg protein). Interestingly, the concentration of the 1-stearoyl-2-oleoyl-glycerol (18:0/18:1) and 1,2-dioleoyl-glycerol (18:1/18:1) increased through the whole course of palmitate-oleate co-treatment, reaching their maximum at 1.5 μg/mg protein and 2.4 μg/mg protein, respectively (Fig. 9). Co-treatments with one of the two TFAs induced yet another course of DG accumulation The overall DG kept increasing and reached their maximum after 16-hour long incubation, where elaidate produced a 44.1 μg/mg protein and vaccenate a 34 μg/mg protein overall level, which is twice the amount compared to oleate. This difference is mostly caused by the 1,2-dipalmitoyl-glycerol (16:0/16:0), the 1-palmitoyl-2-oleoyl-glycerol (16:0/18:1) and the 1,2-dioleoyl-glycerol (18:1/18:1), as their levels got increased by more than 3 times in elaidate and approximately 2 times in vaccenate co-treatment, except the 1,2-dipalmitoyl-glycerol, where the levels doubled in both cases. Comparing the two TFAs to each other, it is apparent that the elaidate co-treatment produced more DGs in almost all cases and the amount of stearoyl-2-oleoyl-glycerol (18:0/18:1) is nearly twice of the concentration observed in vaccenate (Fig. 9).

3.6. Ceramide levels

Ceramides as toxic lipid intermediates, which are composed of one fatty acyl chain and a sphingosine, are closely related to lipotoxicity. Therefore, we determined the concentration of those ceramides, which contain one of the administered FAs by LC-MS/MS measurement. The most abundant ceramides in BSA-treated control cells were palmitoyl- and stearoyl-sphingosines (16:0 and 18:0) with about 1 µg/mg protein and 250 ng/mg protein levels, respectively, while oleoyl-sphingosine (18:1) was barely detectable (about 10 ng/mg protein) (Figs. 10 and 11). Palmitate treatment caused a 6-fold increase in the overall ceramide levels, compared to control cells, and the increase reached its maximum after an 8-hour long incubation. This change was due to palmitoyl- and stearoyl-sphingosines (16:0 and 18:0) as their levels increased by about 3.9 µg/mg protein and 2.1 µg/mg protein levels, respectively, while change in the oleoyl-sphingosine (18:1) was much smaller (about 14,8 ng/mg protein).

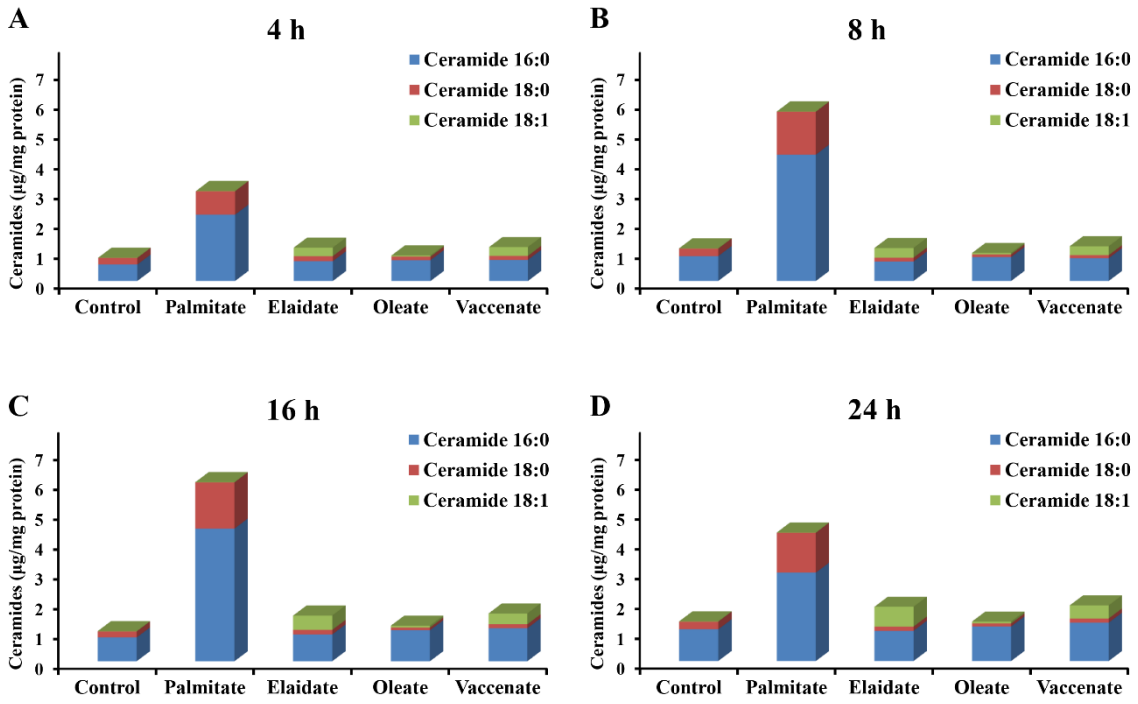

Figure 10. Changes in ceramide levels after single treatments. The cells were treated with palmitate, elaidate, oleate or vaccenate (250 µM) at 70-80% confluence, in 6-well plates. The amount of three different ceramide species was measured by LC-MS/MS in the cell samples prepared after 4, 8, 16 or 24 hour treatments. The detected ceramides contained either a palmitate (16:0) or a stearate (18:0) or a mono-unsaturated FA of 18 carbons (18:1). Data were normalized to the total protein content of the samples, and are shown as mean values of three independent experiments with two parallels. S.D. was below 10% of the mean values in all cases.

