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

PhD thesis outline

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Introduction

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

The cell damage caused by chronic FA surplus is referred to as lipotoxicity, and it has a key role in the pathology of certain obesity-related metabolic disorders. Lipotoxicity induced in pancreatic β-cells is of particular importance as it hinders the compensation of insulin resistance, which intensifies the primary damage through further increase in FA release from the adipocytes. Several *in vitro* studies have been performed to investigate the molecular mechanism of β-cell lipotoxicity by using various insulinoma cell lines and the major endogenous saturated and monounsaturated FAs, i.e., palmitate (16:0) and oleate (18:1 cis-Δ9), respectively. It has been revealed that noxious ER stress aggravated by the accumulation of toxic lipid intermediate such as ceramides, is a key factor in the escalation of apoptosis and autophagy. Lipotoxic ER stress activates signaling pathways that lead to pro-apoptotic transcriptional changes via XBP-1 mRNA cleavage and phosphorylation of eIF2α. Severe malfunction of the ER causes a further activation of SAPK/JNK and diverse caspase cascades, which also enhance β-cell apoptosis.

Saturated palmitate and cis-unsaturated oleate have been widely investigated and a remarkably greater toxicity of the former FA has been repeatedly demonstrated. In contrast, very little is known about the toxicity of dietary trans-fatty acids (TFAs) and

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

Not only has it been repeatedly demonstrated that saturated FAs are more harmful than the unsaturated ones, the ability of oleate to reduce the toxicity of palmitate has also been reported, and it has also become a widely investigated phenomenon. Administration of oleate and palmitate at the same time was shown to be less toxic than palmitate alone in various cell types. It has also been observed that activation of the UPR triggered by palmitate can be prevented or at least diminished with the co-administration of oleate. These observations raised a very interesting question, whether or not the TFAs also alleviate the palmitate induced toxicity like oleate does.

Objectives

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

- to compare the toxicity of the two major dietary TFAs (i.e., elaidate and vaccenate) with that of the best studied endogenous saturated and cis-unsaturated FAs (i.e., palmitate and oleate) in RINm5F cells,
- to determine the change in FA profile and in the ceramide and diglyceride levels after palmitate, oleate, elaidate or vaccenate treatment,
- to test the potential mitigating effect of elaidate and vaccenate on palmitate-induced toxicity,
- to investigate how oleate, elaidate and vaccenate influence palmitate-induced alterations in the FA profile and the intracellular accumulation of ceramides and diglycerides.

Methods

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.

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 0.25 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.

Cell viability was assessed by using the Colorimetric (MTT) Kit for Cell Survival and Proliferation according to the manufacturer's instructions. MTT-derived formazan was measured with spectrophotometer. Cell viability was expressed as the percentage of viable cells in the total cell population.

Apoptotic and necrotic cells were detected by using fluorescence microscopy. Cells with green fluorescence (Annexin V labeling) were considered as apoptotic while those with red or both green and red fluorescence (propidium iodide DNA staining) were considered as necrotic. Apoptosis index was calculated as (number of apoptotic cells) / (number of all cells counted) × 100.

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. Spliced and unspliced XBP-1 sequences were amplified by PCR and the

products were purified by PEG precipitation. For a better visibility, PstI restriction endonuclease cleavage was carried out by using PstI enzyme. The unspliced XBP-1 is cut in two fragments by PstI while the spliced variant remains uncut. 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 0.05 were considered to be statistically significant.

Results

Viability, apoptosis, stress and ER stress

- Palmitate was found to decrease cell viability in a dose dependent manner. Cell viability in samples treated with oleate was much higher, as expected based on previous observations. **The comparison between the three observed unsaturated FAs did not show any difference.** Neither cis- nor trans-unsaturated FAs reduced the viability significantly. In combination treatment with oleate, the damage caused by palmitate was lessened significantly in accordance with several earlier observations. **Importantly, in case of the trans-unsaturated elaidate and vaccenate co-treatments, significant viability improvements could also be observed. It is also remarkable that no obvious difference was observed between the protective effect of the two trans-unsaturated FAs.**

- To reveal the contribution of apoptotic cell death in the change of cell viability, apoptotic index was determined. Palmitate treatment caused an increase in apoptotic index. **The three unsaturated FAs alone caused a small and statistically not significant elevation in the apoptotic index when compared to the control level.** A distinct change can be observed upon unsaturated FA co-treatments compared to palmitate-treated cells, as **the simultaneously added unsaturated FAs lessened the amount of apoptotic cells significantly.**

- The amount of cleaved caspase-3 was found to increase in palmitate-treated cells compared to control levels. **The single treatment with either of the unsaturated FAs caused only a slight elevation in the level of caspase-3 compared to the control cells.** The cells co-treated with palmitate and either of

the unsaturated FAs contained lower amount of the cleaved caspase-3 enzymes compared to palmitate alone treatment. Oleate co-administration caused a remarkable decrease compared to palmitate. **An attenuation of palmitate-induced caspase cleavage was also seen upon co-treatment with one of the TFAs, yet it was statistically significant only in case of elaidate.**

- After palmitate treatment, an enhanced splicing was indicated by the sXBP-1/uXBP-1 ratio. However, the cis- and trans-unsaturated FAs only caused a minor and non-significant elevation of the ratio compared to control. The addition of oleate next to palmitate caused the ratio to diminish compared to palmitate alone. **The TFA co-treatment also resulted in a moderation of sXBP-1/uXBP-1 ratio, where elaidate and vaccenate caused a significant lessening in the ratio compared to palmitate.**

