Assessment of fatty acid profile and biosynthetic lipid intermediates in cultured cells and its application to study the potential role of enhanced electron transfer in the control of fatty acid desaturation

PhD thesis

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List of abbreviations

AP-1	activating protein 1
ATF-6	activating transcription factor 6
BSA	bovine serum albumin
cDNA	complementary DNA
CHOP	C/EBP homologous protein
CoA	coenzyme A
CYB5	cytochrome b5
CYB5R	cytochrome b5 reductase
DG	diglyceride
DGAT	diacylglycerol acyltransferase
ER	endoplasmic reticulum
ERK1/2	extracellular signal-regulated kinases
FA	fatty acid
FAME	fatty acid methyl ester
FAT/CD36	fatty acid/translocase
FFA	free fatty acid
FID	flame ionization detection
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GC	gas chromatography
HEK293T	human embryonic kidney 293T cell
HPLC	high performance liquid chromatography
IKK	inhibitory-κB kinase

IL-1R	interleukin 1 receptor
IRE-1α	inositol requiring enzyme 1α
IRS	insulin receptor substrate
JNK	c-Jun aminoterminal kinase
MRM	multiple reaction monitoring
MS	mass spectrometry
NASH	non-alcoholic steatohepatitis
NCB5OR	NAD(P)H cytochrome b5 oxidoreductase
NF-κB	nuclear factor κB
PERK	protein kinase R-like ER kinase
PKC	protein kinase C
PLC	phospholipase C
ROS	reactive oxygen species
RT-PCR	reverse transcription polymerase chain reaction
SCD1	stearoyl-CoA desaturase 1
TFA	trans fatty acid
TG	triglyceride
TLR-4	Toll-like receptor 4
TNFR	tumor necrosis factor receptor
UPR	unfolded protein response

1. Introduction

1.1. Elevated fatty acid levels in overnutrition

Obesity and its connected diseases, such as insulin resistance and type II diabetes, non-alcoholic steatohepatitis (NASH) and cardiovascular diseases have become pandemics. According to a report by WHO in 2014, 1.9 billion of the humans older than 18 years are obese. This fact and the growing death rate caused by obesity connected diseases are focusing the attention of researchers on the investigation of the mechanism of these pathologies.

Depot fat stored in lipid droplets in the adipocytes contains both endogenously synthetized fatty acids (FAs) and FAs ingested with animal fat [1] and with natural or hydrogenated plant oils [2]. Dietary fats are usually absorbed in the intestines as resynthesized complex lipids packed in chylomicrons and transported to the adipose tissue through the lymphatic and systemic circulation [3]. The other nutrients, such as sugars or amino acids are also largely converted into FAs and then into complex lipids in the liver. These lipids are packed into very low-density lipoproteins, and they are also transported to the fat tissue through the systemic circulation. FAs are liberated from triglycerides (TGs) by lipoprotein lipase presented in the capillaries of the adipose tissue, and then they get into the adipocytes. Chronic oversupply of these lipoprotein-derived FAs eventually causes adipocyte hypertrophy, a basic feature of obesity, which in case of genetic predisposition, can lead to local inflammation in the adipose tissue [4]. Cells produce adipokines and leukotrienes, and so activate the inflammatory macrophages and lymphocytes. Cytokines secreted by macrophages interfere with insulin signaling and cause an intensification in TG turnover. This effect along with the constant oversupply of nutrients leads to an increase in the free fatty acid (FFA) level of the serum [5]. While the FFAs can serve as nutrients for aerobic cells primarily in starvation and physical exercise, a chronic and continuous elevation of serum FFA level is deleterious to a wide variety of cells and tissues.

Several *in vitro* studies have been performed to investigate the molecular mechanism of lipotoxicity on various cell lines by using the major endogenously synthetized saturated FA palmitate (16:0) and unsaturated FA oleate (18:1 cΔ9) at high concentrations. It has been demonstrated that the key events are the FFA-induced endoplasmic reticulum (ER)

stress [6], changes in mitochondrial functions [7] and the altered lipid metabolism with accumulation of toxic intermediates [8].

1.2. Toxicity of fatty acids

1.2.1. Molecular background of lipotoxicity

The cell nutrient FFAs can also serve as signal molecules by connecting to the cell surface receptors, thus a chronic elevation of the FFA levels can disturb cellular functions in both ways. By connecting to the Toll-like receptor 4 (TLR-4), interleukin 1 receptor (IL-1R) and tumor necrosis factor receptor (TNFR) on the cell surface, saturated FAs induce the production of inflammatory cytokines (i.e. interleukin-6 and tumor necrosis factor-α) [9]. The produced cytokines activate nuclear factor-κB (NF-κB) and activating protein 1 (AP-1) transcription factors through the activation of inhibitory-κB kinase (IKK) complex and c-Jun aminoterminal kinase (JNK) and induce an inflammatory response. Palmitate can also trigger Ca^{2+} signaling by activating the fatty acid/translocase (FAT/CD36) receptor on the cell surface [10].

FAs enter the cytosol via a protein-mediated transport [11] and they are activated with coenzyme A (CoA). A permanent FA-CoA surplus intensifies mitochondrial and peroxisomal β-oxidation, which causes an elevation in the level of reactive oxygen species (ROS).

The Fa-CoA excess may cause disturbance in the normal functions of the ER as well. Long chain FA-CoA molecules, especially the saturated ones can cause a change in the Ca^{2+}-homeostasis of the organelle's luminal environment through two major routes. Palmitoyl-CoA has a membrane-permeabilizing effect [12], which contributes to ER Ca^{2+} depletion along with the signaling through FAT/CD36 receptor. Since Ca^{2+} is essential for the proper functioning of chaperons and foldases in the ER lumen, the ion release may cause a disturbance in the protein folding and quality control. The ER transmembrane stress receptors, i.e., inositol requiring enzyme 1α (IRE-1α), protein kinase R-like ER kinase (PERK) and the activating transcription factor 6 (ATF-6) detect the increasing amount of immature, unfolded proteins. Through these sensors, the shift in the balance of protein translation and protein folding, and the consequent intraluminal accumulation of immature proteins induce the unfolded protein response (UPR) [13]. In case of a chronic

stress, the UPR can cause apoptosis via PERK and IRE1 pathways by inducing transcription and translation of proapoptotic factors such as the C/EBP homologous protein (CHOP) [14] and activation of JNK [15]. The oxidative stress along with the ER-stress also enhances the inflammation through the activation IKK and JNK [16, 17].

Insulin receptor substrate (IRS) proteins further take part in insulin signaling through their tyrosine phosphorylation after they connect to the activated insulin receptor. However, while the tyrosine phosphorylation is an intrinsic part of the IRS function, the serine phosphorylations in the same polypeptide chains inhibit this functioning. The stress kinases JNK, IKK and protein kinase C (PKC) can serine phosphorylate the IRS, thus inflammation and the oxidative and ER-stress inhibit the insulin signaling pathway at this point [18, 19]. A chronic stress, such as the one caused by permanently elevated FFA levels, often leads to insulin resistance in the body. β-cells increase insulin production to compensate the developed insulin resistance, however, the long-term pressure and the deleterious effects of lipotoxicity on β-cells themselves can hinder the proper functioning of these insulin producing cells and it can lead to a decrease in the β-cell mass. These synergistic effects may lead to the development of type II diabetes.

1.2.2. Differences between the toxicity of saturated and unsaturated fatty acids

Saturated FAs such as palmitate are known to be more toxic than unsaturated ones [20-22]. Dietary trans fatty acids (TFA) namely elaidate of industrial origin (18:1 tΔ9) or naturally occurring vaccenate (18:1 tΔ11) have controversial assessment. *In vivo* studies suggest that chronic intake of TFAs may be associated with type II diabetes [23]. On the contrary, Kleber et al. found no correlation between the TFA intake and adverse cardiac outcomes [24]. However, *in vitro* investigation on a rat insulinoma cell line revealed that TFAs show greater similarity to oleate that to palmitate regarding either the short-term toxicity or the lipid metabolism [25]. Recently, a growing attention is focused on the investigation of the potential protective effects of oleate against palmitate toxicity. It has been shown that co-administration of oleate with palmitate can prevent activation of the unfolded protein response in β-cells [26]. In neuronal cells, oleate-pretreatment blocked phosphorylation of extracellular signal-regulated kinases (ERK1/2) and JNK and the nuclear translocation of NF-κB promoted by palmitate. It has been proved that in muscle cells, oleate protects against palmitate-induced mitochondrial DNA damage and decline

in ATP level, and also that treatment with oleate and an oleate-palmitate mixture does not induce mitochondrial ROS production in contrast with palmitate alone [16]. In hepatocytes and a hepatoma cell line, oleate addition alleviated palmitate-induced toxicity, restored the cell viability, and reduced oxidative and ER-stress [27]. Furthermore, in case of rats with high fat diet, a half replacement of the diet with olive oil also decreased NASH injury. It has been proved that TFA co-administration can also attenuate the toxic effects of palmitate to some degree, in rat insulinoma cell cultures [28].

1.3. Lipid metabolism

1.3.1. Complex lipids

Beside the intensified β-oxidation, the enhanced incorporation of FAs into complex lipids provides an alternative for the cells to control the amount of FA-CoA. Thus, the build-up of FA-CoA causes a significant intracellular accumulation of several lipid species as well. The adverse effects of the excess FFAs are usually attributed to the accumulation of ectopic fat in different cells such as hepatocytes or β-cells [5]. However, since TGs are neutral and non-toxic lipids, incorporation of FA-CoA into TGs can ameliorate the toxic effects of FA surplus [29]. In the synthesis of TGs, a key enzyme is the diacylglycerol acyltransferase (DGAT), which has two isoforms, DGAT-1 and DGAT-2. DGAT-1 knock-out mice have normal weight when they are fed chow but are unable to gain weight on high-fat diet [30]. This indicated that a second DGAT may exist. DGAT-2 has a specific affinity twice as high to oleoyl-CoA than to palmitoyl-CoA [31]. This is in accordance with the observation that the synthesis of TGs has a genetically determined pattern. Normally, a saturated FA chain is connected to the first carbon of glycerol, and an unsaturated FA chain occupies the second position [32].

Diglycerides (DGs), the esters of a glycerol and two FA chains, are central intermediates of TG and phospholipid metabolism. 1,2-DGs are formed from plasma membrane glycerolipids via a hydrolytic cleavage by phospholipase C (PLC), and they activate PKC isoenzymes, thus they serve as signal messengers. Activation of PKC leads to an ER Ca^{2+} depletion thus it may also contribute to the ER-stress [10]. In case of an excess of FAs, DGs produced by both PLC-mediated signaling and by stimulated esterification of glycerol can also have an effect on insulin secretion [33]. In the synthesis and metabolism of phospholipids, DGs are central intermediates thus alterations in their structure have

effects on the composition of cellular membranes. It has been shown that decreased phospholipid unsaturation can induce the UPR [34], similarly to the disturbed protein maturation that is considered as a fundamental feature of the ER stress. In CHO cells, it has been proven that exogenous palmitate is rapidly incorporated into membrane phospholipids (mostly into phosphatidylcholine) [35]. It has been also revealed that the increased saturation of membrane lipid species causes an obvious change in ER structure and membrane integrity. Computational analyses showed that unsaturated FAs can prevent changes in the membrane fluidity so they can act as membrane stabilizer molecules [36].

When comparing the toxicity of saturated and unsaturated fatty acids, oleate has been proved to induce a more pronounced TG accumulation than palmitate [20, 27]. On the contrary, palmitate treatment caused a significant DG build-up in a human hepatoma cell line [37, 38] and rat insulinoma cell cultures [25] as well. In neuronal cells, oleate pretreatment and also co-treatment increased TG levels and prevented the palmitate-induced DG accumulation [39].

