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

Anna Somogyi

Molecular Medicine Doctoral School

Semmelweis University

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

Dr. Blanka Tóth Ph.D.

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

Dr. Olivér Ozohanics Ph.D.

Complex Examination Committee

Head: Dr. Péter Enyedi, MD. D.Sc.

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

Dr. Viola Horváth, Ph.D.

Budapest

2021

Introduction

Lipotoxicity has received a growing attention in the past decade, and it became evident that elevated free fatty acid (FFA) levels are 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 and It has been demonstrated that the key events are the FFA-induced endoplasmic reticulum (ER) stress, changes in mitochondrial functions and the altered lipid metabolism with accumulation of toxic intermediates. Several publications proved that saturated FAs such as palmitate are more toxic than unsaturated ones. Dietary trans FAs, i.e., elaidate and vaccenate have controversial assessment. In addition to the milder toxicity of unsaturated FAs, the potential protective effects of oleate against palmitate toxicity is receiving growing attention. Co-administration of oleate with palmitate could prevent activation of the unfolded protein response in β-cells, blocked phosphorylation of extracellular signal-regulated kinases (ERK1/2) and c-jun-N-terminal kinase (JNK) in neuronal cells, and it protected oleate protects against palmitate-induced mitochondrial DNA damage and decline in ATP level in muscle cells. In case of trans FAs, their co-administration with palmitate also attenuated the toxic effect of palmitate to in rat insulinoma cells.

Recent publications show that the accumulation of triglycerides (TG) might have a protective role. Since TGs are neutral and non-toxic complex lipids, channeling the excess of FFAs into TG synthesis can be an effective way to ameliorate the toxic effect of FFAs. However, during the synthesis of triglycerides, normally, a saturated fatty acyl group is attached the first position of glycerol and an unsaturated fatty acyl group occupies the second position. Thus, the surplus of saturated or trans fatty acids may block the triglyceride synthesis. Increased levels of saturation of membrane lipids can induce ER stress and hence it may cause severe malfunction of cells or even apoptosis. Unsaturated FAs can prevent changes in the membrane fluidity so they can act as membrane stabilizer molecules according to computational analysis.

Ceramides, the FA amides of sphingosine, are backbones of sphingolipids and have roles in cell signaling. The *de novo* synthesis and the salvage pathways use up 3 molecules of FA-CoAs, thus the formation of these lipids is also an effective way of decreasing the elevated FFA level. Publications showed that in rat insulinoma cells and primary rat pancreatic islets, ceramide analogue increased β-cell apoptosis, and it induced JNK phosphorylation and caspase 3 cleavage which caused a collapse in the mitochondrial membrane potential. It has been also revealed that oleate prevents the palmitate-induced ceramide accumulation in CHO cells and in rat insulinoma cells.

During the metabolism of fatty acids (FA), FA-CoA molecules can be elongated or desaturated by ER-associated enzymes. In humans, one of the key enzymes of desaturation is the stearoyl-CoA desaturase 1 (SCD1) which inserts a cis double bond at the Δ9 position into saturated fatty acids. SCD1 inserts a cis double bond at the Δ9 position into saturated FAs. Since unsaturated FAs are essential for the synthesis of TGs, SCD1 has a crucial role in providing the appropriate substrate supply. 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.

SCD1 receives electrons from NAD(P)H via cytochrome b5 reductase (CYB5R) and cytochrome b5 (CYB5). NCB5OR is an oxidoreductase which has a cytochrome b5 reductase like and cytochrome b5 like domain as well, thus it may also play a role in fatty acid desaturation. NCB5OR knock-out mice have lipoatrophy and insulin dependent diabetes which suggest that in these mice, the desaturation in damaged. Primary hepatocytes yielded from NCB5OR knock-out mice have increased sensitivity to palmitate in contrast to hepatocytes from wild type mice. 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.

Objectives

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., diglycerides 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.

Methods

HepG2 human hepatocarcinoma cells were cultured in Minimum Essential Medium Eagle containing Earle's Salts and sodium bicarbonate, supple-mented with 2 mM L-glutamine, 1 % non-essential amino acids, 10 % FBS and 1 %, at 37 °C in humidified atmosphere, containing 5 % CO_2. RINm5F rat insulinoma cells were cultured in RPMI 1640 medium, supplemented with 10% FBS and 1% antibiotics, at 37 °C in humidified atmosphere containing 5% CO_2. HEK239T human embryonic kidney cells were cultured in DMEM medium supplemented with 10% FBS and 1% antibiotics at 37 °C in a humidified atmosphere containing 5% CO_2.

The coding sequence of human CYB5, CYB5R, SCD1, and NCB5OR were PCR amplified by using appropriate primers. The template cDNA had been produced by reverse transcription of mRNA isolated from HepG2 cells. The purified PCR products were cloned into pcDNA3.1 plasmids between their appropriate restriction sites.