DOI:10.14753/SE.2020.2425

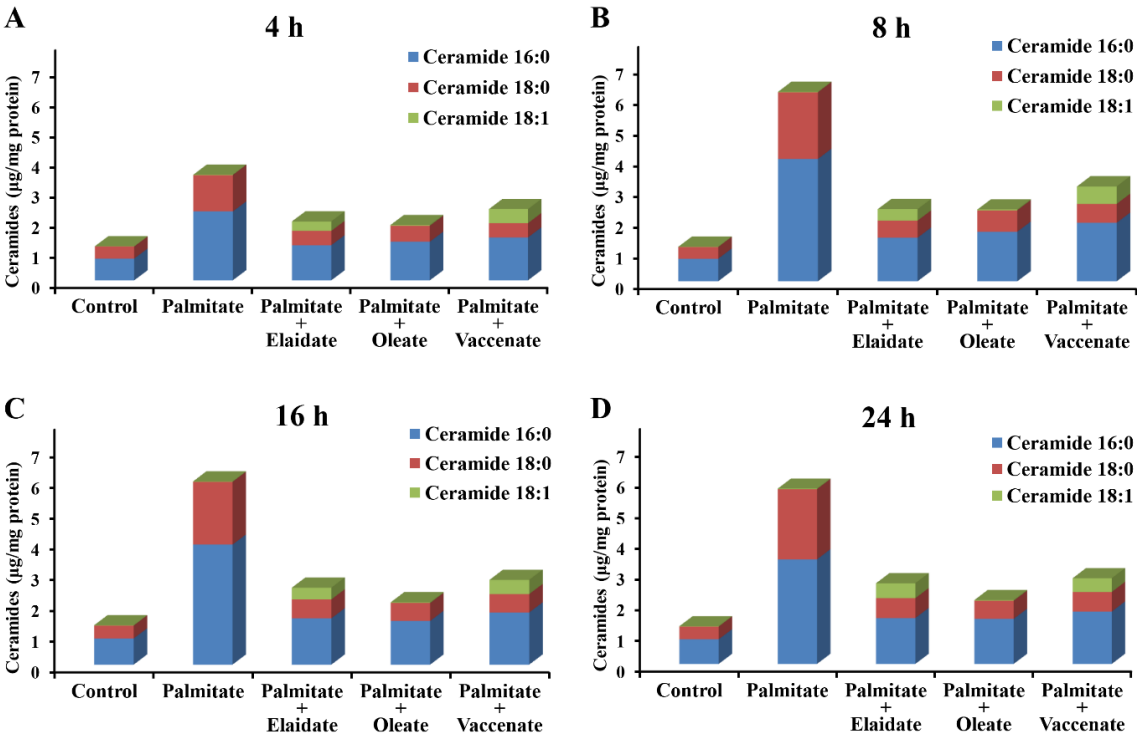

Figure 11. Effect of unsaturated fatty acid co-treatment on ceramide accumulation. The cells were treated with palmitate or in combination with palmitate and elaidate, oleate or vaccenate (250 µM) at 70-80% confluence, in 6-well plates. The amount of three different ceramide species was measured by LC-MS/MS in the cell samples prepared after 4, 8, 16 or 24 hour treatments. The detected ceramides contained either a palmitate (16:0) or a stearate (18:0) or a mono-unsaturated FA of 18 carbons (18:1). Data were normalized to the total protein content of the samples, and are shown as mean values of three independent experiments with two parallels. S.D. was below 10% of the mean values in all cases.

In line with its smaller cytotoxicity, oleate increased the overall ceramide level much less. Although the accumulation of oleoyl-sphingosine (18:1) can be observed in the cells (66.0 ng/mg protein v.s. 12.2 ng/mg protein at 24 h in oleate-treated v.s. control cells, respectively), the concentration of the most abundant ceramide species did not change (palmitoyl-sphingosine, 16:0) or decreased to less than half of control level (stearoyl-sphingosine, 18:0) (Fig. 10). The formation of elaidyl- and vaccenyl-sphingosines in TFA treated cells was an order of magnitude above that of oleoyl-sphingosine upon oleate treatment, which is a conspicuous difference between cis-oleate and the two TFAs. An increase of 658.4 ng/mg protein of elaidyl-sphingosine, 431.4 ng/mg protein of vaccenyl-sphingosine and 53.8 ng/mg protein of oleoyl-sphingosine was found at 24 h in the elaidate-, vaccenate- and oleate-treated cells, respectively. These

differences were also reflected by the alterations of the overall ceramide level, since the three unsaturated FAs had otherwise similar effects on the other investigated ceramide species. The amount of these ceramides nearly doubled in the TFA-treated cells and increased by only by 30% in oleate-treated cells in the longest incubations (Fig. 10).

The simultaneous addition of oleate to palmitate-treated cells caused a much smaller accumulation in the overall ceramide levels. Oleate mitigated the formation of palmitoyl- and stearoyl-sphingosines (16:0 and 18:0), however, despite the excess of oleate only a slight elevation (26.5 ng/mg protein) can be observed in case of the oleoyl-sphingosine (18:1) (Fig. 11). The TFAs, had a somehow similar effect like oleate, as they were almost as effective as oleate in the reduction of palmitoyl- and stearoyl-sphingosine (16:0 and 18:0) levels, but a much more pronounced elevation in elaidyl- and vaccenyl-sphingosines (18:1) was observed. Their amount reached to as high as 487 ng/mg protein in the case of elaidate, and 448 ng/mg protein concentration in the case of vaccenate co-treatments after 24 hours (Fig. 11).

4. Discussion

A well-balanced diet is a major contributing factor to health or disease. Some of the most relevant obesity-related pathologies are type 2 diabetes, the metabolic syndrome and cardiovascular diseases. Several components in the diet can modulate the development of these pathological conditions, such as dietary fats, specifically, saturated and mono-unsaturated FAs. Excessive palmitic acid, the most abundant FA in food, has been shown to be effective in causing ER stress [68] and, consequently, the accumulation of TGs rich in palmitic acid in the cells [69]. Evidence suggests a crucial role of palmitic acid in β-cell failure via serine phosphorylation of insulin receptor substrate (IRS) proteins [70]. This leads to the development of insulin resistance, which forces the β-cells to increase the degree of insulin secretion, which in turn leads to an adaptive ER stress in these cells. The increased demand and the presented lipotoxicity, which also affects the β-cells, coupled with the ER stress is a condition which could eventually lead to β-cell apoptosis [23]. While the different species of dietary FAs contribute differently to lipotoxicity, a great amount of experiments demonstrated that saturated FAs, particularly palmitic acid, are the most able to trigger lipotoxicity [33, 71, 72]. In comparison, unsaturated FAs are less toxic. Oleic acid is a mono-unsaturated FA, which is naturally formed in the human body by stearoyl-CoA desaturase [73, 74]. Just like that of palmitate, the toxicity of oleate has been thoroughly investigated and it has been proved that it is less pronounced compared to the former [18]. One of the configurational isomers of oleate is elaidate, which can be produced by industrial processing of plant oils, as some of the cis double bonds get rearranged to trans position during industrial hydrogenation. The global consumption of these products, such as margarine, drew attention to these TFAs. They have been branded as the worst type of FAs, which are at least partly responsible for the health risks associated with the consumption of fast-foods [59, 75]. Considering these hypotheses, some countries restricted or prohibited the TFA content of nutrition, despite that the observations are based on contradictory *in vivo* experiments, and in the lack of sufficient *in vitro* investigations [76, 77]. It is also interesting that the TFA produced by bacterial isomerization, namely vaccenate, which is consumed with dairy products such as butter and ruminant meat, is deemed natural and is excepted from the above mentioned restrictions [78, 79].

In our study, we compared the toxicity of the above mentioned two TFAs to two most typical saturated and cis-unsaturated FAs, palmitate and oleate, using a previously established cellular model of lipotoxicity in an insulinoma cell line [34]. Viability tests were undertaken by administering BSA-conjugated FAs to the cells in toxic concentrations (250 and 500 μM) for 24 h. In our experiment, the effects of palmitate were in line with the literature, as it decreased the viability of the cells by at least 60% and 85%, at the two concentrations respectively. The induction of apoptosis and ER stress was investigated at 500 μM concentration after 8 h of FA incubation. The toxic effects of palmitate were obvious and complied with the data previously observed. The unsaturated FAs were administrated in the same conditions and while the effects of oleate were similar to those already described in publications, interestingly the TFA treatments brought similar results to that of oleate regarding viability, apoptosis and ER stress, in single treatments. Even more noticeably, no conspicuous difference was observed between elaidate and vaccenate, despite the observations reported in *in vivo* studies.