- The eIF2α phosphorylation was assessed, by comparing the levels of phosphorylated and overall eIF2-α levels. Palmitate caused a significant increase in the P-eIF2α levels compared to control cells, **while the single treatments with unsaturated FAs were deemed insignificant.** Co-treatment with cis-unsaturated oleate resulted in a remarkable decrease in the palmitate-induced phosphorylation. **The trans-unsaturated vaccenate was almost as potent as oleate, while co-administration with trans-elaidate resulted only in a non-significant decrease in the P-eIF2α levels.** The chaperon induction comes with a delay as generally the transcriptional regulations do. **Accordingly, no elevation was detected in the levels of BiP and PDI as long-term ER stress markers.**

- The isoforms of the activated JNK were detected by immunoblotting and as expected, palmitate raised the phosphorylation of the JNK five-fold compared to

control samples, **while the three unsaturated FAs caused a much smaller increase in JNK phosphorylation.** The co-treatment with the cis-unsaturated oleate remarkably reduced the palmitate-induced JNK activation and the level of P-JNK was slightly, but not significantly higher than in the control. **Interestingly, however, neither of the TFAs affected the palmitate-triggered JNK phosphorylation significantly.**

Changes in the fatty acid profile and in the accumulation of ceramides and DGs:

During a 24-hour long treatment, the incorporation of the FAs and the ceramide and DG accumulation were assessed in different times.

- **The investigation proved that all the FAs were incorporated into the cells efficiently**. Although the same concentration of palmitate was applied in single and co-treatments, **intracellular amount of palmitate in co-treatments did not reach at any given time the values observed after palmitate alone treatment.** As a result of desaturation and elongation, an elevation can be observed in stearate and cis-palmitoleate levels after palmitate treatment. Interestingly, **the addition of both TFAs caused an obvious accumulation of their degradation intermediate, which was in a striking contrast to oleate.**

- The LC-MS/MS measurement revealed an increase in the amount of the most important DG types containing 16 and 18 long carbon chains from saturated and mono-unsaturated FAs. Altogether, the palmitate treatment caused a remarkable increase in the overall DG levels compared the control cells. **The effects of oleate treatment differed greatly from those of palmitate.** The increase in overall DG level was the largest at 4 h of oleate treatment, when its amount tripled. It is also notable that the amounts of the four DGs which are the most

markedly elevated in palmitate-treated cells, were always found to be decreased in oleate-treated cells. **More or less the same changes can be observed in the cells after TFA treatment.** The overall DG amount stayed far below the level observed after palmitate treatment and was more similar to that induced by oleate.

- **After oleate co-treatment almost all of the DG levels showed a less pronounced increase**, and the biggest difference was observed in the levels of the two most abundant DGs, where a lower level compared to palmitate treated cells could be seen. Co-treatments with one of the two TFAs induced yet another course of DG accumulation. **The overall DG kept increasing and reached their maximum after a 16-hour long incubation, which was twice the amount detected in oleate-co-treated samples. Comparing the two TFAs to each other, it is apparent that the elaidate co-treatment resulted in higher levels of almost all DG species.**

- Palmitate treatment caused a dramatic increase in the overall ceramide levels, compared to control cells. In line with its smaller cytotoxicity, oleate increased the overall ceramide level much less, and the two TFAs had similar effects on the investigated ceramide species. Nevertheless, t**he formation of elaidyl- and vaccenyl-sphingosines in TFA treated cells was an order of magnitude above that of oleoyl-sphingosine upon oleate treatment**, which is a conspicuous difference between cis-oleate and the two TFAs. .

- The simultaneous addition of oleate to palmitate-treated cells caused a much smaller accumulation of ceramides altogether. **The TFAs, had similar effect**, and they reduced palmitoyl- and stearoyl-sphingosine accumulation almost as effectively as oleate, **but a much more pronounced elevation in elaidyl- and**

vaccenyl-sphingosines compared to oleoyl-sphingosines was still observed.

Conclusions

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

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

- All of the FAs were incorporated and metabolized by the cells; however, there is a significant difference between the catabolism of cis and trans unsaturated FAs as the products of the first step of β-oxidation of TFAs were obviously accumulated, but no such phenomenon was seen in case of oleate.

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

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

- The presence of oleate reduced the amounts of ceramides and DGs containing saturated FAs chains remarkably in co-treatments. While the TFAs also reduced the ceramide and DGs levels in co-treatments, over the time, the DG levels reached a remarkably high amount, which began to approach the levels observed in palmitate treatments. These data further support the role of ceramides in β-cell lipotoxicity and they also reveal an important difference between the metabolism of oleate and TFAs.

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

Abbreviations:

BiP/GRP78: binding immunoglobulin protein/glucose-regulated protein 78, **BSA**: bovine serum albimun, **cDNA**: complementary deoxyribonucleic acid, **eIF2-α**: eukaryotic initiation factor 2-α, **FBS**: fetal bovine serum, **GAPDH**: glyceraldehyde 3-phosphate dehydrogenase, **GC**: gas chromatography, **HPLC-MS/MS**: high performance liquid chromatography-mass spectrometry/mass spectrometry, **JNK**: c-Jun aminoterminal kinase, **MTT**: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, **RNA**: ribonucleic acid, **SDS**: sodium dodecyl sulfate, **PDI**: protein disulfide isomerase, **XBP-1**: X box-binding protein-1

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