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Diet induced altered fatty acid composition and the activation of their metabolizing enzymes in mice

by

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INTRODUCTION

Dietary fat is a macronutrient which delivers substrates to generate metabolic energy and mediators to influence biochemical pathways regulating lipid metabolism and inflammatory responses. More importantly dietary fat provides fatty acids (FA) to build and maintain cellular membranes surrounding cells and cellular compartments. The main FAs incorporated into cellular membranes belong to the family of saturated fatty acids (SAFA) and monounsaturated fatty acids (MUFA). Additionally polyunsaturated fatty acids (PUFAs) can be found in cellular membranes. In this thesis the content of MUFA, SAFA and PUFA in membrane phospholipids was investigated whether determined by gene expression of specific metabolizing enzymes (SCD1, ELOVL6, FADS1, FADS2 and ELOVL5) or by diet derived FAs. Stearoyl-CoA desaturase-1 (SCD1) and elongase 6 (ELOVL6) are major metabolizing enzymes of MUFA/SAFA metabolism. The regulation of their gene expression is strictly associated with the MUFA and SAFA content in phospholipids (PL) of cellular membranes. PUFAs on the other hand are metabolized by fatty acid desaturase 1 (FADS1), fatty acid desaturase 2 (FADS2) and elongase 5 (ELOVL5) but their content in PL seems mainly determined by dietary fat. This thesis aims to investigate associations of FA composition incorporated into PL to gene expression of FA metabolizing enzymes and dietary fat intake.

THEORETICAL BACKGROUND

Monounsaturated fatty acids (MUFAs) and ***Saturated fatty acids (SAFAs)*** – MUFAs contain one double bound which can appear in different positions. They are incorporated into cell membranes by up to 45% depending on type of tissue and cell compartment and they are therefore one of the most abundant FAs in cellular membranes. The most important members of the MUFA-family distributed in human cellular membranes are palmitoleic acid (POA, 16:1n7), oleic acid (OA, 18:1n9) and vaccenic acid (VC, 18:1n7). Oleic acid (OA), as the main fatty acid in olive oil, contributes to health benefits such as reduction of accumulation of hepatic triglyceride and improved glucose response in insulin resistant subjects. MUFAs as well as SAFAs are not essential but can be obtained from diet. The typical Mediterranean diet pattern contains high amounts of MUFAs due to usage of olive oil as the main dietary fat source often combined with a high intake of polyunsaturated fatty acids (PUFAs) deriving foods like fish and vegetables serving in general health benefits. SAFAs are found in animal derived fats like lard and butter and in plant derived fats like coconut oil. They are distributed in animal tissues by 30-40% in phospholipids. The most common SAFAs are palmitic acid

(PA, 16:0) contained in animal tissue between 15 and 25% and stearic acid (SA, 18:0) and contribute to animal tissue between 10 and 20%. PA (16:0) and SA (18:0) are also the most abundant SAFAs in human cellular membranes. Consumption of diets high in SAFAs as in Western diet are not recommended because associations to development of metabolic and cognitive disorder. However despite their negative health impact SAFAs are crucial for regular cell function. **MUFA/SAFA metabolism and metabolizing enzymes -** The d*e-novo* synthesis of SAFAs is strictly regulated by e.g. dietary influences. However, MUFAs and SAFAs derived from diet can also directly enter into the endogenous MUFA/SAFA pool. Dietary MUFAs and SAFAs are further indirectly involved in the regulation of gene expression of enzymes belonging to the *de-novo* synthesis. ***Stearoyl-CoA desaturase 1 (SCD1)*** – SCD1 (Δ-9-desaturase) belongs to the desaturase family, mainly expressed in white adipose tissue (WAT) and strongly inducible in liver. SCD1 inserts an additional double bound into SAFAs and is the rate-limiting enzyme for endogenous MUFAs derived from SAFAs. PA and SA are main substrates which are desaturated into POA and OA respectively. ***Elongase 6 (ELOVL6)*** – ELOVL6 elongates SAFAs like PA and MUFAs such as POA and OA by two carbons. The metabolic products of these reactions are SA, VC and eicosenoic acid (EA, 20:1n9). **Specific MUFA/SAFA ratios of SCD1 and ELOVL6** - Enzymatic activity of fat metabolizing enzymes was found to correlate with specific FA substrate/product ratio. The ratios of 18:1n9/18:0 and 16:1n7/16:0 describe the enzymatic activity of SCD1 known as “desaturation index” or “SCD1 index”. Any alterations in gene expression pattern of SCD1 are proposed to correlate with these ratios. ELOVL6 specific FA ratios 18:0/16:0 and 18:1n7/16:n7 decrease in ELOVL6-/- models due to less metabolic products of ELOVL6 18:0 and 18:1. Reduction of these specific FA ratios was observed after an n3-PUFA enriched diet.