Saturated FA oversupply is assumed to increase the amount of DGs containing two saturated fatty acyl chains, which can block the following steps of TG synthesis in the cells. Thus, effects which can channel the FA-CoA surplus into TG pool (such as presence of unsaturated FA or an enhanced desaturase activity in the cells) may prevent saturated FA-induced lipotoxicity. But when the TG synthesis is blocked, the FA-CoA excess may be diverted towards the synthesis of other, potentially toxic lipid intermediates such as ceramides.

Ceramides are FA amides of sphingosine. These compounds form the hydrophobic backbones of sphingolipids, and they also play roles in cell signaling. Ceramides are known to participate in signaling pathways influencing cell growth [40] cellular senescence [41], and they can induce apoptosis as well [42-44]. The FA-CoA supply can affect the intracellular ceramide synthesis in several steps. Ceramides can be synthetized in two different ways, the *de novo* synthesis and the so-called salvage pathway [45]. During the *de novo* synthesis, the first step is a fusion of palmitoyl-CoA and serine which results in a 3-dehydrosphinganine, which is reduced to sphinganine in the next step. Ceramide synthase acylates sphinganine with another FA-CoA to produce

dihydroceramide, which can be desaturated to form a ceramide. In the salvage pathway, sphingosine, which is an intermediate of sphingolipid degradation is recycled through an acylation by ceramide synthase resulting in another molecule of ceramide. As it can be seen, several FA-CoA molecules can enter the ceramide synthesis pathways; hence it is likely that any alteration in the FA-CoA supply of the cells can modify the level of the different types of ceramides. Ceramides are involved in FA-induced toxicity via different pathways. In rat insulinoma cells and primary rat pancreatic islets, ceramide analogue (C2-ceramide) increased β-cell apoptosis, furthermore, it induced JNK phosphorylation and caspase-3 cleavage [46]. Regarding the mitochondrial dysfunctions, C2-ceramide caused a collapse in the mitochondrial membrane potential which led to a cytochrome c release, and the ROS level was also increased. According to publications, mitochondrial ROS production occurred through *de novo* synthesis of ceramides in L6 myotubes as well [16]. It has been also revealed that oleate prevents the palmitate-induced ceramide accumulation in CHO cells [29] and in rat insulinoma cells [28].

These observations highlight the importance of a balanced availability of saturated and unsaturated FAs in the cells.

1.3.2. Role of Stearoyl-CoA desaturase in lipid metabolism

The end product of the *de novo* biosynthesis of FAs is mainly the saturated palmitate containing 16 carbon atoms. However, for the synthesis of the substrates for complex lipids, palmitate needs to be modified, i.e., elongated or desaturated [47]. In the human body, stearoyl-CoA desaturase (SCD1) is a key enzyme of the FA metabolism. It inserts a cis double bond at the Δ9 position into saturated FAs (i.e., 16:0 palmitate or 18:0 stearate) that yields the corresponding mono-unsaturated FAs (i.e., 16:1 cisΔ9 palmitoleate or 18:1 cisΔ9 oleate, respectively) [48, 49]. The substrates may come from *de novo* synthesis or from the hydrolysis of complex lipids, and they can be of endogenous or dietary FAs. As mentioned above, unsaturated FA chains are essential for the synthesis of TGs, thus SDC1 has a crucial role in providing the appropriate substrate supply in the process. SCD1 is co-localized with DGAT-2 in the ER-membrane which also contributes to a harmonized functioning [47].

The phenotype of SCD1 knock-out mice clearly shows the importance of the desaturase in TG synthesis. SCD1 knock-out mice are unable to accumulate depot fat and they are

resistant to the obesity caused by a high-fat/high-carbohydrate diet because they have a limited capacity to synthetize complex lipids such as TGs [50]. *In vitro* experiments show that overexpression of SCD1, thus enhancing the desaturating activity of the cells, provides a protection against the saturated FA-induced toxicity while a decreased function of SCD1 aggravates the damage [51-53]. Reduced level of SCD1 in mouse insulinoma cells caused a more pronounced stress upon palmitate treatment [54]. SCD1-overexpressing CHO cells became completely resistant to palmitate-induced apoptosis compared to wild-type CHO cells and the caspase-3 activity was also reduced in response to palmitate treatment [29]. Moreover, SCD1-overexpressing cells treated with palmitate had a 5-fold increase in TG levels compared to control cells. This shows that SCD1 has an essential role in lipid metabolism. The ratio of the unsaturated (e.g., oleate) and the saturated (e.g., stearate) FAs influence the membrane fluidity, moreover, the produced unsaturated FAs are needed for the synthesis of complex lipids.

SDC1 is an iron-containing enzyme which is localized in the ER membrane. Desaturation occurs through a monooxygenation and a dehydration of the FA chain. Unlike other desaturases (such as Δ5 and Δ6 desaturases) which are natural fusion proteins thus contain a cytochrome b5-like and a desaturase domain, the Δ9 desaturase enzymes do not have this cytochrome b5-like domain [55, 56]. SCD1 receives electrons for its catalytic activity from NAD(P)H via NAD(P)H cytochrome b5 reductase (CYB5R) flavoprotein and cytochrome b5 (CYB5) hemoprotein electron-carriers [57, 58]. These proteins form the classic electron transfer chain in the ER membrane [59].

The NAD(P)H cytochrome b5 oxidoreductase (NCB5OR or CYB5R4) enzyme was first described by Hao Zhu et al. [60]. The enzyme is part of the ferredoxin–$NADP^+$-reductase superfamily. All members of this superfamily have a FAD prosthetic group binding site and a NAD(P)H coenzyme binding site; however, the NCB5OR is the only member which has three well defined domains. The CYB5-like domain is localized on the N-terminal end of the protein and it has a heme group, and the CYB5R-like domain, localized on the C-terminal end, binds a FAD prosthetic group and it can also bind NAD(P)H. The hinge sequence (CS: CHORD-SGT1) links the two domains and it is responsible for the overall NAD(P)H-oxidoreductase activity. Both the CYB5-like and the CYB5R-like domains show homology with the microsomal CYB5 hemoprotein and

CYB5R flavoprotein, however, NCB5OR is a soluble protein that does not have a membrane-anchoring region.

NCB5OR has been proved to be a cytosolic enzyme that does not have access to the luminal ER redox environment [61]. This means that it uses the same electron supply as the CYB5R – CYB5 couple. When comparing the phenotype of CYB5 knock-out and NCB5OR knock-out mice, obvious differences can be seen. The unsaturated/saturated fatty acid ratio of CYB5 knock-out mice does not change significantly [62], while in case of NCB5OR knock-out, the desaturation is damaged, and the mice have lipoatrophy and insulin-dependent diabetes [63]. It was found that primary hepatocytes yielded from NCB5OR knock-out mice have increased sensitivity to palmitate and a firm ER-stress was seen after FA treatments in contrast to hepatocytes from wild type mice. These observations and the fact that NCB5OR has CYB5-like and a CYB5R-like domains suggest that NCB5OR may act as an alternative electron donor in Δ9 desaturation [64] (Fig 1).

In case of an oversupply of saturated FAs, expression of SCD1 or/and the associated electron transfer proteins may be induced to alleviate the toxic effects. Because of the presence of an alternative electron transfer chain, and the protective role of NCB5OR against palmitate toxicity, we may assume that there is a need of an enhancement of the chain.

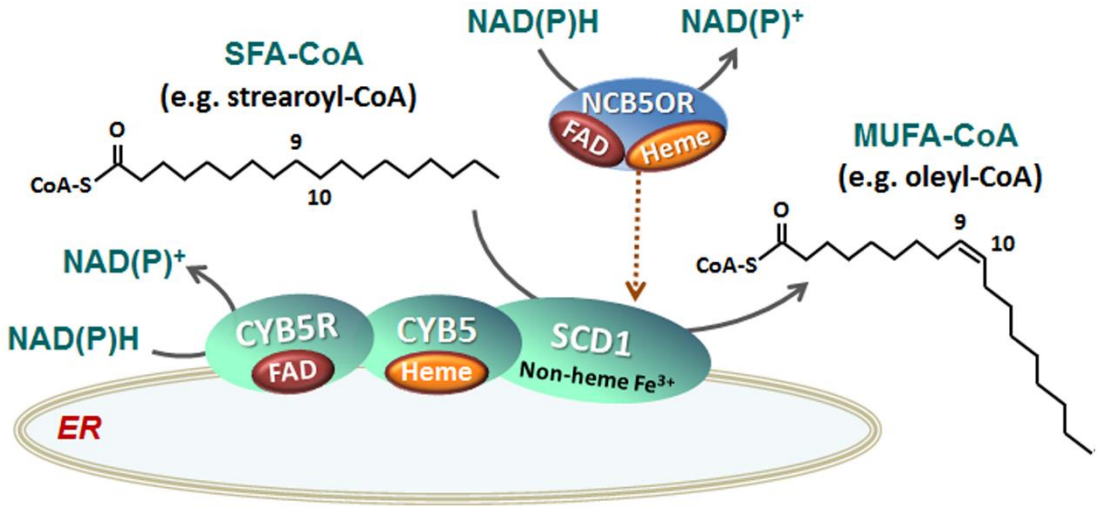

Figure 1. Electron transfer chains of microsomal fatty acyl-CoA desaturation. Saturated fatty acids can be desaturated by integral enzymes of the endoplasmic reticulum (ER) membrane. The first double bond is formed at carbon 9 in the saturated acyl-CoAs by a non-heme iron containing protein, stearoyl-CoA desaturase (SCD1). SCD1 converts saturated fatty acyl-CoA-s (FA-CoA), such as stearoyl-CoA (18:0) or palmitoyl-CoA (16:0) to Δ9 mono-unsaturated derivatives, oleyl-CoA (18:1 cisΔ9) or palmitoleyl-CoA (16:1 cisΔ9), respectively. Cytosolic NAD(P)H feeds the process with electrons either through the flavoprotein cytochrome b5 reductase (CYB5R) and the hemoprotein cytochrome b5 (CYB5) or through the CYB5R-like and CYB5-like domains of NAD(P)H cytochrome b5 oxidoreductase (NCB5OR). ([65])

1.4. Analytical methods for the determination of lipids in biological samples

1.4.1. Analysis of lipid profile by using gas chromatography

Gas chromatography (GC) is a common tool to determine the lipid composition of different food samples such as milk or margarine, or biological matrices (such as blood or tissues). For GC measurements, the components must be volatile thus, the sample preparation process usually has to involve a derivatization step. In most of the cases, the lipids are converted into FA esters, in particular, fatty acid methyl-esters (FAME) [66].

In multiple-step sample preparations, the first step is the lipid extraction. The extraction method developed by Folch et al. [67] and improved by Bligh et al. [68] was the most common and frequently used method for lipid extraction. Both of these methods use chloroform and methanol as extracting agents and since that publication, several research has used this mixture [69-71]. The next step is the methyl-ester formation which can be catalyzed by acids or bases. Several publications suggest a saponification before the esterification. Saponification does not lead to a chemical equilibrium; thus, the

conversion may be near 100%, and the soaps can be easily and rapidly esterified by an acidic catalyzer [72].

In the one-step sample preparation, lipid extraction and derivatization occur simultaneously. Esterification may be performed without the saponification step [71, 73, 74], or with saponification as well [71]. In these methods, the produced FAMEs are usually extracted into heptane [71], benzene [73] or hexane [74].