Cellular FA uptake is determined primarily by the availability of FFA, while fatty acyl-CoA utilization is limited by the capacities of the relevant metabolic pathways, therefore FFA abundance leads to high levels of fatty acyl-CoA in the cells. Ceramides and DGs are biosynthetic intermediates that are implicated in lipotoxicity, especially in β-cells. Increased uptake of FFAs is a major mechanisms underlying triglyceride accumulation [80]. TG synthesis provides a major route to drain the intracellular acyl-CoA pool. However, the efficient completion of this pathway requires the insertion of an unsaturated FA at carbon-2 of glycerol, and hence TG synthesis is largely hindered by an overwhelming supply of saturated FAs [81]. This provides a plausible explanation for the higher toxicity of unbalanced saturated FAs. Ceramides represent a class of lipids which combines cell toxicity with ER stress and apoptosis. They are implicated in insulin resistance, inflammation, oxidative stress and cell death and may be generated *de novo* from serine and palmitoyl-CoA, via serine palmitoyltransferase [82]. An alternative pathway is based on the action of neutral sphingomyelinase, which catalyzes the release of ceramides from membrane sphingomyelin [83, 84].

Our investigation on palmitate-treated cells revealed an accumulation of DGs containing two saturated fatty acyl chains, which are rarely present in control cells. While the mono-unsaturated FAs evoked a much milder DG increase, and the accumulation of

DGs remained far behind the levels observed after palmitate treatment, the TFAs induced a much greater accumulation in the DG content compared to oleate, and the rise is mostly limited to DGs containing one or two chains of the given unsaturated FA. It is possible that the accumulation of these DGs is caused by an interference of TG synthesis, as the TFA chains might disturb the third acylation during the process, but the exact process is yet to be determined. Beside these changes, we observed a several-fold increase in the ceramide content after palmitate treatment. The unsaturated FAs, either cis or trans, caused a much smaller change in the overall ceramide levels, although the presence of ceramides containing TFA chains was noteworthy, and interestingly was much more pronounced than the production of oleoyl-sphingosine. To explain the difference, further studies are needed, but it can be due to a different affinity to sphingosine acyltransferase or a problem in the utilization of ceramides containing TFAs. Either way, a prolonged TFA consumption can lead to higher intracellular ceramide concentrations, which ultimately can cause a more prominent toxicity. Both in the toxicity and the incorporation into complex lipids, the TFAs in our investigations were found to be more similar to oleate than to palmitate. This seems to confirm the connection between the accumulation of ceramides and DGs and the intensity of cell damage. These findings do not support either the deleterious health effects of TFAs in general, or the often suggested difference between the industrial elaidate and the natural vaccenate. It is true, however, that diabetes is not the only pathology, which has been suspected to be associated to TFA consumption, and β-cells are not the only potential targets of TFA toxicity. Our study also revealed differences between the metabolism of the investigated unsaturated FAs, which were not reflected in the short-term toxicity but might be important in the long run for the health effects of chronic consumption of dietary FAs.

In a recent study, the incorporation of TFAs in cellular lipids has been reported. The investigation revealed that in endothelial (HUVEC) and hepatoma (HepG2) cells, the TFAs suppress TNF-α-induced inflammatory gene expression [85]. In our study, the FA profile revealed that the beneficial trans-palmitoleate (16:1 trans-Δ9) [61, 86], is not only ingested with food, but it is also produced endogenously as a partially degraded intermediate of vaccenate (18:1 trans-Δ11) [87]. It is noteworthy that vaccenate, the major TFA in ruminants, has been shown to reach high proportions also in industrially partially hydrogenated vegetable oils [88]. We also found elevated amount of trans-palmitoleate

(16:1 trans-Δ9) and its positional isomer (16:1 trans-Δ7), in the TFA-treated cells. They are the shortened derivatives of vaccenate and elaidate likely produced as intermediates of β-oxidation. However, in cells treated with oleate the analogous cis intermediate of β-oxidation (16:1 cis-Δ7) was not elevated in spite of higher intracellular oleate contents. A possible reason for this phenomenon might be due to a potentially different affinity of mitochondrial long-chain acyl-CoA dehydrogenase (LCAD) to cis or trans unsaturated FAs. The difference in the affinity of 5-trans-tetradecenoyl-CoA and 5-cis-tetradecenoyl-CoA to LCAD was shown in a study [89]. This might explain the poorer oxidation and greater accumulation of intermediates of TFAs compared to those of oleate.

The cis-unsaturated oleate has been repeatedly shown to reduce the damage caused by the saturated palmitate [81], so another goal of our investigation was to compare this ability to that of trans-unsaturated elaidate and vaccenate. The experimental parameters were the same as described above, while each of the unsaturated FAs were simultaneously administered with palmitate and at the same concentration. Regardless of the doubled FA dose, the cells were successfully rescued from cellular damage caused by palmitate after the co-treatment with oleate, as expected. The co-treatment with oleate protected the cells from lipoapoptosis and from stress, as shown by the lack of caspase-3 cleavage, the phosphorylation of JNK, the cleavage of XBP-1 mRNA and phosphorylation of eIF2α, as two crucial parts of the UPR. The co-treatment with oleate significantly reduced the levels of those ceramides that contain saturated FA chains. This shows that oleate is able to mitigate the ceramide accumulation caused by palmitate. Similar effect was seen in the DG accumulation, as the overall DG level was lower, just like the level of DGs containing saturated chains although the incorporation of oleate in this case was much more noticeable. All things considered, the protective effect of oleate was evident in our experiments, and it is in agreement with the data found in the literature.

The effect of TFA co-treatments were rather similar to that of oleate. One of the obvious differences was observed in the levels of phosphorylated JNK, which was not reduced as effectively, indicating remaining overall stress in the cells. Another interesting observation was that elaidate did not manage to attenuate the ER stress as successfully as the other two unsaturated FAs, but the viability and apoptosis analysis did not show such differences. Although the TFAs prevented the accumulation of ceramides containing saturated FA chains and minimized the overall ceramide accumulation like oleate, an

explicit amount of unsaturated ceramides was detected after TFA co-treatment. The described reduction of ceramide levels corresponded well with decreased intensity of apoptosis. In the first 8 h of the incubation with FAs, a similar observation can be made in the DG content, but in later times, the total DG levels after TFA co-treatment approached the amounts observed in palmitate treatments. This phenomenon is partly caused by the increase in DGs containing TFA chains. Based on these observations, we believe that the accumulation of ceramides is an important step in the development of β-cell lipotoxicity and lipoapoptosis. These results also indicate that TFAs are not the most harmful type of FAs, at least in concern with β-cells and diabetes.