Polyunsaturated fatty acids (PUFAs) - PUFAs, mainly n3- and n6-PUFAs, are essential FAs which cannot be generated in humans and have to be provided by diet. Higher n3-PUFAs such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are characteristic for animal fat such as fish oil or seal fat while n6-PUFAs like linoleic acid (LA) are rather found in plant oils such as corn oil, safflower oil, soybean oil, sunflower oil and plant oil based dietary fats like margarine. Sunflower oil contains mainly linoleic acid (18:2n6), an n6-PUFA which is one of the main dietary fats consumed in Western diet. The intake of n3-PUFAs originating especially from fish oil is recommended because of their

positive correlation to reduce the risk for cardiovascular diseases and their immunomodulatory function. However dietary oils high in n6-PUFAs like LA are preferably consumed. The imbalance of dietary n3-PUFAs and n6-PUFAs ratio is also in discussion to promote the development of chronic diseases. n6-PUFA-rich dietary oils are in general considered a healthier substitute to dietary fats rich in SAFAs assuming to reduce possible health risks caused by SAFA-rich diets. **PUFA metabolism**-n3- and n6-PUFAs are mainly metabolized by fatty acid desaturase 1 (FADS1), fatty acid desaturase 2 (FADS2) and elongase 5 (ELOVL5). The metabolism starts with the desaturation of n3-PUFA ALA and n6-PUFA LA by FADS2. FADS2 desaturates ALA to 18:4n3 and LA to GLA. 18:4n3 and GLA are further elongated by ELOVL5 to 20:4n3 and DHGLA respectively followed by a desaturation by FADS1 to EPA and AA respectively. A further elongation by ELOVL5 leads to DPA in the n3-PUFA pathway and to 22:4n6 in the n6-PUFA pathway. FADS2 desaturates then DPA to DHA and 22:4n6 to 22:5n6. The available amounts of n3- and n6-PUFAs by dietary intake influence directly this metabolic competition. **PUFA metabolizing enzymes**-The enzymatic activity of FADS1, FADS2 and ELOVL5 is believed to determine partially the endogenous content of PUFAs. Also dietary supplementation with PUFAs can alter the endogenous PUFA pool. Dietary supplementation with n3-PUFA, for example, increases the content of n3-PUFAs EPA and DPA in humans derived directly from diet or by increased enzymatic activity of metabolizing enzymes. On the contrary supplementation with dietary fat high in n6-PUFA LA, as typical in Western diet, did not increase the n6-PUFA metabolite AA in plasma/serum phospholipids. However, dietary AA supplementation did increase AA content in phospholipids in plasma of mice. This suggests a regulation of endogenous available AA derived from n6-PUFAs like LA by the metabolizing enzymes FADS1 or FADS2, which are rate limiting for the conversion of GLA to AA. **FADS1** – FADS1 has been found rate limiting for endogenous AA content. A significant increase of 20:3n6 and reduction of 20:4n6 can be observed in phospholipids in FADS1-/- mice compared to their wild type counterparts. **ELOVL5** – ELOVL5 is crucial for the 18:4n3 content, an elongation product of 18:3n6. In ELOVL5-/- mice elongation of 18:3n6 and 18:4n3 by 88-90% was reduced. **FADS2** – The knock-out of FADS2 lead to a reduction of enzymatic products (n3-PUFA EPA and DHA) and higher level of substrates (n3-PUFA ALA) in phospholipids in mice receiving a canola or flaxseed diet both rich in LA. **Specific PUFA ratios for FADS1, FADS2 and ELOVL5-** The most frequently reported FA ratio to reflect FADS1 enzymatic activity is 20:4n6/20:3n6. This ratio was reported to increase after n3-PUFA rich diet suggesting an increase of enzymatic activity in healthy subjects. The ratio 18:3n6/18:2n6 is