There is a wide variety of possibilities for chromatographic separation and for detection of FAMEs. When the aim is to separate cis and trans isomers, we need a strongly polar stationary phase. This is usually a propyl-nitrile-polysiloxane stationary phase [75, 76], or a poly-(90%-bispropyl-nitrile-10%-propyl-nitrile-phenyl-siloxane) phase [77]. The most common detection techniques used in the analysis of FAMEs is flame ionization detection (FID) [69, 74, 78] or mass spectrometry (MS) [78, 79]. When gas chromatography is coupled to mass spectrometry, the ion source is usually an electron ionization source. Since the fragmentation pattern is unique to each molecule, the analytes can be identified by using this ion source.

1.4.2. Quantitation of complex lipids by using high performance liquid chromatography

Ceramides can be measured by different techniques. Lesser-used methods involve enzymatic measurements with diacylglycerol kinase assay [80, 81] and thin-layer chromatography [82, 83]. In some studies, high performance liquid chromatography (HPLC) coupled to UV detection was used for the analysis [84, 85]. These methods usually cannot distinguish different types of ceramides thus only total ceramide level can be determined. Quantitation of different forms of ceramides can be performed by using tandem MS in multiple reaction monitoring (MRM) mode. The outstanding sensitivity and specificity of tandem MS allows the detection of the compounds without a prior separation step [86, 87]; however, the selectivity, specificity and the robustness can be further improved by coupling the MS to an HPLC [88, 89].

DGs can also be measured by the diacylglycerol kinase assay [90], by thin layer chromatography [37] or by capillary gas chromatography [91]. The main problem to be faced when detecting DGs by MS is the poor proton affinity of the compounds and the

consequent lack of a stable charge during ionization. This problem can be usually solved by derivatization or adduct formation [92, 93].

In the investigation of lipotoxicity, simple, precise and robust analytical methods are essential for the proper lipid profiling of cells. However, the current lipid measurements usually use time-consuming sample preparation methods and potentially carcinogenic extracting agents. Furthermore, the simultaneous determination of a wide array of different complex lipid species such as ceramides and DGs needs a newly developed methodology.

2. Objectives

High levels of FAs cause several cellular malfunctions such as ER stress or altered lipid metabolism which can cause an accumulation of toxic intermediates. The toxicity of saturated FAs is known to be more pronounced than that of the unsaturated ones. Furthermore, the co-administration of unsaturated FAs with saturated ones can attenuate the toxic effects of the latter both *in vitro* and *in vivo*. Unsaturated FAs are essential for the synthesis of complex lipids such as TG. Without the sufficient amount of unsaturated FAs, this biosynthesis may be blocked which can lead to the build-up of FA-CoA and the consequent accumulation of ceramides. *In vitro* experiments revealed that overexpression of SCD1, thus enhancing the desaturating activity of the cells, protects against palmitate toxicity. SCD1 receives electrons from NAD(P)H via CYB5R and CYB5 for its functioning. The oxidoreductase NCB5OR has a CYB5R-like and a CYB5-like domain as well thus it may serve as an alternative electron donor. In addition, in NCB5OR knock-out mice, the desaturation is damaged, and cells obtained from NCB5OR knock-out animals have increased palmitate sensitivity. Because of the presence of an alternative electron transfer chain, and the protective role of NCB5OR against palmitate toxicity, we may assume that the electron supply needs an enhancement in certain conditions. Based on this hypothesis, we aimed to investigate the dependence of the cellular desaturation activity on the expression level of the participating proteins. Our specific aims were the following:

- To develop suitable analytical methods for the assessment of uptake, incorporation and metabolism of the relevant saturated and cis or trans unsaturated fatty acids in cultured cells.

  Our studies on the metabolic background of lipotoxicity were to be enhanced by an improved GC-FID method to analyze the FA content of the cell including esterified and non-esterified FAs and by an HPLC-MS/MS method to quantitate the amount of potentially toxic lipid intermediates (i.e., DG and ceramides).
- To investigate the possible alterations in cellular FA desaturation upon the overexpression of the participating protein components, i.e., SCD1, CYB5R, CYB5 or NCB5OR or their combinations.

We intended to analyze the FA content of the cultured cells by the newly developed methodology and use the ratio of unsaturated/saturated FA levels as an indicator of the desaturation activity.

– To elucidate the rate-limiting step in the overall process of FA-CoA desaturation for a better understanding of the physiological and pathological role of the alternative electron supply pathways.

  Demonstration of any condition (i.e., enhanced desaturation because of an SCD1 overexpression or a saturated FA overload) that makes the desaturation-associated electron transfer rate-limiting would provide a mechanistic explanation to the proposed anti-lipotoxic action of NCB5OR.

3. Results

3.1. Development of an HPLC-MS/MS method for the simultaneous quantitative analysis of ceramides and diglycerides in cultured cells

3.1.1. Optimization of tandem mass spectrometry

During the high-performance liquid chromatography coupled to tandem mass spectrometry measurements, we aimed to determine the levels of those ceramides and DGs which contain the most common endogenous FAs, i.e., palmitic (16:0), stearic (18:0), palmitoleic (16:1 cisΔ9) and oleic (18:1 cisΔ9) acids in different combinations. We used a non-physiological Cer17:0 ceramide as an internal standard.

We recorded a full scan mass spectrum for each ceramide and DG standard solution (5 µg/ml). We successfully detected protonated ceramide ions in positive ionization mode. In case of DGs, the poor ionization ability is a problem to be faced. Thus, we used a 10 mM ammonium-acetate solution as mobile phase which enables the formation of ammonium-adducts. Precursor ions separated in the first quadrupole (Q1) were fragmented with N_2 in the second quadrupole (q2) and the product ions were scanned in the third quadrupole (Q3). We chose two product ions (or daughter ions) for each precursor (or mother ion) and these mass transitions were used during the measurements. This so-called multiple reaction monitoring (MRM) mode provides the high sensitivity, selectivity and efficiency of the triple quadrupole MS technique. In case of ceramides, one typical daughter ion for each measured compound had the m/z of 264. According to The Human Metabolome Database, this fragment is a common fragment ion for ceramides [94]. Previous studies suggested that this ion was generated by the loss of one acyl group and two molecules of water [87, 88]. The ion produced from the mother ion through the loss of one molecule of water was chosen as the other transition.

As mentioned above, the mother ion for DGs was their ammonium-adduct. The two daughter ions were produced by the loss of either an acyl group and the NH_3 or one molecule of water. For dioleoyl-glycerol (DG 18:1-18:1), the transition from the loss of water could not be used since the m/z of the product ion would have been the same as the one produced from DG 18:0-18:2. Thus, one transition yielded by the loss of one oleoyl-group and one ammonia was used. The MRM transitions used in our method are presented in Table 1.

Table 1. Quantitative HPLC/MS/MS parameters for ceramide and diglyceride analysis

Compound	Symbol	Mass transition (m/z)	Retention time (min)
N-palmitoyl-sphingosine	Cer16:0	538.60–264.30 538.60–520.60	7.2
N-heptadecanoyl-sphingosine	Cer17:0	552.61–264.30 552.61–534.60	8.0
N-stearoyl-sphingosine	Cer18:0	566.52–264.30 566.52–548.60	8.8
N-oleoyl-sphingosine	Cer18:1	564.66–264.30 564.66–546.50	6.5
Dipalmitoyl-glycerol	DG 16:0-16:0	586.50–313.30 586.50–551.50	10.2
Palmitoyl-palmitoleoyl-glycerol	DG 16:0-16:1	584.50–311.30 584.50–313.30	9.1
Palmitoyl-stearoyl-glycerol	DG 16:0-18:0	614.60–313.30 614.60–341.30	11.7
Palmitoyl-oleoyl-glycerol	DG 16:0-18:1	612.50–313.30 612.50–339.30	10.7
Palmitoleoyl-oleoyl-glycerol	DG 16:1-18:1	610.50–311.30 610.50–339.30	9.9
Distearoyl-glycerol	DG 18:0-18:0	642.60–341.30 642.60–607.60	12.9
Stearoyl-oleoyl-glycerol	DG 18:0-18:1	640.60–339.30 640.60–341.30	12.0
Dioleoyl-glycerol	DG 18:1-18:1	638.80–339.30	11.4

3.1.2. Liquid chromatography

Compounds were separated on a Kinetex® 5 µm, C8 100 Å, LC Column, 100 x 3 mm column. The flow rate was 0.5 ml/min with a gradient elution of methanol (mobile phase A) and 10 mM ammonium-acetate (mobile phase B): 0 min at 90 % A; 1 min at 90 % A; 9 min at 95 % A; 10.5 min at 98 % A; 11.5 min at 98 % A; 12 min at 90 % A; 14 min at 90 % A. Fig 2. shows a typical chromatogram of a control cell sample containing the measured ceramides and DGs.

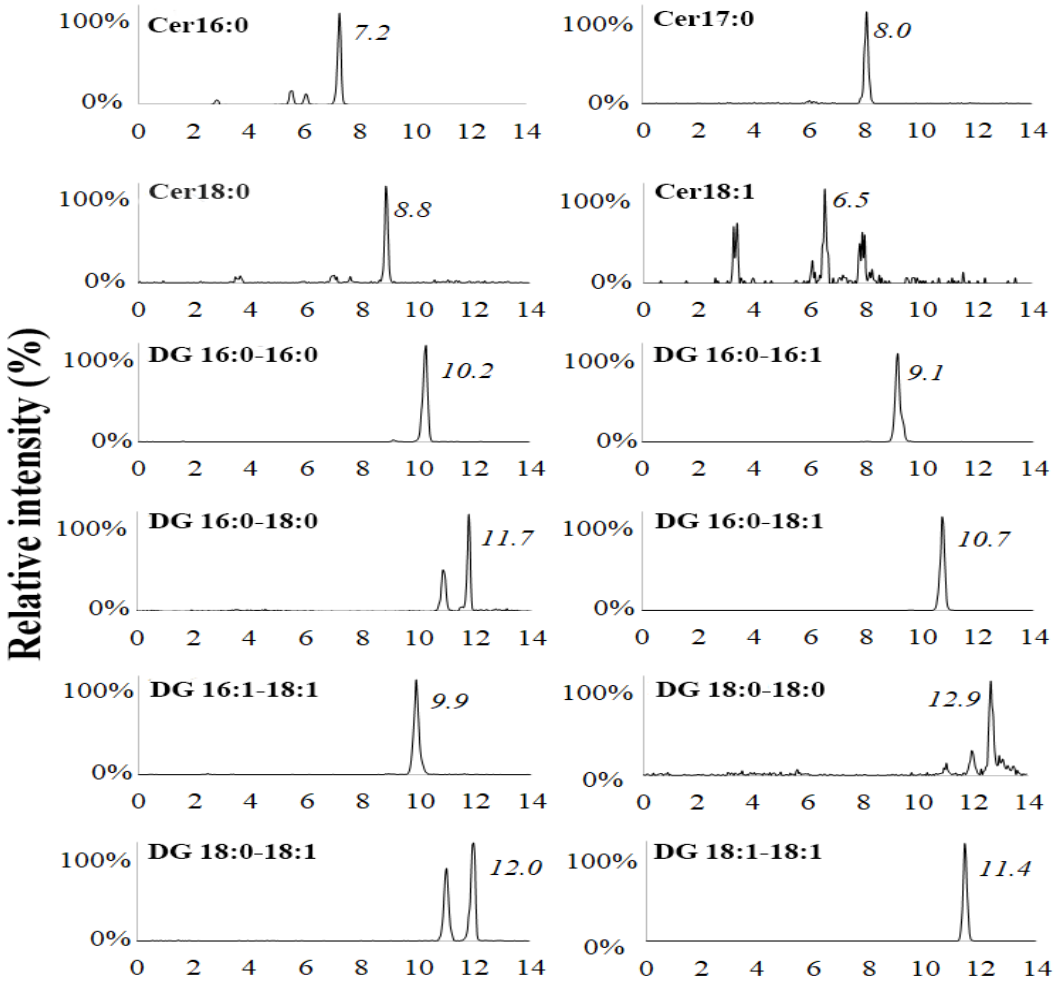

Figure 2. Typical chromatograms of a control cell sample containing the measured ceramides and diglycerides. A specific identification of co-eluting compounds can be achieved using the MRM mode.