For transient transfection, 6 × 105 HEK293T cells were seeded per well in six-well plates. Cells were transfected with 2 μg of DNA using 10 μL of Lipofectamine 2000 reagent in 250–250 μL of Opti-MEM, according to the manufacturer’s instructions. Complexes were added to cells in serum- and antibiotics-free DMEM, and cells were harvested 24 h after transfection.

Palmitate, elaidate, oleate and vaccenate were diluted in isopropanol and conjugated with fatty acid free BSA in 1:4 ratio, at 37 °C for 1 hour. The working solution for treatments was prepared in FBS-free and antibiotic-free medium at 0.05, 0.25 or 0.5 mM final concentration. The culture medium had been replaced by FBS-free and antibiotic-free medium for 1 hour before the treatments.

For Western blot analysis cells were washed twice with PBS and harvested in lysis buffer by scraping. The lysates were centrifuged in a benchtop centrifuge. Protein concentration of the supernatant was measured. Samples were electrophoresed in SDS polyacrylamide gels and transferred to PVDF membranes. Primary and secondary

antibodies were applied overnight at 4 °C and for 1 h at room temperature, respectively. Equal protein loading was validated by detection of GAPDH. Western blot detection was carried out with chemiluminescence detection.

Total RNA was purified from the cells and cDNA was produced by reverse transcription. Equal loading was validated by detection of GAPDH. DNA samples were separated by electrophoresis in agarose gel and visualized by EtBr staining.

For fatty acid, ceramide and DG analysis, cells were washed once with PBS, then harvested in PBS by scraping. The samples were then sedimented in a benchtop centrifuge, and the supernatants were discarded. The cells were suspended in PBS, and the protein concentration was measured.

Methanol containing 2 W/V% NaOH was added to the cell suspension in the crimp vials, the samples were incubated at 90 °C for 30 min, and then cooled to room temperature. Methanol containing 13-15% of boron trifluoride was added to the samples, and the vials were incubated at 90 °C for 30 min. After cooling to room temperature, saturated NaCl solution and n-hexane were added. Fatty acid methyl esters were extracted to the upper phase containing n-hexane, and this phase was transferred to a vial for GC analysis.

For HPLC-MS/MS analysis the cells were pelleted by centrifugation and resuspended in methanol containing ceramide 17:0 internal standard. The samples were homogenized with an ultrasonic sonotrode and centrifuged. The supernatants were transferred to vials for HPLC-MS/MS analysis.

The results of western blot analyses and DNA gel electrophoresis were carried out by densitometry using ImageQuant 5.2 software and are shown as relative band densities normalized to a GAPDH as a reference protein. Data are presented in the diagrams as mean values ± S.D. and were compared by ANOVA with Tukey's multiple comparison post hoc test using GraphPad Prism 6 software. Differences of a P value below 0.01 were considered to be statistically significant.

Results

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

- We developed a sensitive and reproducible method, which allows the **simultaneous detection of a wide array of ceramides and diglycerides** (DGs) by **using** reverse-phased high performance liquid chromatography and electrospray tandem mass spectrometry (**HPLC-ESI-MS/MS**) after a simplified sample preparation.
- The compounds were separated in a C8 column with a gradient elution by methanol and ammonium acetate buffer in 14 minutes and they were detected by a triple quadrupol in a multiple reaction monitoring mode. The common analytical lipid extraction employing a methanol/chloroform mixture was replaced by a shorter and simpler procedure, which uses methanol and an ultrasonic agitation. Non-physiological ceramide analogue containing heptadecanoate (17:0), was used as internal standard.
- After optimization of the preparation process, the methodology was tested on hepatocarcinoma cell cultures. **Cellular accumulation of biosynthetic lipid intermediates was detected in the cells after treatment with BSA-conjugated palmitate.** We observed a **5 times elevation in level of ceramides containing palmitate or palmitate-derivate stearate**. Regarding the DGs, the amount of DGs containing at least one palmitate chain increase and **there was an obvious alteration in the ratio of fully saturated DGs and at least partly unsaturated DGs (from 0.17 to 1.51)**. 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.