frequently proposed to represent FADS2 enzymatic activity which increases with n3-PUFA enriched diet, but also reduces in healthy subjects after fish oil supplemented diet. Several FA ratios are proposed to represent the enzymatic activity of ELOVL5. Increased ratios of 20:3n6/18:3n6, 22:4n6/20:4n6, 22:5n3/20:5n3, 20:5n3/18:3n3, 22:5n3/18:3n3, 22:5n3/20:5n3 were observed by ELOVL5 overexpression in transfection models.

Gene expression of MUFA and SAFA metabolizing enzymes SCD1 and ELOVL6 - Regulation of SCD1 and ELOVL6 gene expression is mediated through specific TFs like nuclear hormone receptors (NHRs) activated by dietary and synthetic ligands. Dietary inducers of SCD1 gene expression are SAFAs, cholesterol, glucose and fasting. A diet rich in PUFA or conjugated LA on the other hand suppresses the gene expression of SCD1. This suppression is mediated through SREBP-1c shown in liver of wild type and obese mice. The ELOVL6 gene is also a SREBP-1c target gene. Promoter studies found SREBP-1c specific binding sequences within the ELOVL6 promoter. **Gene expression of PUFA metabolizing enzymes ELOVL5, FADS1 and FADS2** – Genes of ELOVL5, FADS1 and FADS2 can be regulated by NHRs which act via SREBP-1c. FADS1 and FADS2 gene expression is induced by high carbohydrate diet or by combination of high carbohydrate diet with 18:1n9 and high SAFA. Also gene expression of ELOVL5 is reported to be induced by high carbohydrate diet. Although FADS1 and FADS2 are mainly target genes of SREBP-1c additional regulation by activation of LXRα for FADS1, FADS2 and ELOVL5 in secondary manner via the SCREBP-1a pathway has been reported. Diets high in PUFAs like EPA, DHA and AA suppress the expression of FADS1 and FADS2 in liver assumed by the activation of PPARα.

Nuclear Hormone Receptors (NHRs) regulated by Fatty Acids - Nuclear hormone receptors (NHRs) are transcription factors which after stimulation induce or suppress gene expression of their specific target genes. Activation of NHRs can be achieved by specific synthetic ligands or by natural ligands derived from diet. FAs are potential activators for the group of NHRs peroxisome proliferator activated receptor (PPAR) (α, β/δ, γ), but also for the NHR liver-X-receptor (LXR) and several other TFs like the sterol regulatory element-binding protein (SREBPs). LXR and SREBP1c can be activated by dietary SAFAs. NHRs retinoic acid receptor (RAR) and retinoid X receptor (RXR) can be activated by FAs but remain more sensitive to forms of vitamin A. PUFAs like DHA, AA and DPA were found to bind to the ligand domain of the RXR. RAR, PPARα, β/δ, γ and LXR are required to build heterodimers with RXR for their functionality. **NHRs and Vitamin A -** Vitamin A is a fat soluble vitamin derived from plants as pro-vitamin β-carotene and from animals as retinyl-ester (RE). Main

metabolic active forms of vitamin A 9-cis retinoic acid (9-cis-RA) and all-*trans* retinoic acid (ATRA) are accounted for most of the physiological functions of vitamin A. NHRs activated by active forms of vitamin A are retinoid X receptor (RXR) and retinoic acid receptor (RAR). RAR is more sensitive to ATRA as a ligand while RXR responds to ATRA and 9-cis-RA. Recently 9-cis-13,14-dihydroretinoic acid has been presented as a more potential metabolic forms of vitamin A. RA content is crucial for the activity of RXR and RAR. RA is catabolized to inactive metabolites all-*trans*-4-oxo-retinoic acid and all-*trans*-4-hydroxy-retinoic acid by the enzyme cytochrome Cyp26A1. **Connecting vitamin A and fatty acid regulatory pathways by CYP26A1 -** Regulatory pathways of vitamin A and FAs are crosslinked to each other by the activation of RXR. RXR is crucial to form heterodimers with other NHRs regulating fatty acid controlled target genes. An activation of RXR can be achieved by several forms of RA; e.g. as recently published by 9-cis-13,14-dihydroretinoic acid. The clearance of RA is dependent on enzymatic activity of CYP26A1. This enzyme is induced by vitamin A but also by n3-PUFAs like DHA which could lead to an increased clearance of RA. Less available RA could lead to a reduced activation of RXR which could affect the gene expression of its target genes including fat metabolizing enzymes.