As previously alluded, the TG synthesis is a possible way for the cells to drain acyl-CoA surplus. Some studies suggest that the outstanding toxicity of saturated FAs is due to the inability of the cells to process fully saturated DGs [4]. Based on these observations some theories suggest that the ability of unsaturated FAs to mitigate palmitate toxicity is due to their help for the TG synthesis to proceed. These assumptions are supported by experimental data obtained in different cell types, such as CHO cells and primary mouse embryonic fibroblasts [4], mouse myoblasts [90], human mesenchymal stromal cells and osteoblasts [65]. On the other hand, palmitate treatment in HepG2 cells resulted in controversial data regarding the deleterious or protective role of TG deposition in β-cell lipotoxicity [64, 91, 92]. After the comparison of FA profiles, our data suggest, that this assumed mechanism might not play a central role in the protective effect of the investigated unsaturated FAs in RINm5F cells, since the incorporation of palmitate into the cells decreased after co-treatments. The observed difference was almost as much as the increase in the amount of unsaturated FAs. These findings suggest that the reduction in ceramide and DG accumulation can be attributed, at least partly, to a lower palmitate uptake in the co-treated cells. The possible interference between the cellular uptake of different FAs and/or the saturation of FA uptake in our experimental conditions deserves further investigation.

5. Conclusions

The toxic effect of saturated FAs, such as palmitate, is widely accepted, and so is the advantage of mono- and polyunsaturated FAs. The ability of cis unsaturated FAs to attenuate palmitate toxicity was also described previously. However, neither the toxicity nor the possible protective effect of dietary TFAs was properly addressed and clarified. During our investigation, we wanted to deepen our knowledge, and to suggest some explanation on the contradictory in vivo data on the effects of elaidate and vaccenate. Based on our results, the conclusions are:

- Compared to the two most studied endogenous FAs, the toxicity of the two major dietary TFAs (i.e., elaidate and vaccenate) was below that of saturated palmitate, but slightly above that of the unsaturated oleate.

- All of the FAs were incorporated and metabolized by the cell; however, there is a significant difference between the metabolism of cis and trans unsaturated FAs as, unlike that of oleate, the products of the first step of β-oxidation of TFAs were obviously accumulated.

- The accumulation of ceramides and DGs after the addition of any of the unsaturated FAs remained far below that induced by palmitate. The incorporation of oleate into ceramides was inconspicuous. Either of the investigated TFAs induced a ceramide and DG accumulation, which was significantly greater than that induced by oleate. These results strengthen the correlation between ceramide and DG accumulation and the intensity of cell damage.

- The simultaneous addition of oleate with palmitate reduced the cellular damage caused by the latter. The co-treatment with TFAs exerted an almost similar protection against the deleterious effects of the saturated FA, nevertheless the phosphorylated JNK levels were slightly higher, indicating an elevated stress intensity in the cells, and the attenuation of ER stress by elaidate was weaker compared to the other two unsaturated FAs.

- The presence of oleate reduced the amounts of ceramides and DGs containing saturated FAs chains remarkably in co-treatments. While the TFAs also reduced the ceramide and DGs levels in co-treatments, over the time, the DG levels reached a remarkably high amount, which began to approach the levels observed in palmitate

treatments. These data further support the role of ceramides in β-cell lipotoxicity and they also indicate an important difference between the metabolism of oleate and the TFAs.

TFA consumption is not only related to diabetes, and its role is suspected also in cardiovascular diseases or other metabolic disorders. Because of this, other cell lines which are possibly targets of TFA toxicity should be investigated through similar experiments. We observed remarkable metabolic differences between TFAs and oleate. These effect were not reflected in the short-term toxicity, but considering the fact, that the consumption of dietary FAs is a long-term condition, these differences may have important roles in the health effects over the time.

DOI:10.14753/SE.2020.2425

6. Summary

Permanently high free FA levels are deleterious to many cell types including the insulin producing pancreatic β-cells. This lipotoxicity is largely due to an increased intracellular acyl-CoA supply and the consequent accumulation of biosynthetic lipid intermediates, such as ceramides and diglycerides, which in turn causes ER stress and apoptosis. Toxicity of the saturated palmitate (16:0) exceeds that of cis-unsaturated oleate (18:1 cis-Δ^9), moreover simultaneous addition of oleate can decrease the effect of palmitate. While the toxicity of and interaction between palmitate and oleate have been widely investigated, very little data is available on the cell damages caused by elaidate (18:1 trans-Δ^9) and vaccenate (18:1 trans-Δ^{11}), although the potential health effects of these dietary TFAs received great publicity. We aimed to compare the effects of these four FAs at high concentrations (250-500 μM) on cell viability, apoptosis, ER stress, JNK phosphorylation alone or in combination treatment, in RINm5F insulinoma cells. Changes in the overall FA profile and the ceramide and diglyceride contents of the cells were also determined by using GC-FID and LC-MS/MS analysis. We observed a marked toxicity of palmitate, which was attenuated by a simultaneous addition of oleate. In spite of their efficient uptake and incorporation in the cells, the two TFAs were found to be scarcely toxic in our experiments. Similarly to oleate and unlike palmitate, they reduced cell viability only at higher concentration, and their impact on ER stress, apoptosis and autophagy was not significant. Palmitate also caused a several fold increase in both ceramide and diglyceride levels, while much smaller elevations were induced by the unsaturated FAs, either cis or trans; however, the incorporation of TFAs in ceramides was strikingly more pronounced compared to oleate. These results show an obvious correlation between the severity of cell damage and the accumulation of lipid intermediates caused by different FA species. This pioneering study on cellular effects of TFAs does not support a short term toxicity of these dietary compounds in insulinoma cells; nevertheless, it revealed some metabolic characteristics that might underlie a long term toxicity.

7. References

1. Alves-Bezerra, M. & Cohen, D. E. (2017) Triglyceride Metabolism in the Liver, *Compr Physiol.* **8**, 1-8.
2. Kazantzis, M. & Stahl, A. (2012) Fatty acid transport proteins, implications in physiology and disease, *Biochim Biophys Acta.* **1821**, 852-857.
3. Fain, J. N. (2006) Release of interleukins and other inflammatory cytokines by human adipose tissue is enhanced in obesity and primarily due to the nonfat cells, *Vitam Horm.* **74**, 443-477.
4. Zámbó, V., Simon-Szabó, L., Szelényi, P., Kereszturi, E., Bánhegyi, G. & Csala, M. (2013) Lipotoxicity in the liver, *World J Hepatol.* **5**, 550-557.
5. Csala, M. (2016) [Hyper-free fatty acidemia - insulin resistance and β-cell death], *Orv Hetil.* **157**, 733-739.
6. Ye, J. (2013) Mechanisms of insulin resistance in obesity, *Front Med.* **7**, 14-24.
7. Volpe, C. M. & Nogueira-Machado, J. A. (2013) The dual role of free fatty acid signaling in inflammation and therapeutics, *Recent Pat Endocr Metab Immune Drug Discov.* **7**, 189-197.
8. Yin, J., Peng, Y., Wu, J., Wang, Y. & Yao, L. (2014) Toll-like receptor 2/4 links to free fatty acid-induced inflammation and beta-cell dysfunction, *J Leukoc Biol.* **95**, 47-52.
9. Papachristodoulou, D. (2019) Energy metabolism in *Medical Sciences* (Jeannette Naish, D. S. C., ed) pp. 57-102, Elsevier, Poland.
10. Febbraio, M. & Silverstein, R. L. (2007) CD36: implications in cardiovascular disease, *Int J Biochem Cell Biol.* **39**, 2012-2030.
11. Xu, S., Nam, S. M., Kim, J. H., Das, R., Choi, S. K., Nguyen, T. T., Quan, X., Choi, S. J., Chung, C. H., Lee, E. Y., Lee, I. K., Wiederkehr, A., Wollheim, C. B., Cha, S. K. & Park, K. S. (2015) Palmitate induces ER calcium depletion and apoptosis in mouse podocytes subsequent to mitochondrial oxidative stress, *Cell Death Dis.* **6**, e1976.
12. Watson, S. P. & Lapetina, E. G. (1985) 1,2-Diacylglycerol and phorbol ester inhibit agonist-induced formation of inositol phosphates in human platelets: possible implications for negative feedback regulation of inositol phospholipid hydrolysis, *Proc Natl Acad Sci U S A.* **82**, 2623-2626.