3.1.3. Sample preparation

The most common method, developed by Bligh et al. uses a chloroform:methanol (1:2 v/v) mixture for the extraction of lipids [68]. However, the consecutive steps may cause a substantial loss of sample, furthermore, the carcinogenic properties of chloroform are well-known. Hence, we developed a short, chloroform-free sample preparation method, and we compared the two extractions (Fig 3). The new method uses pure methanol as an extraction agent. Washed cells were suspended in methanol followed by a sonication of 15-20 sec. Then the samples were centrifuged (13400 rpm, 10 min, 24 °C), and the supernatant was directly infused from HPLC vials. In the original method, we used a chloroform:methanol (1:2 v/v) mixture. Cells were suspended in 90 µl of methanol, and 45 µl of chloroform was added to the samples. After sonication and

centrifugation, the supernatant was dried and reconstituted in 90 µl of methanol. These solutions were sonicated and centrifuged, and the supernatant was transferred to HPLC vials.

We found no significant difference between the efficiency of the two extraction methods, thus we decided to use the new and shorter, chloroform-free sample preparation. After the optimization, we determined the percentage of ceramide and DG recoveries by adding known amount of the compounds to pooled cell suspensions. The recovery was between 79.1% and 86.4%.

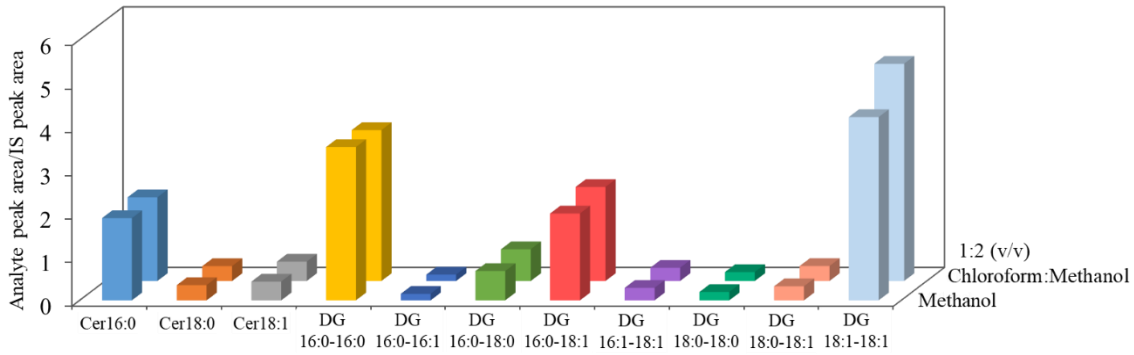

Figure 3. Comparison of the two different extraction methods. Methanol: Washed cells were suspended in methanol, sonicated for 15-20 sec, and then centrifuged at 13400 rpm for 10 minutes. The supernatant was directly injected from HPLC vials. Chloroform:Methanol 1:2 (v/v): 45 µl chloroform was added to 90 µl cell suspension in methanol. The samples were sonicated and centrifuged as above. The supernatant was then dried, reconstituted in 90 µl methanol, sonicated, centrifuged, and the supernatants were transferred to vials for HPLC analysis. Relative ceramide and diglyceride peak areas compared to the internal standard peak area are presented as mean values; n=10. RSD% were below 10% in all cases.

3.1.4. Effect of palmitate treatment on ceramide and DG levels in HepG2 cells

The optimized methodology was tested on HepG2 human hepatocarcinoma cells. At 70-80% confluence, cells were treated with BSA-conjugated palmitate at a concentration of 250 µM for 8 hours, and samples were prepared from the collected cells as described (10.3311/PPch.15357). An obvious, approximately 5-fold elevation of Cer16:0 ceramide (192 ± 26 vs. 976 ± 232 ng/mg protein; control vs. palmitate-treated, respectively) and Cer18:0 ceramide (15.3 ± 4 vs. 86 ± 15 ng/mg protein; control vs. palmitate-treated, respectively) was observed after the treatment (Fig 4). The observed elevation in stearoyl-

sphingosine (Cer18:0) is likely caused by the cellular build-up of stearoyl-chains through the elongation of palmitate.

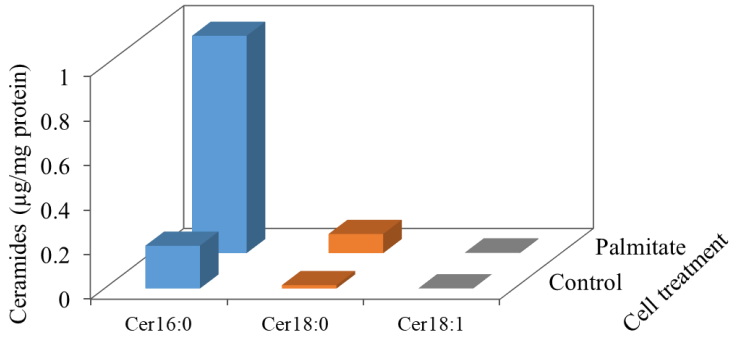

Figure 4. Effect of palmitate treatment on ceramide levels. Cells were treated with BSA (control) or BSA-conjugated palmitate (250 µM) at 70-80% confluence for 8 h. Ceramide levels were measured by LC-MS/MS and normalized to 1 mg protein. Data are shown as means; n=10, RSD% were below 10% in all cases.

We detected a significant accumulation of several DGs containing palmitate derivatives as well (Fig 5). The concentration of dipalmitoyl-glycerol (DG 16:0-16:0) increased 18.5-fold (0.71 ± 0.23 vs. 13.08 ± 2.91 µg/mg protein; control vs. palmitate-treated, respectively) while the palmitoyl-palmitoleoyl-glycerol (DG 16:0-16:1) level elevated 8-fold (0.36 ± 0.06 µg/mg vs. 2.96 ± 0.39 µg/mg; control vs. palmitate-treated, respectively) and palmitoyl-stearoyl-glycerol (DG 16:0-18:0) level increased 6.5-fold (0.12 ± 0.05 vs. 0.77 ± 0.21 µg/mg protein; control vs. palmitate-treated, respectively). As mentioned before, 18:0 FA is an elongation product of palmitate, and 16:1 is also produced in the cells through desaturation of a palmitate molecule.

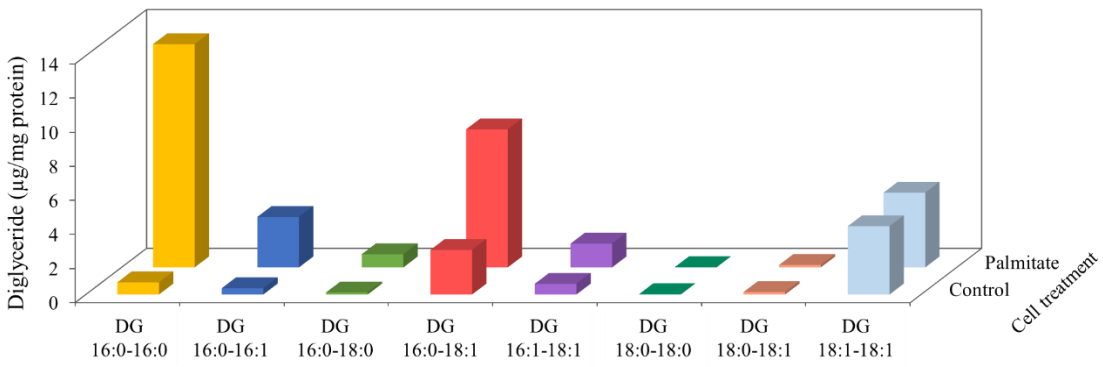

Figure 5. Effect of palmitate treatment on diglyceride levels. Cells were treated with BSA (control) or BSA-conjugated palmitate (250 µM) at 70-80% confluence for 8 h.

Diacylglycerol levels were measured by LC-MS/MS normalized to 1 mg protein. Data are mean values; n=10. RSD% were below 10% in all cases.

The palmitate treatment had a remarkable effect on the ratio of fully saturated DGs (constituted of saturated FAs only) and partly or fully unsaturated DGs (containing at least one unsaturated FA group). The ratio changed considerably, from 0.11 to 0.82 (Fig 6).

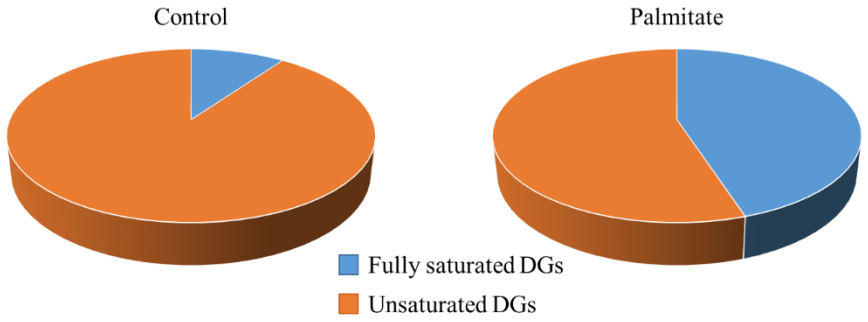

Figure 6. Alteration in the ratio of fully saturated and at least partly unsaturated diglycerides upon palmitate treatment. Cells were treated with BSA (control) or BSA-conjugated palmitate (250 μM) at 70-80% confluence for 8 h. The amounts of saturated (DG 16:0-16:0, DG 16:0-18:0, DG 18:0-18:0) and at least partly unsaturated (DG 16:0-16:1, DG 16:0-18:1, DG 16:1-18:1, DG 18:0-18:1, DG 18:1-18:1, DG 16:1-18:1) DGs were measured by LC-MS/MS.

3.2. Development, validation and application of a GC-FID assay to study cellular incorporation of dietary trans fatty acids

3.2.1. GC-FID assay development

Sample preparation has been developed by improving previously published, fast, one-step methods [71]. Briefly, after harvesting the cells in PBS, the cell suspensions were transferred to glass vials, and mixed with a solution of 2% NaOH in methanol. The NaOH is used for saponification before the esterification step. After a saponification at 90 °C for 30 min, we cooled the samples. For esterification, we added methanol containing boron trifluoride and incubated the samples at 90 °C for 30 min. After cooling, the lipids were extracted with n-hexane and a saturated NaCl solution. The upper phase containing the FAMEs dissolved in hexane was transferred to glass vials for evaporation and resolution in hexane. This evaporation-resolution is essential to eliminate boron trifluoride and

prevent the formation of dative bonds between the BF_3 and the stationary phase, thus, prevent the degradation of the column.

We used a Supelco 37 Component FAME Mix during our preliminary measurements to determine the FAMEs in our samples. First, we identified the peaks of the standard mix by mass spectrometric detection. We also performed preliminary FA treatments with 50 µM of palmitate, oleate, elaidate or vaccenate. Since TFAs are exogenous, control cells do not contain all measured FAs. Then, using flame ionization detection we compared the chromatogram of the FAME Mix and of the cell cultures. According to the retention times, all but one peak could be identified. The exemption was vaccenate (18:1 tΔ11) methyl ester, which was present only in the vaccenate treated cells, and it was not included in the FAME Mix, so it was identified by using a separate standard.