Fatty Acids determine Phospholipids (PLs) and Membrane Composition - FAs fulfil one of their major roles in providing the basic structures for the construction of cellular membranes. Cellular membranes are assemblies of PLs, cholesterol and glycoproteins forming a lipid-bilayer that compartments cells and endogenous cell bodies like endoplasmic reticulum or lipoproteins. PLs are triglycerides esterified with FAs on two hydroxyl positions and a phosphate residue on position one, which classifies as e.g. PL-choline. In cellular membranes PLs form double layers consisting of two PL-layers. PLs are grouped into several subclasses like phosphatidylcholine (PC), sphingomyelins and phosphatidylethanolamine (PE) which are the most abundant PLs in membranes depending on the cell type. Lyso-phosphatidylcholine (LPC) contains only one esterified FA, while in other PLs two FAs are found. The constant turnover of cellular membranes allows adaptation of their composition depending on the type of dietary fat available. Alteration of composition of membranes by e.g. dietary fat influence their fluidity, signal transduction, functionality of membrane bound enzymes, cellular metabolic rate and transport proteins leading to altered cellular signals but is also of further interest as biomarker for dietary fat intake and for association with development of chronic diseases.

MATERIALS AND METHODS

Animal Studies - All studies were performed *in vivo* on a mouse model. In the phase of acclimatization 6-8 weeks old C57BL6 female mice were fed for two weeks with general animal chow diet. 8 animals were housed per cage at an average temperature of 22°C with a daily 12h light and 12h dark cycle. The animals had access to water and food ad libitum. After the two weeks of acclimatization the specific treatment or diets were applied. Animal experiments were performed at the Laboratory Animal Core Facility of the University of Debrecen in accordance to the ethical guidelines of Hungary.

Study 1 – Influence of Dietary Fat - Six to eight-week-old female C57BL6 mice were fed first for two weeks with regular chow. After the acclimatization period, the animals were divided into different feeding groups (n = 6 each group) and received for 4 weeks diets containing each a different kind of a dietary fat. **Study 2 – Synergism of dietary fat and Vitamin A -** After the acclimatization period animals received a vitamin A deficient (0 RE/kg diet) diet containing 5% sunflower oil as dietary lipid for 10 weeks, which represented a diet with a normal fat content. Animals were divided into different feeding groups (n = 6 per group) and were fed for 4 weeks with specific diets containing different amounts of dietary fat and equal amounts of vitamin A (2,500 RE/kg diet, normal vitamin A). Additionally the normal fat/normal vitamin A diet group was augmented by two other normal fat diet groups receiving different amounts of vitamin A, 0 RE/kg diet (vitamin A deficient) and 326,500 RE/kg diet (vitamin A high). **Study 3 - Regulation by NHRs -** After the acclimatization period, animals were gavaged daily for one week with specific synthetic ligands dissolved in 25% Cremophor EL/water (v/v).

Sample Preparation - Animals were anaesthetized by halothane and the blood was taken by cardiac puncture. Blood and organs were obtained. Organs were frozen immediately in liquid nitrogen and plasma was immediately removed and frozen after centrifuging of the blood samples at max. speed for 10 minutes. Organs and plasma were stored at -80°C.

Analysis of Gene Expression by QRT-PCR - Hepatic gene expression was measured by expression level of mRNA of the desired target gene. RNA was extracted from tissue, transcribed into cDNA and amplified by QRT-PCR.

Analysis of PLs in plasma and FA composition in diet by GC – The analysis of frozen plasma (50 µl) and diet samples via GC was performed by Prof. Dr. Tamas Decsi and Dr.