13. Moore, P. C., Ugas, M. A., Hagman, D. K., Parazzoli, S. D. & Poitout, V. (2004) Evidence against the involvement of oxidative stress in fatty acid inhibition of insulin secretion, *Diabetes.* **53**, 2610-2616.
14. Jayadev, S., Liu, B., Bielawska, A. E., Lee, J. Y., Nazaire, F., Pushkareva, M., Obeid, L. M. & Hannun, Y. A. (1995) Role for ceramide in cell cycle arrest, *J Biol Chem.* **270**, 2047-2052.
15. Venable, M. E., Lee, J. Y., Smyth, M. J., Bielawska, A. & Obeid, L. M. (1995) Role of ceramide in cellular senescence, *J Biol Chem.* **270**, 30701-30708.
16. Shimabukuro, M., Zhou, Y. T., Levi, M. & Unger, R. H. (1998) Fatty acid-induced beta cell apoptosis: a link between obesity and diabetes, *Proc Natl Acad Sci U S A.* **95**, 2498-2502.
17. Zhu, Q., Yang, J., Zhu, R., Jiang, X., Li, W., He, S. & Jin, J. (2016) Dihydroceramide-desaturase-1-mediated caspase 9 activation through ceramide plays a pivotal role in palmitic acid-induced HepG2 cell apoptosis, *Apoptosis.* **21**, 1033-1044.
18. Boslem, E., MacIntosh, G., Preston, A. M., Bartley, C., Busch, A. K., Fuller, M., Laybutt, D. R., Meikle, P. J. & Biden, T. J. (2011) A lipidomic screen of palmitate-treated MIN6 beta-cells links sphingolipid metabolites with endoplasmic reticulum (ER) stress and impaired protein trafficking, *Biochem J.* **435**, 267-276.
19. Veret, J., Coant, N., Berdyshev, E. V., Skobeleva, A., Therville, N., Bailbe, D., Gorshkova, I., Natarajan, V., Portha, B. & Le Stunff, H. (2011) Ceramide synthase 4 and de novo production of ceramides with specific N-acyl chain lengths are involved in glucolipotoxicity-induced apoptosis of INS-1 beta-cells, *Biochem J.* **438**, 177-189.
20. Galadari, S., Rahman, A., Pallichankandy, S., Galadari, A. & Thayyullathil, F. (2013) Role of ceramide in diabetes mellitus: evidence and mechanisms, *Lipids Health Dis.* **12**, 98.
21. Pagadala, M., Kasumov, T., McCullough, A. J., Zein, N. N. & Kirwan, J. P. (2012) Role of ceramides in nonalcoholic fatty liver disease, *Trends Endocrinol Metab.* **23**, 365-371.

22. Konstantynowicz-Nowicka, K., Harasim, E., Baranowski, M. & Chabowski, A. (2015) New evidence for the role of ceramide in the development of hepatic insulin resistance, *PLoS One.* **10**, e0116858.
23. Chandra, J., Zhivotovsky, B., Zaitsev, S., Juntti-Berggren, L., Berggren, P. O. & Orrenius, S. (2001) Role of apoptosis in pancreatic beta-cell death in diabetes, *Diabetes.* **50 Suppl 1**, S44-47.
24. Ishizuka, N., Yagui, K., Tokuyama, Y., Yamada, K., Suzuki, Y., Miyazaki, J., Hashimoto, N., Makino, H., Saito, Y. & Kanatsuka, A. (1999) Tumor necrosis factor alpha signaling pathway and apoptosis in pancreatic beta cells, *Metabolism: clinical and experimental.* **48**, 1485-1492.
25. Welsh, N. (1996) Interleukin-1 beta-induced ceramide and diacylglycerol generation may lead to activation of the c-Jun NH2-terminal kinase and the transcription factor ATF2 in the insulin-producing cell line RINm5F, *J Biol Chem.* **271**, 8307-8312.
26. Mandl, J., Meszaros, T., Banhegyi, G., Hunyady, L. & Csala, M. (2009) Endoplasmic reticulum: nutrient sensor in physiology and pathology, *Trends Endocrinol Metab.* **20**, 194-201.
27. Yilmaz, E. (2017) Endoplasmic Reticulum Stress and Obesity, *Adv Exp Med Biol.* **960**, 261-276.
28. Pincus, D., Chevalier, M. W., Aragon, T., van Anken, E., Vidal, S. E., El-Samad, H. & Walter, P. (2010) BiP binding to the ER-stress sensor Ire1 tunes the homeostatic behavior of the unfolded protein response, *PLoS Biol.* **8**, e1000415.
29. Gardner, B. M. & Walter, P. (2011) Unfolded proteins are Ire1-activating ligands that directly induce the unfolded protein response, *Science.* **333**, 1891-1894.
30. Credle, J. J., Finer-Moore, J. S., Papa, F. R., Stroud, R. M. & Walter, P. (2005) On the mechanism of sensing unfolded protein in the endoplasmic reticulum, *Proc Natl Acad Sci U S A.* **102**, 18773-18784.
31. Walter, P. & Ron, D. (2011) The unfolded protein response: from stress pathway to homeostatic regulation, *Science.* **334**, 1081-1086.