In the final measurements, we used a Zebron ZB-88 capillary column with a (propyl-nitrile)-aryl-dimethyl-polysiloxane stationary phase (60 m × 0.25 mm i.d., 0.20 µm film thickness) with hydrogen carrier gas at 35 cm/s velocity and a purge flow of 3.0 ml/min. The split rate was 5, the injector and the detector temperature were 250 °C, and the oven temperature was ramped from 100 °C to 220 °C at a rate of 4 °C/min. In each measurement, we injected 1 µl of the sample. With this method, we separated 10 FAMEs in a 25-min run (Fig 7).

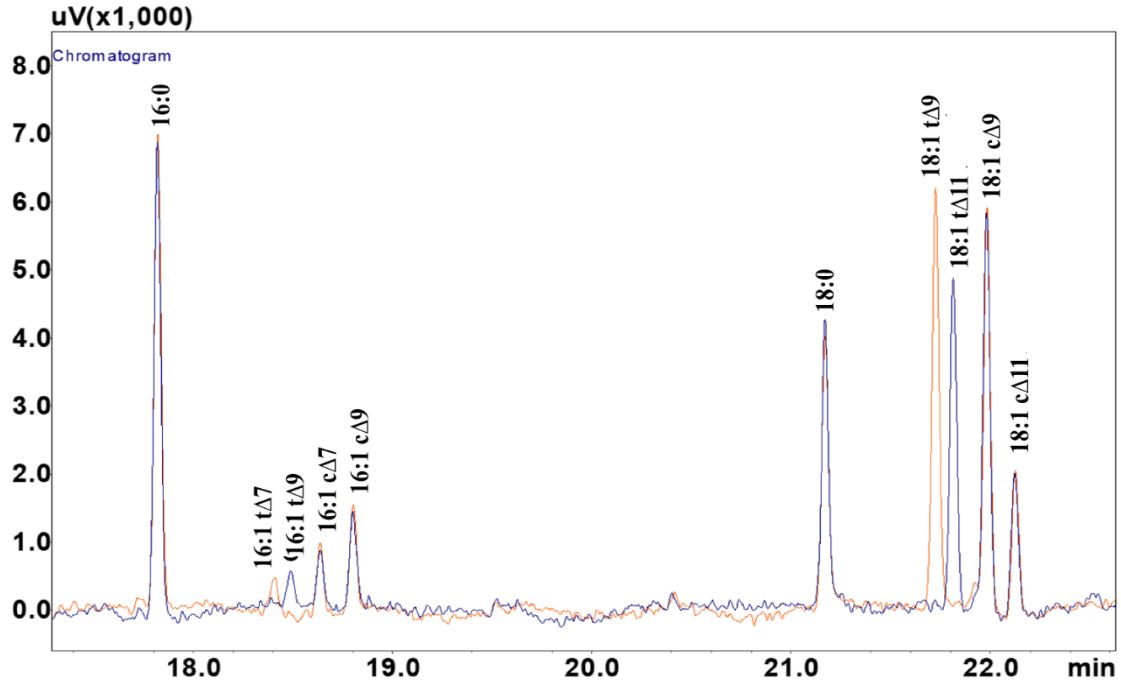

Figure 7. Typical chromatograms of FAMEs in cell samples. RINm5F cells were incubated in a medium containing BSA-conjugated elaidate (red line) or vaccenate (blue line) at 50 μM. Medium was eliminated, and cell samples were withdrawn after 6 h long incubations. Esterified and non-esterified cellular fatty acids were derivatized to methyl esters, and they were separated on a Zebron ZB-88 capillary column with a (propyl-nitrile)-aryl-dimethyl-polysiloxane stationary phase (60 m × 0.25 mm i.d., 0.20 μm film thickness). Ten saturated or cis- or trans-unsaturated FAMEs were detected by flame ionization.

3.2.2. Validation of the GC-FID assay

After optimization, the assay was validated. We checked the specificity, linearity, lower limits of detection and quantification (LOD and LOQ, respectively), resolution, method precision, robustness and recovery.

To check the specificity, we performed our sample preparation method with the solvent and without cell suspension, and we found no peak with the same retention times as the analyzed FAMEs. For the linearity measurements, we prepared standard solutions of FAME standards at eight different concentrations from 1 to 200 μg/ml. Our method was linear in this concentration range with an R^2=0.998 for each compound. The LODs and LOQs were calculated from the mean values of the integration of three general noise peaks and the slope of the calibration curves. The LOD was 0.1 μg/ml for each standard, and the LOQs were between 0.2–0.5 μg/ml.

We used GC Solution software to determine the resolution values for the standard solutions with the highest concentration. Theoretically, the resolution value between two adjacent peaks should be minimum 1.5 for the best evaluation. As shown in Table 2, this was not reached in one case, between the 18:1 tΔ9 (elaidate) and 18:1 tΔ11 (vaccenate) peaks. However, since both FAs are exogenous, there was no elaidate and vaccenate in the same sample at the same time in our experiments. Thus, this does not have effect on the sample evaluation, only on the calibration. We also determined the resolution values for the peaks in cell samples (Table 3). The minimum value for resolution was 2.2; this clearly shows that the evaluation can be processed without any problem.

To investigate the precision of the retention time and the peak area, we injected the same, 10 μg/ml standard solution (for each FAME) five times. The relative standard deviations (RSD%) were <5% for both parameters (Table 4). For the method precision, we prepared and injected three standard solutions from each type of investigated compound

(dipalmitoyl-glycerol for DGs, cis-palmitoelate for FFAs, stearoyl-sphingosine for ceramides and trioleoyl-glycerol for TGs). The measured concentrations and RSD% for each compound are shown in Table 5.

Table 2. Resolution values for the 200 μg/ml standard solution

	Resolution
16:0	n/a
16:1 cΔ9	12.490
18:0	26.930
18:1 tΔ9	5.374
18:1 tΔ11	1.038
18:1 cΔ9	2.037

Table 3. Resolution values for the peaks in cell samples

	16:0	**16:1 tΔ7**	**16:1 tΔ9**	**16:1 cΔ7**	**16:1 cΔ9**	**18:0**	**18:1 tΔ9**	**18:1 tΔ11**	**18:1 cΔ9**	**18:1 cΔ11**
16:0		9.4	10.6	12.7						
16:1 tΔ7	9.4			3.6						
16:1 tΔ9	10.6			2.3						
16:1 cΔ7	12.7	3.6	2.3		2.5					
16:1 cΔ9				2.5		36.2				
18:0					36.2		8.6	10.0	12.8	
18:1 tΔ9						8.6			4.0	
18:1 tΔ11						10.0			2.6	
18:1 cΔ9						12.8	4.0	2.6		2.2
18:1 cΔ11									2.2	

Table 4. Precision of retention times and peak areas

	Retention time		Peak area	
	Mean [min]	**RSD [%]**	**Mean [µVs]**	**RSD [%]**
16:0	17.767	0.006	30352	3.99
16:1 cΔ9	18.748	0.007	25357	3.91
18:0	21.115	0.004	32773	3.83
18:1 tΔ9	21.666	0.004	23560	3.76
18:1 tΔ11	21.757	0.005	19832	4.11
18:1 cΔ9	21.925	0.006	24945	3.79

Table 5. Method precision

	Concentration [µg/ml]	**RSD%**
DG 16:0	22.30	0.46
FFA 16:1 cΔ9	6.03	1.81
Ceramide 18:0	12.46	3.33
TAG 18:1 cΔ9	37.23	5.07

The robustness of the method was determined by changing three parameters of the chromatographic method in two direction each during the measurement of standard solutions: the ramping of the temperature, the velocity of the carrier gas and the oven temperature. The measured concentrations after the changes are shown in Table 6. As all RSD% are below 8.2%, we can confidently say that our method is robust.

Table 6. RSD% of the measured concentrations in the determination of robustness

	3 °C/min	**5 °C/min**	**34 cm/sec**	**36 cm/sec**	**98 °C**	**102 °C**
	RSD%	**RSD%**	**RSD%**	**RSD%**	**RSD%**	**RSD%**
16:0	6.17	5.57	5.62	3.60	7.90	2.90
16:1 cΔ9	6.24	5.63	5.57	3.60	8.16	3.02
18:0	6.34	5.72	5.73	3.66	7.64	2.88
18:1 tΔ9	6.45	5.63	5.80	3.62	7.97	3.02
18:1 tΔ11	6.61	5.83	5.37	3.50	7.22	2.70
18:1 cΔ9	6.68	5.66	5.47	3.62	7.65	2.71

As mentioned above, we used representative compounds for the most important types of lipids. We performed the recovery measurements with these four compounds as well. Standards were dissolved in chloroform in a volumetric flask which was then filled with methanol. Table 7 shows the concentration of spiking solutions. Then, standard solutions were added to samples containing cell suspension and 2% NaOH in methanol, or to samples containing standard solution and 2% NaOH in methanol. We prepared the samples as described from six standard solutions (Table 8) and three unspiked and three spiked samples (Table 9). Regarding the standard solutions, the recoveries for all four compounds were >90%. From cell samples, the recovery of FFA representative cis-palmitoleate was 82%, however, when analyzing biological samples, this is still in the adequate range.

Table 7. Concentration of spiking solutions for the determination of recovery

	m_{std} [mg]	**m_{FAME} (α=100%) [mg]***	**C_{FAME} in spiking solution [µg/ml]**	**$V_{spiking\ sol.}$ [µl]**	**Mass of added FAME [µg]**	**C_{FAME} in the final 200 µl of hexane [µg/ml]**
DG 16:0	0.32	0.30	152.12	10.00	1.52	**6.08**
FFA 16:1 cΔ9	0.85	0.90	448.42	10.00	4.48	**17.94**
Ceramide 18:0	0.28	0.15	73.84	10.00	0.74	**2.95**
TAG 18:1 cΔ9	0.57	0.57	286.28	10.00	2.86	**11.45**

*In case of a 100% conversion during the esterification step.

Table 8. Recovery measurements for standard solutions

	c_{std} [µg/ml]	**Mass in 100 µl**	**$C_{100\%}$* [µg/ml]**	**C [µg/ml]**	**RSD%**	**Recovery [%]**
DG 16:0	30.42	3.04	12.17	10.91	4.92	**90**
FFA 16:1 cΔ9	89.68	8.97	35.87	35.52	2.35	**99**
Ceramide 18:0	14.77	1.48	5.91	5.31	8.32	**90**
TAG 18:1 cΔ9	57.26	5.73	22.90	21.46	6.09	**94**

*In case of a 100% conversion during the esterification step.

Table 9. Recovery measurements for cell samples

	Unspiked cells		Spiked cells		ΔC (α=100%) [µg/ml]	ΔC [µg/ml]	Recovery [%]
	C [µg/ml]	RSD%	C [µg/ml]	RSD%			
DG 16:0	22.30	0.46	28.08	1.88	6.08	5.78	**95**
FFA 16:1 cΔ9	6.03	1.81	20.77	1.88	17.94	14.73	**82**
Ceramide 18:0	12.46	3.33	15.34	1.69	2.95	2.88	**97**
TAG 18:1 cΔ9	37.23	5.07	48.17	0.90	11.45	10.94	**96**

3.2.3. Incorporation of fatty acids into cultured insulinoma cells

With the presented technique, we studied the incorporation of various FAs into RINm5F rat insulinoma cells. Special attention was paid on the two most important dietary TFAs, i.e., elaidate and vaccenate. 50 µM BSA-conjugated palmitate, oleate, elaidate or vaccenate was used for the 6-hour long treatment, and we measured the changes in the cellular FA profile.