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

- **A simple and validated method of gas chromatography with flame ionization detection was developed for the quantification of 10 saturated and unsaturated FAs.** The sample preparation uses a fast one-step reaction avoiding the use of chloroform. Calibration was linear between 0-200 μg/ml for each FAs with an R^2 >0.99. Recovery was 82% for unesterified FAs and >95% for complex lipids such as ceramides, DGs and TGs. Limit of detection and limit of quantitation were below 0.5 μg/ml. Robust method precision were achieved (RSD% was below 6% for each lipid classes).
- The method was tested on palmitate-, oleate-, elaidate- and vaccenate-treated rat insulinoma cells. **Our analysis showed that all 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. The accumulation of two of its metabolites, the desaturation product 16:1 cΔ9 cis-palmitoleate and the elongation product 18:0 stearate was detected as well.
- Regarding treatment with the endogenous cis unsaturated 18:1 cΔ9 oleate, we detected a doubling in its level, too. However, there was no increase in any of its metabolic products.
- The measurement revealed that both exogenous trans unsaturated FAs, the 18:1 tΔ9 elaidate, and the 18:1 tΔ11 vaccenate were also internalized effectively. 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.**

Investigation of Fatty Acid Desaturation and the Associated Electron Transfer Chain

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

- **The cells were transiently transfected with verified constructs of pcDNA3.1 expression vector containing human *SCD1* or *NCB5OR* cDNA** and were harvested and processed 24 h after transfection. **A large increase in the mRNA and the protein level of both SCD1 and NCB5OR** was observed after transfection.
- **The FA content** of the cells **was analyzed** with the developed GC-FID method, and **the extent of FA desaturation was evaluated by calculating the ratio of the two major mono-unsaturated FAs** (main SCD1 products 16:1 cΔ9 cis-palmitoleate and 18:1 cΔ9 cis-oleate) **over the two major saturated FAs** (main SCD1 substrates 16:0 palmitate and 18:0 stearate). After the **overexpression of SCD1**, the FA composition of the cells changed pronouncedly. An obvious elevation was observed in the level of palmitoleate and oleate, and **there was a marked elevation in the unsaturated/saturated ratio** as well. 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.**
- **Cells were treated with BSA only as controls or 50 μM BSA conjugated palmitate for 15 h** to test the potential effects of SCD1 and NCB5OR overexpression under a palmitate oversupply. As a control, BSA did not cause any change in FA composition, whereas **the concentration of palmitate and stearate increased obviously after palmitate treatment**. Alongside with this, **the unsaturated/saturated ratio of cellular FAs decreased slightly,** which

shows that the desaturating capacity was indeed challenged by the palmitate overload.

- **Overexpression of SCD1 caused a marked elevation in the level of palmitoleate and oleate, and in the extent of desaturation. However, NCB5OR had no effect** on these parameters.

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

- **The cells were transiently transfected with verified constructs of pcDNA3.1 expression vector containing human *CYB5* or *CYB5R* cDNA or their combination** and were harvested and processed 24 h after transfection. A large **increase in the mRNA and the protein level of both CYB5 and CYB5R** was observed after transfection.
- **The overexpression of CYB5 and CYB5R or their combination did not cause change in the FA composition of the cells**, and the extent of desaturation remained below that of the control cells.
- One potential cause of the inability of the electron transfer proteins to cause an enhancement in the activity of SCD1 may be that the potential of the electron transfer chain is not saturated. **Thus, to study the effect of CYB5R, CYB5, CYB5R + CYB5, or NCB5OR overexpression at highly accelerated desaturation, we co-transfected the cells with them along with SCD1.** The **elevation in the mRNA and protein levels are clearly seen** after the double and triple co-transfection as well compared to control. Although the extent of **desaturation was elevated compared to the control cells, we could not detect the expected synergistic elevation in the extent of desaturation in any of the co-transfections.**

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 determination of 14 ceramides and diglycerides. Our studies revealed 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.

Abbreviations:

FFA: free fatty acid, **ER**: endoplasmic reticulu, **TG**: triglyceride, **DG**: diglyceride, **FA-CoA**: fatty acid coenzyme A, **JNK**: c-jun aminoterminal kinase, **SCD1**: stearoyl-CoA desaturase 1, **CYB5R**: cytochrome b5 reductase, **CYB5**: cytochrome b5, **NCB5OR**: NAD(P)H cytochrome b5 oxidoreductase, **GC-FID**: gas chromatography – flame ionization detection, **HPLC-MS/MS**: high performance liquid chromatography – tandem mass spectrometry, **cDNA**: complementary DNA, **BSA**: bovine serum albumin, **SDS**: sodium dodecyl sulphate, **GAPDH**: glyceraldehyde 3-phosphate dehydrogenase.

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)

   IF: 1.257

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)

   IF: 1.257

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)

   IF: 3.057

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
   FOOD AND CHEMICAL TOXICOLOGY 124 pp. 324-335. , 12 p. (2019)
   IF: 3.775

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. Anna Somogyi; György Horvai; Miklós Csala; Blanka Tóth
   Analytical Approaches for the Quantitation of Redox-active Pyridine Dinucleotides in Biological Matrices
   PERIODICA POLYTECHNICA-CHEMICAL ENGINEERING 60 : 4 pp. 218-230. , 13 p. (2016)
   IF: 0.556