32. Karaskov, E., Scott, C., Zhang, L., Teodoro, T., Ravazzola, M. & Volchuk, A. (2006) Chronic palmitate but not oleate exposure induces endoplasmic reticulum stress, which may contribute to INS-1 pancreatic beta-cell apoptosis, *Endocrinology.* **147**, 3398-3407.
33. Cunha, D. A., Hekerman, P., Ladriere, L., Bazarra-Castro, A., Ortis, F., Wakeham, M. C., Moore, F., Rasschaert, J., Cardozo, A. K., Bellomo, E., Overbergh, L., Mathieu, C., Lupi, R., Hai, T., Herchuelz, A., Marchetti, P., Rutter, G. A., Eizirik, D. L. & Cnop, M. (2008) Initiation and execution of lipotoxic ER stress in pancreatic beta-cells, *J Cell Sci.* **121**, 2308-2318.
34. Simon-Szabo, L., Kokas, M., Mandl, J., Keri, G. & Csala, M. (2014) Metformin attenuates palmitate-induced endoplasmic reticulum stress, serine phosphorylation of IRS-1 and apoptosis in rat insulinoma cells, *PLoS One.* **9**, e97868.
35. Lei, K. & Davis, R. J. (2003) JNK phosphorylation of Bim-related members of the Bcl2 family induces Bax-dependent apoptosis, *Proc Natl Acad Sci U S A.* **100**, 2432-2437.
36. Harding, H. P., Zhang, Y. & Ron, D. (1999) Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase, *Nature.* **397**, 271-274.
37. Shi, Y., Vattem, K. M., Sood, R., An, J., Liang, J., Stramm, L. & Wek, R. C. (1998) Identification and characterization of pancreatic eukaryotic initiation factor 2 alpha-subunit kinase, PEK, involved in translational control, *Mol Cell Biol.* **18**, 7499-7509.
38. Ameri, K. & Harris, A. L. (2008) Activating transcription factor 4, *Int J Biochem Cell Biol.* **40**, 14-21.
39. Su, N. & Kilberg, M. S. (2008) C/EBP homology protein (CHOP) interacts with activating transcription factor 4 (ATF4) and negatively regulates the stress-dependent induction of the asparagine synthetase gene, *J Biol Chem.* **283**, 35106-35117.
40. Ye, J., Rawson, R. B., Komuro, R., Chen, X., Dave, U. P., Prywes, R., Brown, M. S. & Goldstein, J. L. (2000) ER stress induces cleavage of membrane-bound ATF6 by the same proteases that process SREBPs, *Mol Cell.* **6**, 1355-1364.

41. Hong, M., Luo, S., Baumeister, P., Huang, J. M., Gogia, R. K., Li, M. & Lee, A. S. (2004) Underglycosylation of ATF6 as a novel sensing mechanism for activation of the unfolded protein response, *J Biol Chem.* **279**, 11354-11363.
42. Martindale, J. J., Fernandez, R., Thuerauf, D., Whittaker, R., Gude, N., Sussman, M. A. & Glembotski, C. C. (2006) Endoplasmic reticulum stress gene induction and protection from ischemia/reperfusion injury in the hearts of transgenic mice with a tamoxifen-regulated form of ATF6, *Circ Res.* **98**, 1186-1193.
43. Lee, A. S. (2005) The ER chaperone and signaling regulator GRP78/BiP as a monitor of endoplasmic reticulum stress, *Methods.* **35**, 373-381.
44. Oakes, S. A. & Papa, F. R. (2015) The role of endoplasmic reticulum stress in human pathology, *Annu Rev Pathol.* **10**, 173-194.
45. Han, D., Lerner, A. G., Vande Walle, L., Upton, J. P., Xu, W., Hagen, A., Backes, B. J., Oakes, S. A. & Papa, F. R. (2009) IRE1alpha kinase activation modes control alternate endoribonuclease outputs to determine divergent cell fates, *Cell.* **138**, 562-575.
46. Hollien, J. & Weissman, J. S. (2006) Decay of endoplasmic reticulum-localized mRNAs during the unfolded protein response, *Science.* **313**, 104-107.
47. Lizarbe, M. A., Barrasa, J. I., Olmo, N., Gavilanes, F. & Turnay, J. (2013) Annexin-phospholipid interactions. Functional implications, *Int J Mol Sci.* **14**, 2652-2683.
48. Green, D. R. & Llambi, F. (2015) Cell Death Signaling, *Cold Spring Harb Perspect Biol.* **7**.
49. Porter, A. G. & Janicke, R. U. (1999) Emerging roles of caspase-3 in apoptosis, *Cell Death Differ.* **6**, 99-104.
50. Spierings, D., McStay, G., Saleh, M., Bender, C., Chipuk, J., Maurer, U. & Green, D. R. (2005) Connected to death: the (unexpurgated) mitochondrial pathway of apoptosis, *Science.* **310**, 66-67.
51. Yamamoto, K., Ichijo, H. & Korsmeyer, S. J. (1999) BCL-2 is phosphorylated and inactivated by an ASK1/Jun N-terminal protein kinase pathway normally activated at G(2)/M, *Mol Cell Biol.* **19**, 8469-8478.
52. Rozpedek, W., Pytel, D., Mucha, B., Leszczynska, H., Diehl, J. A. & Majsterek, I. (2016) The Role of the PERK/eIF2alpha/ATF4/CHOP Signaling Pathway in

Tumor Progression During Endoplasmic Reticulum Stress, *Curr Mol Med.* **16**, 533-544.

53. Lai, E., Bikopoulos, G., Wheeler, M. B., Rozakis-Adcock, M. & Volchuk, A. (2008) Differential activation of ER stress and apoptosis in response to chronically elevated free fatty acids in pancreatic beta-cells, *Am J Physiol Endocrinol Metab.* **294**, E540-550.
54. Oh, Y. S., Bae, G. D., Baek, D. J., Park, E. Y. & Jun, H. S. (2018) Fatty Acid-Induced Lipotoxicity in Pancreatic Beta-Cells During Development of Type 2 Diabetes, *Front Endocrinol (Lausanne).* **9**, 384.
55. Maedler, K., Oberholzer, J., Bucher, P., Spinas, G. A. & Donath, M. Y. (2003) Monounsaturated fatty acids prevent the deleterious effects of palmitate and high glucose on human pancreatic beta-cell turnover and function, *Diabetes.* **52**, 726-733.
56. Thorn, K. & Bergsten, P. (2010) Fatty acid-induced oxidation and triglyceride formation is higher in insulin-producing MIN6 cells exposed to oleate compared to palmitate, *J Cell Biochem.* **111**, 497-507.
57. Jenkins, T. C. (1993) Lipid metabolism in the rumen, *J Dairy Sci.* **76**, 3851-63.
58. Kuhnt, K., Baehr, M., Rohrer, C. & Jahreis, G. (2011) Trans fatty acid isomers and the trans-9/trans-11 index in fat containing foods, *Eur J Lipid Sci Technol.* **113**, 1281-1292.
59. Salmeron, J., Hu, F. B., Manson, J. E., Stampfer, M. J., Colditz, G. A., Rimm, E. B. & Willett, W. C. (2001) Dietary fat intake and risk of type 2 diabetes in women, *Am J Clin Nutr.* **73**, 1019-1026.
60. Bassett, C. M., Edel, A. L., Patenaude, A. F., McCullough, R. S., Blackwood, D. P., Chouinard, P. Y., Paquin, P., Lamarche, B. & Pierce, G. N. (2010) Dietary vaccenic acid has antiatherogenic effects in LDLr-/- mice, *J Nutr.* **140**, 18-24.
61. Kleber, M. E., Delgado, G. E., Lorkowski, S., Marz, W. & von Schacky, C. (2016) Trans-fatty acids and mortality in patients referred for coronary angiography: the Ludwigshafen Risk and Cardiovascular Health Study, *Eur Heart J.* **37**, 1072-1078.