Our analysis showed that all of the four FAs were internalized and metabolized by the cells. The elevation of total FA content caused by endogenous saturated palmitate was obvious; the level of 16:0 containing lipid doubled during the treatment (to 61.00 ± 12.84 µg/mg protein). The accumulation of two of its metabolites, the desaturation product 16:1 cΔ9 cis-palmitoleate (8.32 ± 1.31 µg/mg protein vs. 10.13 ± 1.97 µg/mg protein; control vs. palmitate treated) and the elongation product 18:0 stearate (20.26 ± 1.29 µg/mg protein vs. 24.31 ± 3.69 µg/mg protein; control vs. palmitate treated) was detected as well (Fig 8).

Regarding treatment with the endogenous cis unsaturated 18:1 cΔ9 oleate, we detected a doubling in its level, too (to 53.41 ± 7.65 µg/mg protein). However, there was no increase in any of its metabolic products (Fig 8).

The measurement revealed that both exogenous trans unsaturated FAs, the 18:1 tΔ9 elaidate, and the 18:1 tΔ11 vaccenate were also internalized effectively, and their concentration increased from zero to 29.65 ± 4.63 µg/mg protein and 33.32 ± 4.63 µg/mg

protein, respectively. At the same time, in contrast with oleate, the β-oxidation intermediates of these TFAs, which were undetectable in the control cell samples, were also accumulated significantly. We found that the concentration of 16:1 tΔ7 FA increased to 2.24 ± 0.58 µg/mg protein in elaidate-treated cells and the concentration of 16:1 tΔ9 FA increased to 2.76 ± 0.59 µg/mg protein in vaccenate-treated cells (Fig 8).

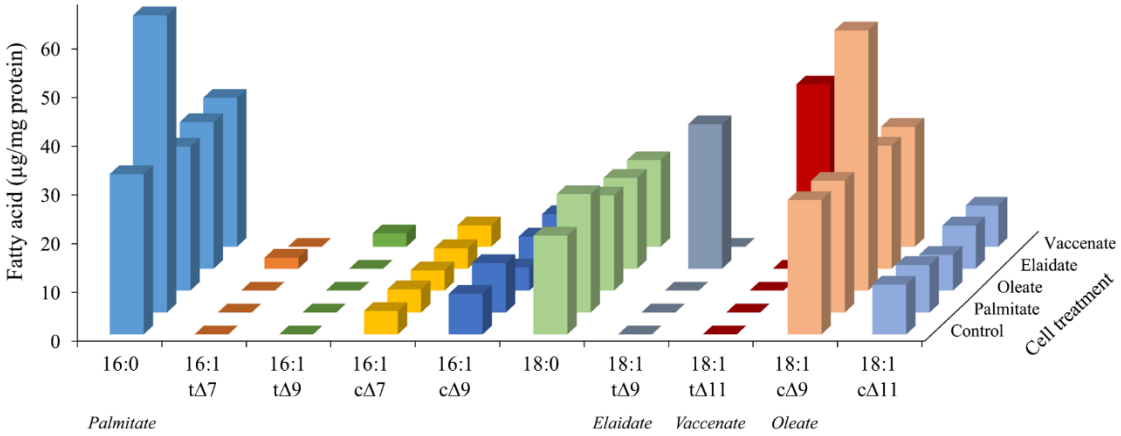

Figure 8. Fatty acid profile of the cells. Cells were incubated in a medium containing FA-free BSA (Control) or BSA-conjugated palmitate, oleate, elaidate or vaccenate (50 µM) as indicated. Medium was eliminated, and cell samples were withdrawn after 6 h long incubation. The amount of 10 saturated and mono-unsaturated FAs was measured by GC-FID after saponification and methylation, and the results were normalized to the total protein content of the samples. Data are shown as means; n = 9. RSD% were below 10% in all cases.

3.3. Investigation of Fatty Acid Desaturation and the Associated Electron Transfer Chain

3.3.1. Changes in the fatty acid profile of HEK293T cells after overexpression of SCD1 or NCB5OR and/or palmitate administration

Transfection efficiency of HEK293T cells was tested and found to be over 80% using pEGFP-N1 expression vector and fluorescent microscopy. Then the cells were transiently transfected with verified constructs of pcDNA3.1 expression vector containing human *SCD1* or *NCB5OR* cDNA. According to our preliminary experiments, the maximum protein concentration in transfected HEK293T cells was reached after 24 h, and maintained between 24 and 48 h, and therefore, cells were harvested and processed 24 h

after transfection. We monitored the efficiency of the transfection and the expression level of the two genes by semi-quantitative RT-PCR analysis (for mRNAs) and Western blot (for proteins). We could detect the endogenous mRNA expression of *SCD1* and *NCB5OR* in control (empty vector transfected) cells, while a large increase in the corresponding mRNA levels was detected upon transfection (Fig 9). Regarding the protein levels, a small amount of endogenous SCD1 protein was revealed, and we could hardly detect the endogenous NCB5OR protein in control cells. After transfection with the expression constructs, both protein levels increased remarkably (Fig 9).

We analyzed the FA content of the cells with the previously described GC-FID protocol. We evaluated the extent of FA desaturation by calculating the ratio of the two major mono-unsaturated FAs over the two major saturated FAs, which also corresponded the main SCD1 products 16:1 cΔ9 cis-palmitoleate and 18:1 cΔ9 cis-oleate and the main SCD1 substrates 16:0 palmitate and 18:0 stearate, respectively. We did not notice any apparent changes in the cellular FA profile after transfection with an empty vector (the unsaturated/saturated ratio was 1.25 ± 0.07 vs 1.25 ± 0.06 in the untransfected vs. mock-transfected cells, respectively) and we used the mock-transfected cells as control in the following experiments. After the overexpression of SCD1, the FA composition of the cells changed pronouncedly (Table 10). An obvious elevation was observed in the level of palmitoleate and oleate, and that of palmitoleate was statistically significant (Table 10). There was a marked elevation in the unsaturated/saturated ratio as well (Fig 9). Regarding the NCB5OR overexpression, we did not detect significant increase neither in the concentration of palmitoleate or oleate, nor in the calculated unsaturated/saturated ratio (Table 10 and Fig 9).

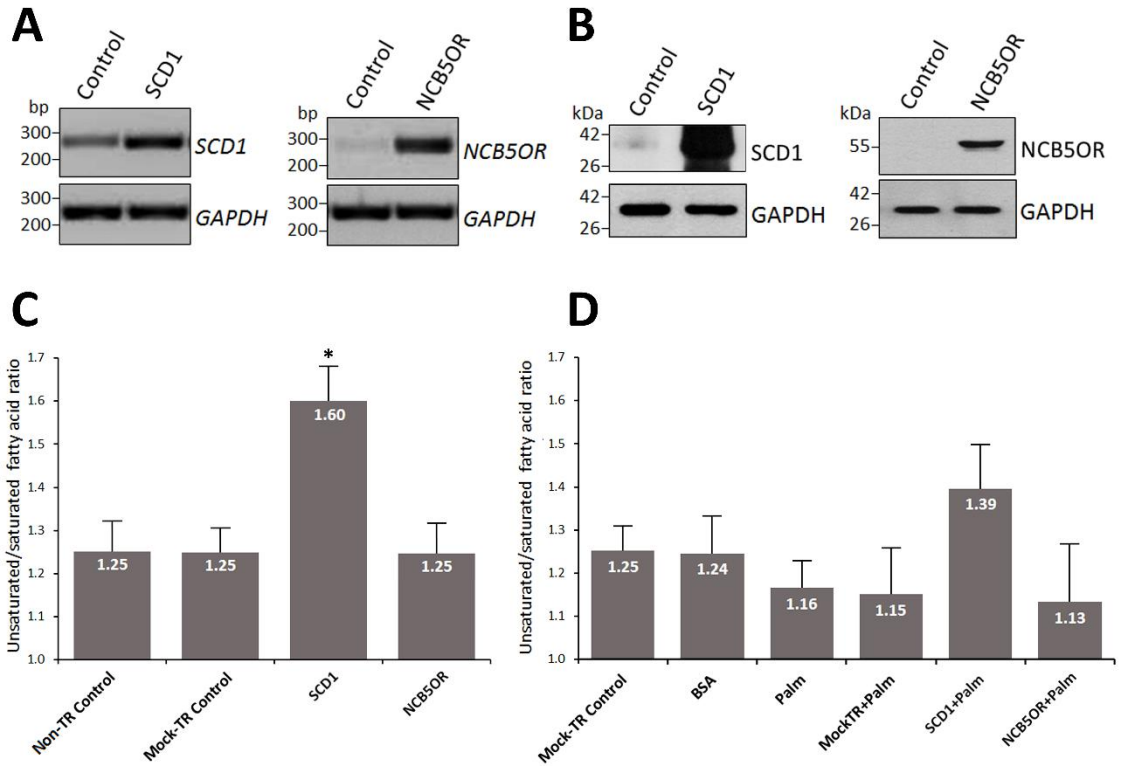

Figure 9. Changes in the fatty acid profile of the SCD1 or NCB5OR transfected HEK293T cells. A. *SCD1* and *NCB5OR* mRNA levels were monitored in transiently transfected HEK293T cells by using semi-quantitative RT-PCR with gene specific primers and by using *GAPDH* as a housekeeping reference gene. A representative ethidium bromide-stained agarose gel image is shown. **B.** The amount of SCD1 and NCB5OR proteins was assessed by Western blot analysis of cell lysates by using specific antibodies and GAPDH as a loading and internal control. A representative immunoblot is shown. **C.** Cells were mock-transfected or transfected with the plasmid expressing *SCD1* or *NCB5OR* genes. They were harvested and FA methyl esters were produced by saponification and methylation 24 h after transient transfection. **D.** The cells (control or 24 h transfected) were treated with BSA or BSA-conjugated palmitate (50 μM) for 15 h. Cells were then harvested, and FA methyl esters were produced by saponification and methylation. **C and D.** The amount of saturated (C16:0, C18:0) and mono-unsaturated (C16:1 cisΔ9, C18:1 cisΔ9) FAs was measured by GC-FID, and the ratio of unsaturated/saturated FAs was calculated. Data are shown in the diagrams as mean values ± S.D.; n=3; *P < 0.01 vs. mock transfected control.

SDC1 and NCB5OR were found to have protective effects against palmitate toxicity in previously published studies [95, 96]. Thus, we decided to test the potential effects of SCD1 and NCB5OR overexpression under a palmitate oversupply. Cells were treated with BSA only as controls or 50 μM BSA conjugated palmitate for 15 h. As a control for palmitate treatment, BSA did not cause any change in FA composition, whereas the concentration of palmitate and stearate increased obviously after palmitate treatment (Table 10). Alongside with this, the unsaturated/saturated ratio of cellular FAs decreased

slightly (Fig 9), which shows that the desaturating capacity was indeed challenged by the palmitate overload. When overexpressing the SCD1, we observed a marked elevation in the levels of palmitoleate and oleate again, and also in the extent of desaturation. However, the overexpression of NCB5OR had no effect on these parameters (Table 10 and Fig 9).