Tamas Marosvölgyi (University of Pecs, H). Diet and plasma samples were analyzed by gas chromatography. For the analysis frozen samples were thawed and the pentadecanoylphosphatidylcholine as internal standard was added. Lipids were extracted by the addition of 3 ml chloroform and 1 ml methanol according to the method of Folch. Lipid extracts were reconstituted in 70 µl chloroform and lipid classes were separated by thin layer chromatography (TLC). Fatty acids were analyzed by high-resolution capillary GC using a Finnigan 9001 gas chromatograph with split injection, automatic sampler and flame ionization detector with a DB-23 cyanopropyl column of 40 m length. The temperature program was set to the following parameters: temperature of injector at 80°C/min up to 280°C, temperature of detector at 280 °C, temperature of column area at 60°C for 0.2 min, temperature increase by 40°C/min up to 180 °C, 5 min isothermal period, temperature increase by 1.5 °C/min up to 200 °C, 8.5 min isothermal period, temperature increase by 40 °C/min up to 240 °C and 13 min isothermal period. The constant linear velocity was 0.3 m/s (referred to 100 °C). Peak identification was confirmed by comparison with authentic mixtures of weighed fatty acid (FA) methyl esters (GLC-463: Nu-Chek Prep, Elysian, MN, USA; and Supelco 37 FAME Mix: Supelco, Bellefonte, PA, USA). In study1 all values were normalized to coconut fat (=1) to compare diet groups with each other and using coconut fat as comparison group.

Analysis of liver and plasma samples by electrospray ionization tandem mass spectrometry (ESI-MS/MS) – PLs were analyzed in plasma and liver. Frozen tissue samples and frozen plasma samples were sent for the analysis of FA composition in specific PLs via ESI-MS/MS to Prof. Dr. Gerd Schmitz and PD Dr. Gerhard Liebisch (University of Regensburg, D). Liver homogenates and plasma were extracted according to the procedure described by Bligh and Dyer et al. in the presence of non-naturally occurring lipid species as internal standards. Lipids were quantified by ESI-MS/MS in positive ion mode as described previously by Brugger et al. and Liebisch et al. The amounts of incorporated FAs were calculated as percentages.

Statistics - Results were displayed as mean and standard error mean. Statistical analysis was performed by Kruskal-Wallis- test followed by paired analysis in study 1 and by ANOVA followed by the Bonferroni post hoc test for study 2 and study 3 using the program SPSS. Statistically significant differences were accepted and displayed from a value of $p < 0.05$ and $p<0.01$.

5. RESULTS

In three *in vivo* studies on mice main enzymes of the SAFA, MUFA and PUFA metabolism were studied and the consequences on fatty acid (FA) contents in phospholipids (PLs). PLs in plasma were analyzed in total in their composition by gas chromatography (GC) and for selected PLs subgroups measured in plasma in liver by electrospray ionization tandem mass spectrometry (ESI-MS/MS). The first study investigated regarding the altered gene expression of enzymes of the MUFA/SAFA and PUFA metabolism by several dietary fats. The study of PUFA rich dietary fat -sunflower oil- in combination with the micronutrient vitamin A examined the synergism of inducing and repressing signals on the gene expression of MUFA and SAFA metabolizing enzymes SCD1 and ELOVL6. Furthermore altered hepatic gene expression of SCD1 and ELOVL6 by activation of selected NHRs was studied.

Study 1 – Influence of Dietary Fat

Fatty acid composition of diets - The composition of selected FAs in diets applied in this study was analyzed by GC. Coconut fat diet displayed more than 90% SAFAs of all measured fatty acids, especially 16:0 (18.2%) and 18:0 (20.5%). Margarine contained a mixture of SAFA (31.3%), MUFA (44.6%) and PUFA (n6-PUFAs 19.0%, n3-PUFAs 5.03%). The characteristic FA in olive oil was the MUFA 18:1n9 (70.4%), while n6-PUFAs (10.6%) and SAFAs (15.2%) were found in less amounts. Sunflower oil mainly contained the n6-PUFA 18:2n6 (67.2%), further MUFA (20.0%) and SAFAs (11.6%). Fish oil displayed highest amounts of n3-PUFAs (37.1%) among the investigated dietary fats, mainly EPA (16.1%) and DHA (13.7%). Further 31.6% SAFA and 24.8% MUFA were measured in fish oil diet.