62. Sluijs, I., Forouhi, N. G., Beulens, J. W., van der Schouw, Y. T., Agnoli, C., Arriola, L., Balkau, B., Barricarte, A., Boeing, H., Bueno-de-Mesquita, H. B., Clavel-Chapelon, F., Crowe, F. L., de Lauzon-Guillain, B., Drogan, D., Franks, P. W., Gavrila, D., Gonzalez, C., Halkjaer, J., Kaaks, R., Moskal, A., Nilsson, P., Overvad, K., Palli, D., Panico, S., Quiros, J. R., Ricceri, F., Rinaldi, S., Rolandsson, O., Sacerdote, C., Sanchez, M. J., Slimani, N., Spijkerman, A. M., Teucher, B., Tjonneland, A., Tormo, M. J., Tumino, R., van der, A. D., Sharp, S. J., Langenberg, C., Feskens, E. J., Riboli, E., Wareham, N. J. & InterAct, C. (2012) The amount and type of dairy product intake and incident type 2 diabetes: results from the EPIC-InterAct Study, *Am J Clin Nutr.* **96**, 382-390.

63. Stender, S., Astrup, A. & Dyerberg, J. (2008) Ruminant and industrially produced trans fatty acids: health aspects, *Food Nutr Res.* **52**.

64. Chen, X., Li, L., Liu, X., Luo, R., Liao, G., Li, L., Liu, J., Cheng, J., Lu, Y. & Chen, Y. (2018) Oleic acid protects saturated fatty acid mediated lipotoxicity in hepatocytes and rat of non-alcoholic steatohepatitis, *Life Sci.* **203**, 291-304.

65. Dalla Valle, A., Vertongen, P., Spruyt, D., Lechanteur, J., Suain, V., Gaspard, N., Brion, J. P., Gangji, V. & Rasschaert, J. (2019) Induction of Stearoyl-CoA 9-Desaturase 1 Protects Human Mesenchymal Stromal Cells Against Palmitic Acid-Induced Lipotoxicity and Inflammation, *Front Endocrinol (Lausanne).* **10**, 726.

66. Sommerweiss, D., Gorski, T., Richter, S., Garten, A. & Kiess, W. (2013) Oleate rescues INS-1E β-cells from palmitate-induced apoptosis by preventing activation of the unfolded protein response, *Biochem Biophys Res Commun.* **441**, 770-776.

67. Simon-Szabo, L., Kokas, M., Greff, Z., Boros, S., Banhegyi, P., Zsakai, L., Szantai-Kis, C., Vantus, T., Mandl, J., Banhegyi, G., Valyi-Nagy, I., Orfi, L., Ullrich, A., Csala, M. & Keri, G. (2016) Novel compounds reducing IRS-1 serine phosphorylation for treatment of diabetes, *Bioorg Med Chem Lett.* **26**, 424-428.

68. Preston, A. M., Gurisik, E., Bartley, C., Laybutt, D. R. & Biden, T. J. (2009) Reduced endoplasmic reticulum (ER)-to-Golgi protein trafficking contributes to ER stress in lipotoxic mouse beta cells by promoting protein overload, *Diabetologia.* **52**, 2369-2373.

69. Moffitt, J. H., Fielding, B. A., Evershed, R., Berstan, R., Currie, J. M. & Clark, A. (2005) Adverse physicochemical properties of tripalmitin in beta cells lead to morphological changes and lipotoxicity in vitro, *Diabetologia.* **48**, 1819-1829.
70. Capurso, C. & Capurso, A. (2012) From excess adiposity to insulin resistance: the role of free fatty acids, *Vascul Pharmacol.* **57**, 91-97.
71. Kwak, H. J., Yang, D., Hwang, Y., Jun, H. S. & Cheon, H. G. (2017) Baicalein protects rat insulinoma INS-1 cells from palmitate-induced lipotoxicity by inducing HO-1, *PLoS One.* **12**, e0176432.
72. Laybutt, D. R., Preston, A. M., Akerfeldt, M. C., Kench, J. G., Busch, A. K., Biankin, A. V. & Biden, T. J. (2007) Endoplasmic reticulum stress contributes to beta cell apoptosis in type 2 diabetes, *Diabetologia.* **50**, 752-763.
73. Enoch, H. G., Catala, A. & Strittmatter, P. (1976) Mechanism of rat liver microsomal stearyl-CoA desaturase. Studies of the substrate specificity, enzyme-substrate interactions, and the function of lipid, *J Biol Chem.* **251**, 5095-5103.
74. Hodson, L. & Fielding, B. A. (2013) Stearoyl-CoA desaturase: rogue or innocent bystander?, *Prog Lipid Res.* **52**, 15-42.
75. Mozaffarian, D., Katan, M. B., Ascherio, A., Stampfer, M. J. & Willett, W. C. (2006) Trans fatty acids and cardiovascular disease, *N Engl J Med.* **354**, 1601-1613.
76. Potera, C. (2015) Food Companies Have Three Years to Eliminate Trans Fats, *Am J Nurs.* **115**, 14.
77. Hyseni, L., Bromley, H., Kypridemos, C., O'Flaherty, M., Lloyd-Williams, F., Guzman-Castillo, M., Pearson-Stuttard, J. & Capewell, S. (2017) Systematic review of dietary trans-fat reduction interventions, *Bull World Health Organ.* **95**, 821-830G.
78. Wang, X., Gupta, J., Kerslake, M., Rayat, G., Proctor, S. D. & Chan, C. B. (2016) Trans-11 vaccenic acid improves insulin secretion in models of type 2 diabetes in vivo and in vitro, *Molecular nutrition & food research.* **60**, 846-857.
79. Alstrup, K. K., Brock, B. & Hermansen, K. (2004) Long-Term exposure of INS-1 cells to cis and trans fatty acids influences insulin release and fatty acid oxidation differentially, *Metabolism: clinical and experimental.* **53**, 1158-1165.

80. Donnelly, K. L., Smith, C. I., Schwarzenberg, S. J., Jessurun, J., Boldt, M. D. & Parks, E. J. (2005) Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease, *J Clin Invest.* **115**, 1343-1351.
81. Palomer, X., Pizarro-Delgado, J., Barroso, E. & Vazquez-Carrera, M. (2018) Palmitic and Oleic Acid: The Yin and Yang of Fatty Acids in Type 2 Diabetes Mellitus, *Trends Endocrinol Metab.* **29**, 178-190.
82. Chavez, J. A., Siddique, M. M., Wang, S. T., Ching, J., Shayman, J. A. & Summers, S. A. (2014) Ceramides and glucosylceramides are independent antagonists of insulin signaling, *J Biol Chem.* **289**, 723-734.
83. Kolesnick, R. N. & Kronke, M. (1998) Regulation of ceramide production and apoptosis, *Annu Rev Physiol.* **60**, 643-665.
84. Morales, A., Lee, H., Goni, F. M., Kolesnick, R. & Fernandez-Checa, J. C. (2007) Sphingolipids and cell death, *Apoptosis.* **12**, 923-939.
85. Da Silva, M. S., Julien, P., Bilodeau, J. F., Barbier, O. & Rudkowska, I. (2017) Trans Fatty Acids Suppress TNF-alpha-Induced Inflammatory Gene Expression in Endothelial (HUVEC) and Hepatocellular Carcinoma (HepG2) Cells, *Lipids.* **52**, 315-325.
86. de Souza, R. J., Mente, A., Maroleanu, A., Cozma, A. I., Ha, V., Kishibe, T., Uleryk, E., Budylowski, P., Schünemann, H., Beyene, J. & Anand, S. S. (2015) Intake of saturated and trans unsaturated fatty acids and risk of all cause mortality, cardiovascular disease, and type 2 diabetes: systematic review and meta-analysis of observational studies, *BMJ.* **351**, h3978.
87. Jaudszus, A., Kramer, R., Pfeuffer, M., Roth, A., Jahreis, G. & Kuhnt, K. (2014) trans Palmitoleic acid arises endogenously from dietary vaccenic acid, *Am J Clin Nutr.* **99**, 431-435.
88. Jean-Louis Sébédio, W. M. N. R. (2008) Analysis of Trans Mono- and Polyunsaturated Fatty Acids in *Trans Fatty Acids* (Albert J. Dijkstra, R. J. H., Wolf Hamm, ed) pp. 102-131, Blackwell Publishing Ltd, Singapore.