Table 10. Fatty acid profiles. HEK293T cells were transiently transfected with empty pcDNA3.1- (Mock-TR control) or pcDNA3.1-_SCD1, pcDNA3.1-_NCB5OR, pcDNA3.1-_CYB5, pcDNA3.1-_CYB5R plasmid constructs, and they were harvested at 24 h post-transfection. Some 24-h-long transfected cells were also treated with BSA or BSA-conjugated palmitate (50 μM) for 15 hours before sample preparation. Saturated (16:0; 18:0) and mono-unsaturated (16:1 cisΔ9; 18:1 cisΔ9 FAs were measured by GC-FID after saponification and methylation. Data were normalized to the protein content of the samples and are shown as mean values ± S.D.; n=3; *P < 0.01 vs. mock transfected control; #P < 0.01 vs. palmitate-treated mock transfected cells.

	Amount of fatty acid (μg/mg protein)			
	16:0 (palmitate)	**16:1 (palmitoleate)**	**18:0 (stearate)**	**18:1 (oleate)**
Non-TR control	35.34 ± 2.64	15.06 ± 0.99	14.42 ± 1.17	47.23 ± 2.72
Mock-TR control	36.73 ± 2.13	16.36 ± 1.13	14.79 ± 1.11	48.01 ± 3.03
SCD1	34.57 ± 3.05	25.52 ± 1.84*	14.56 ± 1.32	53.08 ± 3.63
NCB5OR	37.12 ± 3.12	16.27 ± 1.34	15.22 ± 1.28	48.95 ± 3.03
BSA	37.59 ± 2.28	15.32 ± 1.01	15.09 ± 1.02	50.17 ± 2.94
Palm	44.35 ± 3.44	18.49 ± 1.33	16.57 ± 1.63	52.42 ± 3.86
Mock-TR+ Palm	42.36 ± 4.56	18.95 ± 1.46	14.50 ± 1.78	46.36 ± 3.62
SCD1+Palm	44.63 ± 4.16	30.40 ± 2.39*#	15.03 ± 1.82	52.66 ± 3.16
NCB5OR+ Palm	48.75 ± 4.33*	21.28 ± 1.57	16.57 ± 1.35	52.59 ± 4.06
CYB5	33.98 ± 2.14	14.20 ± 1.20	13.19 ± 1.11	41.08 ± 3.11
CYB5R	38.91 ± 3.19	12.78 ± 1.29	14.14 ± 1.51	46.50 ± 3.85
CYB5+CYB5R	32.45 ± 2.82	12.68 ± 1.79	12.24 ± 0.99	40.89 ± 3.08
SCD1+CYB5	33.44 ± 2.45	21.79 ± 1.86	13.96 ± 1.01	45.11 ± 3.75
SCD1+CYB5R	36.40 ± 2.96	21.83 ± 1.58	15.45 ± 1.53	47.24 ± 3.23
SCD1+CYB5+CYB5R	32.07 ± 3.03	19.06 ± 1.84	11.67 ± 0.89	40.31 ± 2.98
SCD1+ NCB5OR	39.83 ± 3.20	25.38 ± 1.38*	16.42 ± 1.13	54.48 ± 3.27

3.3.2. Fatty acid desaturation in the cells overexpressing various components of the related electron transfer chains

Our results, regarding the lack of effects of NCB5OR overexpression on desaturation, indicate that either NCB5OR is unable to improve the electron supply of SCD1 or that the electron transfer chain is not saturated thus it does not need enhancement to supply

reducing power for SCD1. Therefore, we decided to overexpress the protein members of the classic chain, CYB5 and CYB5R or both in order to further study the level of desaturation.

Human CYB5 and CYB5R cDNAs were inserted into pcDNA3.1 expression vector, and we transiently transfected HEK293T cells with the verified constructs. 24 h after transfection, we examined the efficiency of transfection and the expression level of the two genes at mRNA and protein levels. We could detect the endogenous mRNAs of both genes in mock-transfected cells by RT-PCR, and as expected, the transfected cells showed elevated mRNA levels. The western blot showed a very low or almost undetectable amount of CYB5 and CYB5R, but after transfection, an obvious elevation was observed in their protein level (Fig 10). The FA composition of the cells was not changed after the overexpression of CYB5, CYB5R or their combination, and the extent of desaturation remained below that of the control cells (Table 10 and Fig 10).

According to our measurements, none of the electron transfer chain proteins could enhance the activity of SCD1. One potential cause may be that the potential of the electron transfer chain is not saturated. Hence, we decided to study the effect of CYB5R, CYB5, CYB5R + CYB5, or NCB5OR overexpression at highly accelerated desaturation in SCD1-transfected cells. Although the corresponding single transfection elevated the gene product levels more effectively, the elevation in the mRNA and protein levels are clearly seen after the double and triple co-transfection as well compared to control (Fig 10). Compared to the single SCD1 transfection, the elevation of the unsaturated/saturated ratio was less significant after co-transfections, and the cause is probably the lower protein level mentioned above. However, we could not detect the expected synergistic elevation in the extent of desaturation in any of the co-transfections (Table 10 and Fig 10).

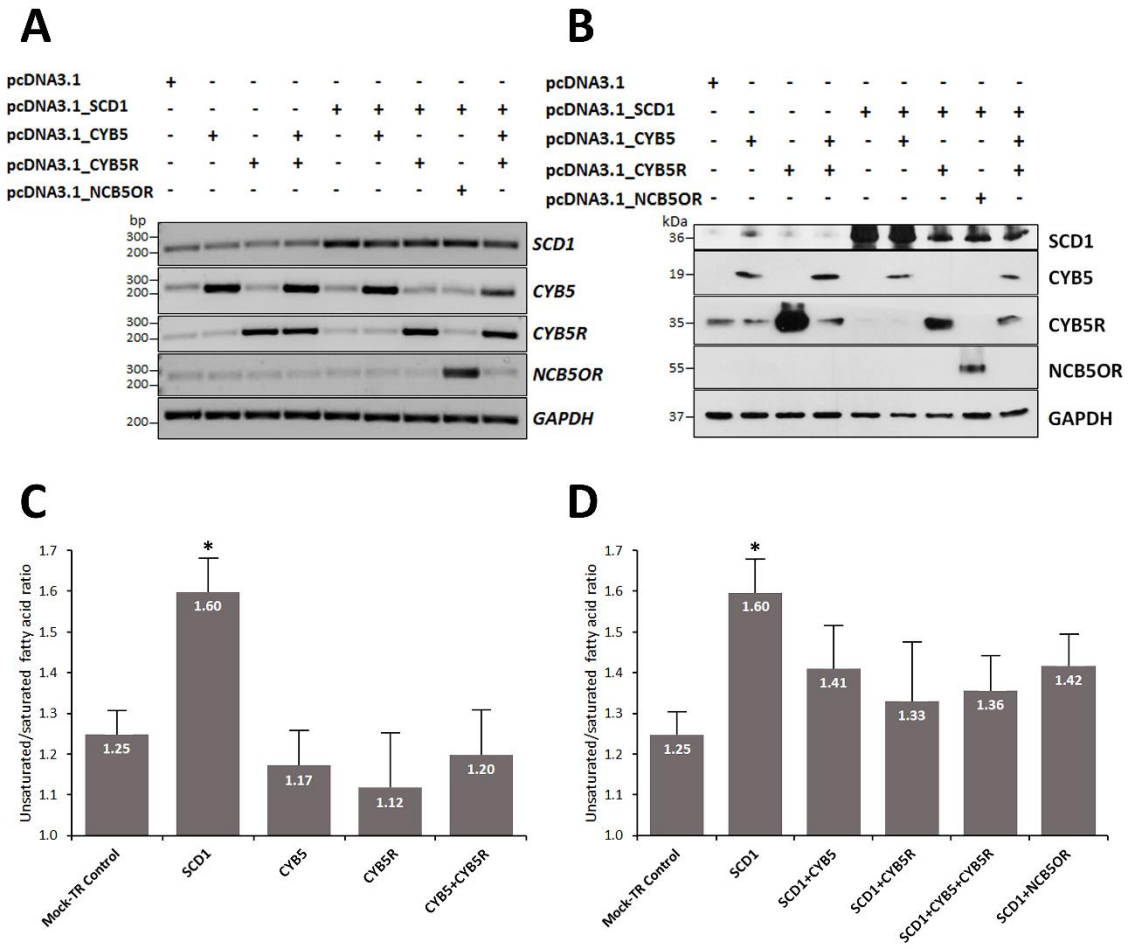

Fig 10. Fatty acid desaturation in the cells overexpressing various components of the related electron transfer chains. HEK293T cells were mock-transfected or transfected with plasmids expressing *SCD1*, *CYB5*, *CYB5R* or *NCB5OR* genes and their combinations. **A.** The mRNA levels were compared by using semi-quantitative RT-PCR with gene specific primers and by using *GAPDH* as a housekeeping reference gene. A representative ethidium bromide-stained agarose gel of three independent experiments is shown. **B.** The amount of SCD1, CYB5, CYB5R and NCB5OR proteins was assessed by Western blot analysis of cell lysates by using specific antibodies and GAPDH as a loading and internal control. A representative immunoblot of three independent experiments is shown. **C and D.** Cells were mock-transfected or transfected with the expression constructs as indicated. They were harvested and FA methyl esters were produced by saponification and methylation 24 h after transient transfection. The amount of saturated (16:0, 18:0) and mono-unsaturated (16:1 cis Δ9, 18:1 cis Δ9) FAs was measured by GC-FID, and the ratio of unsaturated/saturated FAs was calculated. Data are shown in the diagrams as mean values ± S.D.; n=3; *P < 0.01 vs. mock transfected control.

4. Discussion

Chronic elevated FFA levels may lead to type II diabetes [97, 98], NASH [99, 100] and insulin resistance [101-103]. Since FAs have an amphipathic character, they are largely connected to albumin in the plasma. Through the systemic circulation, they reach the cells and penetrate the cell membrane to get metabolized. In the cells, they are conjugated to CoA, and are modified for further utilization or are catabolized to provide energy for the cells. The FA modifications, such as elongation and desaturation, are catalyzed by ER-membrane associated enzyme systems. In the desaturation process, the first and committed event is the insertion of a cis-double bond in the Δ9 position into the FA-chain by SCD1 enzyme [104]. This may be followed by the insertion of additional double bonds at other positions.

The higher toxicity of saturated FAs, such as palmitate, compared to that of unsaturated ones, such as oleate, has been repeatedly demonstrated [22, 105]. Moreover, the protective role of unsaturated FAs was proved *in vitro* in several cell cultures and *in vivo* as well [27, 106, 107]. One reason of the milder toxicity can be the different efficiency of their incorporation into complex lipids. The esterification of saturated FAs into TGs is less efficient in the absence of a proportionate supply of unsaturated ones, thus the co-administration of unsaturated ones can channel the saturated FA-CoA into TG synthesis [108, 109] and lessen the palmitate-induced DG and ceramide accumulation [28, 108, 110]. Since DGs are central intermediates of phospholipid synthesis, the alteration in their structure such as an increasing saturation of the FA chains has direct effects on the composition of membrane lipids. These facts highlight the importance of a balanced unsaturated/saturated ratio in the efficient TG synthesis and in the preservation of the ideal membrane fluidity.

In the analysis of FA composition or the complex lipid content of cell cultures, different analytical methods can be used. Gas chromatography is a common method for the analysis of FA composition of food or biological samples while HPLC-MS/MS technique has an outstanding sensitivity and selectivity which makes it an excellent tool for the analysis of a wide variety of complex cellular compounds with similar structures as well. A major disadvantage of the protocols available at the beginning of my work was the involvement of a time-consuming sample preparation with chloroform as an extracting

agent for lipid analysis [69, 71]. During our research, we developed a GC-FID method for the quantification of endogenous and exogenous FAs and a HPLC-MS/MS method for the quantification of DGs and ceramides. In both measurements, we used rapid sample preparations omitting the carcinogen chloroform. In case of FA profiling, we demonstrated that after the acid-catalyzed esterification, the evaporation of the samples is essential in order to eliminate boron trifluoride and prevent the column from degradation. This validated method is suitable for the quantification of a wide range of FAs including saturated, cis- and trans-unsaturated ones. We also proved that the usage of pure methanol instead of the chloroform/methanol mixture provides the same yield in one step without a lengthy extraction protocol in case of ceramides and DGs.