MUFA/SAFA content in PLs is determined mainly by enzymatic activity of SCD1 and ELOVL6 regulated by dietary fatty acids

SCD1 - The amounts of SAFAs 16:0 and 18:0 measured in diet differed from those measured in PLs, which was true for all experimental diets. The only agreement observed between diet and PLs was for the MUFA 16:1n7 which was lowest in both diet and PLs after sunflower oil supplemented diet. No other agreement between PLs and diet of the MUFA and SAFA composition could be found. A comparison between the hepatic gene expression to specific SCD1 ratios in diet and in PLs was only reflected in the ratio of 18:1n9/18:0 in PLs.

ELOVL6 - Single fatty acids (16:0, 18:0, 16:1n7, 18:1n7) involved in the ELOVL6 metabolic pathway measured in diet did not reflect the values in PLs. The enzyme specific ratios of fatty acids in PLs did not reflect the hepatic gene expression of ELOVL6 except for the fish oil fed group which showed lowest values of these ratios together with hepatic gene expression.

FADS1, FADS2 and ELOVL5 influence PUFA content only marginal, mainly dietary PUFAs determine PUFA content in PLs

FADS1 and FADS2 - 18:2n6 was measured highest in sunflower oil supplemented diet as well as in PLs of this diet group. 20:5n3, 22:5n3 and 22:6n3 were highest in diet and in PLs of the fish diet supplemented group. In similar fashion, 18:3n3 was highest in diet and in PLs of margarine supplemented diet group. FADS1 specific FA ratio calculated in diet did not reflect a similar pattern in PLs. Ratio of 20:4n6/20:3n6 is comparable in diet and PLs in fish oil diet group. FADS2 specific FA ratios showed that 18:2n6/18:3n6 were highest in fish oil supplemented diet also calculated highest in PLs in the fish oil supplemented diet group. Further 20:5n3/18:3n3 highest in fish oil diet group was also highest in PLs. Comparing the hepatic gene expression with specific enzyme ratios in PLs there was an agreement to FADS2 specific FA ratio of 22:6n3/22:5n3. This was lowest in PLs of fish diet group in agreement with lowest gene expression of FADS2 in this diet group.

ELOVL5 - Substrate and product ratio of ELOVL5 was highest in diet and in PLs of coconut fat supplemented diet group. Only the enzyme specific ratio of ELOVL5 22:5n3/20:5n3 was lowest in fish oil supplemented diet and in PLs of animals fed a fish oil supplemented diet. Comparing the hepatic gene expression to enzyme specific ratios in PLs only the lowest amount of 22:5n3/20:5n3 in fish supplemented diet group agreed with the lowest gene expression of ELOVL5 in this feeding group.

CYP26A1 is induced by Fish Oil Diet - Hepatic gene expression of CYP26A1 was significantly increased in fish oil supplemented diet group compared to margarine supplemented diet. A higher gene expression level was also measured in coconut fat supplemented diet group which was significant compared to margarine diet group. Further in the highest hepatic gene expression of CYP26A1 was measured after activation of RAR which was significant compared to control.

Study 2 - Synergism of dietary fat and vitamin A - Inductive effect of vitamin A abolished in combination with sunflower oil