89. Yu, W., Liang, X., Ensenauer, R. E., Vockley, J., Sweetman, L. & Schulz, H. (2004) Leaky beta-oxidation of a trans-fatty acid: incomplete beta-oxidation of elaidic acid is due to the accumulation of 5-trans-tetradecenoyl-CoA and its hydrolysis and conversion to 5-trans-tetradecenoylcarnitine in the matrix of rat mitochondria, *J Biol Chem.* **279**, 52160-52167.
90. Peng, G., Li, L., Liu, Y., Pu, J., Zhang, S., Yu, J., Zhao, J. & Liu, P. (2011) Oleate blocks palmitate-induced abnormal lipid distribution, endoplasmic reticulum expansion and stress, and insulin resistance in skeletal muscle, *Endocrinology.* **152**, 2206-2218.
91. Briaud, I., Harmon, J. S., Kelpe, C. L., Segu, V. B. & Poitout, V. (2001) Lipotoxicity of the pancreatic beta-cell is associated with glucose-dependent esterification of fatty acids into neutral lipids, *Diabetes.* **50**, 315-321.
92. Cnop, M., Hannaert, J. C., Hoorens, A., Eizirik, D. L. & Pipeleers, D. G. (2001) Inverse relationship between cytotoxicity of free fatty acids in pancreatic islet cells and cellular triglyceride accumulation, *Diabetes.* **50**, 1771-1777.

DOI:10.14753/SE.2020.2425

8. Bibliography of the candidate's publications

Publications related to the thesis:

1. Farkas Sarnyai; Anna Somogyi; Zsófia Gór-Nagy; Veronika Zámbó; Péter Szelényi; Judit Mátyási; Laura Simon-Szabó; Éva Kereszturi; Blanka Tóth; Miklós Csala
   Effect of cis- and trans-Monounsaturated Fatty Acids on Palmitate Toxicity and on Palmitate-induced Accumulation of Ceramides and Diglycerides
   INTERNATIONAL JOURNAL OF MOLECULAR SCIENCES 21: 7 Paper: 2626 (2020)

   IF: 4.183

2. Farkas Sarnyai; Mária Berinkeiné Donkó; Judit Mátyási; Zsófia Gór-Nagy; Ildikó Marczi; Laura Simon-Szabó; Veronika Zámbó; Anna Somogyi; Tamás Csizmadia; Péter Lőw; Péter Szelényi; Éva Kereszturi; Blanka Tóth; Miklós Csala
   Cellular toxicity of dietary trans fatty acids and its correlation with ceramide and diglyceride accumulation
   FOOD AND CHEMICAL TOXICOLOGY 124 pp. 324-335, 12 p. (2019)

   IF: 3.775

Publications not directly related to the thesis:

1. Veronika Zámbó; Laura Simon-Szabó; Farkas Sarnyai; Judit Mátyási; Zsófia Gór-Nagy; Anna Somogyi; Péter Szelényi; Éva Kereszturi; Blanka Tóth; Miklós Csala
   Investigation of the putative rate-limiting role of electron transfer in fatty acid desaturation using transfected HEK293T cells
   FEBS LETTERS 594: 3 pp. 530-539, 10 p. (2020)

   IF: 2.675

2. Farkas Sarnyai; Tímea Szekerczés; Miklós Csala; Balázs Sümegi; András Szarka; Zsuzsa Schaff; József Mandl
   BGP-15 Protects Mitochondria in Acute, Acetaminophen Overdose Induced Liver Injury
   PATHOLOGY AND ONCOLOGY RESEARCH (2019)

   IF: 2.433

3. Péter Szelényi; Anna Somogyi; Farkas Sarnyai; Veronika Zámbó; Laura Simon-Szabó; Éva Kereszturi; Miklós Csala
   Microsomal pre-receptor cortisol production is inhibited by resveratrol and epigallocatechin gallate through different mechanisms
   BIOFACTORS 45: 2 pp. 236-243, 8 p. (2019)

   IF: 3.598

4. Zsuzsanna Darula; Farkas Sarnyai; Katalin F. Medzihradszky
   O-glycosylation sites identified from mucin core-1 type glycopeptides from human serum
   GLYCOCONJUGATE JOURNAL 33: 3 pp. 435-445, 11 p. (2016)

   IF: 2.186

5. Veronika Zámbó; Mónika Tóth; Krisztina Schlachter; Péter Szelényi; Farkas Sarnyai; Gábor Lotz; Miklós Csala; Éva Kereszturi
   Cytosolic localization of NADH cytochrome b5 oxidoreductase (Ncb5or)
   FEBS LETTERS 590: 5 pp. 661-671, 11 p. (2016)

   IF: 3.623

DOI:10.14753/SE.2020.2425

9. Acknowledgements

First of all, I would like to thank my supervisor Prof. Dr. Miklós Csala for his effective guidance throughout the course of my Ph.D. studies and for his firm belief in me even at the hardest times. Without him, this dissertation would not have been realized. I would like to express my gratitude to the previous and currently acting director, Prof. Dr. Gábor Bánhegyi and Dr. Gergely Keszler for providing me with the opportunity to work at the Department of Medical Chemistry, Molecular Biology and Pathobiochemistry. I appreciate Prof. Dr. József Mandl for accepting me into the Ph.D. school.

Herein, I would like to thank the present and past members of our laboratory: Éva Kereszturi, Veronika Zámbó, Anna Somogyi, Péter Szelényi and Laura Simon-Szabó for experimental help as well as for their useful comments and suggestions and Valéria Béláné Szénási for her precious technical support. I am also deeply grateful for Dr. Blanka Tóth and Dr. Péter Lőw for their experimental contribution to my work. I would also like to thank all my colleagues working at the Department of Medical Chemistry, Molecular Biology and Pathobiochemistry for their help.

Finally, I would like to express my special thanks to my family for their encouragement and support.