Our experiments on FA-treated cells revealed an incorporation of the purely exogenous trans FAs as efficient as that of their more physiological saturated and cis-unsaturated counterparts in the cells. Our analysis showed an elevation in the levels of lipids containing the specific FAs administered and also in the level of some metabolic products. We found elevated concentrations of the desaturation and elongation products of palmitate proving that the FA was intensely metabolized by the cells. Our results revealed some differences between the metabolism of the cis-unsaturated oleate and the trans-unsaturated elaidate and vaccenate. We found elevated levels of trans-palmitoleate (16:1 tΔ7) and its positional isomer (16:1 tΔ9), which are the β-oxidation products of elaidate and vaccenate, respectively. However, in case of oleate, the elevation of cis-palmitoleate (16:1 tΔ7) was not observed. A possible reason for this phenomenon may be a different affinity of the mitochondrial long-chain acyl-CoA dehydrogenase (LCAD) enzyme to cis- or trans-unsaturated FAs. It has been demonstrated that LCAD has a different affinity to 5-trans-tetradecenoyl-CoA and 5-cis-tetradecenoyl-CoA, and this might explain the poor oxidation of elaidate compared to oleate [111]. This difference may affect the intensity of FA utilization in several anabolic pathways thus it deserves further investigation.

When we treated HepG2 cells with palmitate in toxic concentration, we observed an obvious accumulation of some biosynthetic lipid intermediates. Regarding DG levels, there was a noticeable change in the ratio of DGs containing only saturated FA chains and of the DGs which contained at least one unsaturated FA chain. Accordingly, the most prominent, 18.5-fold elevation was observed in the level of dipalmitoyl-glycerol. The synthesis of TGs follows a genetically determined pattern, where a saturated FA chain is

usually attached to the first position of glycerol and an unsaturated FA chain occupies the second position [32]. In addition, the specific activity of DGAT2 is twice as high with oleoyl-CoA than with palmitoyl-CoA ([31]). Both facts suggest that the further acylation of the fully saturated DGs thus the synthesis of TGs may be hindered when only saturated acyl-CoA is provided. As mentioned above, the incorporation of saturated FAs into DGs has a direct effect on the composition of membrane lipids. It has been revealed in several studies that increased level of membrane saturation triggered ER stress which eventually may cause inflammation, fibrosis and cell death [5, 112]. In CHO cells, it has been proven that exogenous palmitate was rapidly incorporated into membrane phospholipids (mostly into phosphatidylcholine) [35]. It has also been revealed that the increased saturation of membrane lipid species caused an obvious change in ER structure and membrane integrity. Computational analyses have shown that unsaturated FAs can prevent changes in the membrane fluidity so they can act as membrane stabilizer molecules [36].

Ceramide accumulation may be induced in two ways by palmitate overload. The first step of the *de novo* ceramide synthesis is the condensation of a palmitoyl-CoA and a serine; it is thus possible that an oversupply of palmitate may enhance the synthesis of ceramides through sphinganine production. The elongation product of palmitoyl-CoA is stearoyl-CoA, which is used as a substrate in both the *de novo* and the salvage pathways of Cer18:0 ceramide. When two different enzyme inhibitors were used to reveal which pathways played the key role in the accumulation of ceramides, it was found that both of the pathways contribute equally to the increase [113].

The mitigating effect of desaturation against saturated FA-induced lipotoxicity has been demonstrated in several studies [95, 114, 115]. SCD1 receives electrons from NAD(P)H via CYB5R and CYB5. Beside these proteins, NCB5OR can provide an alternative electron transfer route for SCD1. The NCB5OR knock-out mice show a similar lipoatrophy and a decrease in the ratio of unsaturated/saturated FAs as SCD1 knock-out mice [64]. It is an interesting question that the desaturation capacity of the cell is limited by the expression level of SCD1 or by the conductivity of the electron transfer chains.

When we overexpressed SCD1 alone, a notable shift in the ratio of unsaturated and saturated FAs was observed which is an obvious sign of the enhancement of desaturase activity without metabolic tension. In contrast, when the proteins of the classic electron

transfer chain (i.e., CYB5R and/or CYB5) or the alternative electron supplier NCB5OR were overexpressed, no change in the ratio was observed indicating that there was no accelerated desaturation. This suggests that in the wild-type cells, SCD1 functions at its full capacity, and in case of an elevation in the level of SCD1, the substrate and the electron supply allow a more intense desaturation. This is consistent with the rate-limiting role of SCD1 in the Δ9 desaturation. However, we presumed that a higher demand caused by an intensified SCD1 activity, may exhaust the electron supply provided by CYB5R and CYB5 or NCB5OR. The enhanced activity can be provoked by SCD1 overexpression or a palmitate-treatment, and in these experimental conditions, the electron transfer might become rate-limiting.

Therefore, we treated the cells with palmitate to increase the demand of desaturation and potentially exhaust the electron transfer chain. The FA concentration was chosen based on the literature and our own experiments [5, 116]. This 50 μM palmitate concentration is enough to challenge the SCD1 enzyme however, it does not cause any destructive stress to the cells. According to our data, the treatment induced a significant change in the FA profile of HEK293T cells without any decrease in cell viability or metabolic imbalance. However, neither palmitate-treatment nor elevated SCD1 levels allowed overexpression of the electron transfer proteins to further enhance the desaturation in the cells.

These observations show that in our cellular model, in HEK293T cells, SCD1 itself catalyzes the rate limiting reaction of the Δ9 desaturation and this cannot be further facilitated by a reinforcement of the electron supply. Our results are consistent with the publications about the induction of SCD1 expression upon palmitate treatment [117-119], and in the scientific literature, no data is available about the similar induction of the electron transfer proteins after saturated FA treatment.

5. Conclusion

Lipotoxicity has been receiving a growing attention, and it became evident that elevated FFA levels especially those of the saturated ones, are deleterious to cells. Accumulation of toxic intermediates and increased levels of saturation of membrane lipids can induce ER stress and hence it may cause severe malfunction of cells. SCD1 inserts the first double bond into the saturated fatty acyl chains, and hence this enzyme plays a central role in the desaturation of endogenous and exogenous FAs. SCD1 receives electrons from NAD(P)H via CYB5R and CYB5. NCB5OR, an oxidoreductase with a CYB5R-like and CYB5-like domain, may provide an alternative electron transfer chain. Because of the presence of an alternative electron transfer route, and the protective role of NCB5OR against palmitate toxicity, we may assume that there is a need of an enhancement of the chain in case of FA oversupply.

During our research:

- We developed and validated a simple and reliable method using gas chromatography with flame ionization detection for the quantification of 10 saturated and unsaturated FAs**.** Beside the GC-FID analysis, we achieved the simultaneous detemination of 14 ceramides and diglycerides. Our studies revealed that both the saturated and the cis and trans unsaturated fatty acids were incorporated efficiently, and that palmitate treatment caused an obvious elevation in the level of fully saturated diglyerides and a shift in the ratio of fully saturated DGs and partly unsaturated DGs, and that palmitate treatment caused a significant intracellular Cer16:0 and Cer18:0 ceramide accumulation.
- We concluded that the overexpression of SCD1 enhanced the desaturation and increased the unsaturated/saturated FA ratio; however, the overexpression of the proteins of the classic or alternative electron transfer chain could not further facilitate the desaturation.
- In our cellular model, the desaturation-associated electron transfer could not be considered as a rate-limiting step in the desaturation process even under enhanced desaturation of saturated FA overload.

Our research revealed that the regulation of SCD1 expression can play an important role in the protection against lipotoxicity, thus it deserves further investigation. Moreover,

considering the facts that in NCB5OR knock-out mice, the desaturation is damaged, and the mice have lipoatrophy and insulin-dependent diabetes while the electron transfer does not have role in the rate-limiting step of desaturation, it is worthwhile to investigate an alternative protecting mechanism of NCB5OR against lipotoxicity. In addition, the change in the DG structure and the intracellular ceramide accumulation may play an important role in saturated FA toxicity.

6. Summary

It became evident that permanently elevated FFA levels are deleterious to cells. Saturated FAs are particularly harmful while cis unsaturated ones are partly protective. In addition, *in vivo* studies assign harmful effect to dietary TFAs while little is known about the metabolism of these components. SCD1 is a key enzyme of FA metabolism which receives electrons from NAD(P)H via CYB5R and CYB5. NCB5OR is an oxidoreductase with a CYB5R-like and CYB5-like domain, thus it may play a role in FA desaturation. Because of the presence of an alternative electron transfer chain, we may assume that there is a need of an enhancement of the chain in case of FA oversupply. Precise analytical methods for the profiling the cellular lipid components are essential for the thorough investigation of the metabolic stress caused by excess FAs in the cells.

For the analysis of lipid content of the cells, we developed two sensitive and robust methods with simplified sample preparations which avoid the use of chloroform. Our HPLC-MS/MS method is suitable for the simultaneous detection of four ceramides and ten DGs. With this optimized method, we detected cellular accumulation of ceramides and DGs in the cells after an 8 h long treatment with BSA-conjugated palmitate (250 μM). The pronounced ceramide accumulation observed in palmitate-treated cells is consistent with the blockade of the pathway of TG synthesis at the phase of fully saturated DGs. Our GC-FID method enables the quantification of ten saturated and unsaturated FAs. We observed differences between the metabolism of cis and trans unsaturated FAs in RINm5F cells since we detected accumulating catabolic intermediates of the latter ones. After the optimization of the analytical methods, we overexpressed SCD1, CYB5R, CYB5, NCB5OR, or their combinations in human HEK293T cells and analyzed the FA profile and the alteration in the unsaturated/saturated ratio to investigate the influence of the expression level of proteins involved in the desaturation process on the desaturating activity. Our results suggest that the level of the desaturase enzyme itself but not the expression of the associated electron transfer chain components defines the capacity to desaturate FAs in our cellular model. Considering the fact, that NCB5OR has a protecting effect against lipotoxicity, it deserves a further investigation regarding an alternative protecting mechanism.

7. References

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8. Bibliography of the candidate's publications

Publications related to the thesis:

1. Anna Somogyi; Judit Mátyási; Zsófia Gór-Nagy; Farkas Sarnyai; Miklós Csala; Blanka Tóth
   Application of Gas Chromatography – Flame Ionization Detection to Study Cellular Incorporation of Dietary Trans Fatty Acids of Medical Importance
   PERIODICA POLYTECHNICA-CHEMICAL ENGINEERING 65: 2 pp. 149-157., 9 p. (2021)
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2. Anna Somogyi; Mária Berinkeiné Donkó; Farkas Sarnyai; Gergely Becskereki; Miklós Csala; Blanka Tóth
   Simultaneous Quantitative Determination of Different Ceramide and Diacylglycerol Species in Cultured Cells by Using Liquid Chromatography–Electrospray Tandem Mass Spectrometry
   PERIODICA POLYTECHNICA-CHEMICAL ENGINEERING 64: 4 pp. 421-429., 9 p. (2020)
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3. 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)
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Publications not directly 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, 15 p. (2020)
   IF: 4.556

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
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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
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4. Anna Somogyi; György Horvai; Miklós Csala; Blanka Tóth
Analytical Approaches for the Quantitation of Redox-active Pyridine Dinucleotides in Biological Matrices
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