The highest hepatic gene expression of SCD1 and ELOVL6 was observed in mice fed a low fat (LF) compared to mice fed a high fat (HF) diet. The hepatic gene expression of ELOVL6 was significantly higher in mice fed LF diet compared to the other diets. The alteration of gene expression of these two enzymes in liver displayed a tendency of decrease with increasing amount of dietary fat. NF diets were supplemented with different amounts of vitamin A to determine the effect of vitamin A on gene expression and fatty acid composition. Different amounts of dietary vitamin A had no effect on the hepatic expression of SCD1 and ELOVL6. Dietary fat did not affect the total contribution of SAFAs in PLs. However, the content of the FA 16:0 bound in PLs decreased after a HF diet compared to a LF diet. In contrast, mice fed a HF diet showed higher plasma values of the fatty acid 18:0 bound in PLs compared to NF and LF diet. The analysis via GC revealed that the total amount of MUFA was not influenced by dietary vitamin A. However, the ratio of 18:1n9/18:0 decreased significantly in mice fed VA deficient diet compared to those fed the high vitamin A diet. Other ratios were not influenced by dietary vitamin A. LF diets resulted in more MUFAs than SAFAs being incorporated in PLs in both liver and plasma. In contrast, mice fed HF diets incorporated more SAFAs and less MUFAs into PLs. The total amount of plasma SAFAs was not influenced by different amounts of vitamin A. However, when looking at individual SAFAs and specific PL, there was a significant increase in plasma amounts of LPC 18:0 in mice fed with the high VA diet compared to the VA deficient diet. In contrast, plasma amount of LPC 16:0 were higher in animals receiving a deficient vitamin A diet compared to animals receiving a high vitamin A diet. This result was also observed in plasma PLs analysed by GC incorporating lower amounts of 16:0 and higher amounts of 18:0 after a high vitamin A diet. The analysis of PE, PC and LPC species in liver and plasma showed an increase of LPC 18:1 and LPC 16:1 amounts in liver and plasma of mice fed VA deficient diets compared to high vitamin A diet. This increase has also been observed in liver and plasma for PC 32:1 and 34:1 as well as for PE 34:1.

Study 3 - Regulation by NHRs - RXR and LXR but not PPARs induce Gene Expression of SCD1 and ELOVL6 and alter the PL composition accordingly

Hepatic SCD1 and ELOVL6 gene expression significantly increased in animals gavaged with LXR and RXR ligands, while treatment with PPARα, PPARβ/δ and PPARγ ligand resulted in no significant alteration. Application of an RAR ligand led to a slight, non-significant suppression of hepatic SCD1 expression. The ELOVL6 gene expression was not influenced by the application of the RAR synthetic ligand. FA composition plasma PL for substrates, products and product/substrate ratios of SCD1 and ELOVL6 enzymes in the treatment groups are compared to the vehicle group. PA (16:0) percentages decreased significantly in the LXR, RAR and RXR groups, whereas significantly increased in the PPARα group. SA (18:0) percentages decreased significantly in the RXR, whereas increased significantly in the RAR group. Percentages of POA (16:1n7) did not differ, whereas VC (18:1n7), OA (18:1n9) and EA (20:1n9) percentages increased significantly in the LXR and RXR groups. The product/substrate ratios exhibited significant increases following treatment (with the exception of the 20:1n9/18:1n9 ratio). Specifically, significant increases of the 16:1n7/16:0 ratio were observed in the LXR group, of the 18:1n9/18:0 ratio in the PPARα, LXR and RXR groups and of the 18:1n7/16:1n7 ratio in PPARγ and RXR groups. SAFAs and MUFAs of selected PL classes PC, PE and LPC were determined in plasma and liver by ESI-MS/MS. The composition of PE species containing one MUFA (36:1 and 34:1) was mainly influenced by the application of RXR and LXR ligand leading to an increase of bound MUFAs in liver tissue and plasma. The oral gavage of RAR ligand, by contrast, decreased MUFAs (displayed by: 32:1, 34:1) in liver and plasma. As in case of PE, an increase of MUFAs (displayed by: 32:1, 34:1 and 36:1) in PC was determined in the liver and plasma after application of agonists for RXR and LXR, while the activation of RAR, PPARα and PPARγ lead to a decrease of PC 36:1 in the liver and plasma. Furthermore, RAR activation reduced the content of PC 32:1, 34:1 and 36:1 in liver and plasma. The activation of RXR and LXR by their synthetic ligands altered the composition of LPC in liver and plasma in which MUFAs (16:1 and 18:1) were increased. 16:1 in plasma and 18:1 in liver were additionally increased by RXR and PPARα. A reduction of 18:1 in liver and 16:1 in plasma and liver was determined after the gavage of RAR agonist. Additionally, the composition of ceramides and sphingomyelins were investigated and remained unchanged in 16:0, 18:0, 16:1 and 18:1 species. In summary, activation of RXR and LXR by their specific ligands was found to lead to an increase of bound MUFAs.

DISCUSSION

Cellular membranes fulfil the most crucial functions of maintenance of cellular homeostasis, reactivity to metabolic challenges and injuries affecting the integrity of cells and organs. The assembling of PLs and incorporated FAs are determining factors for the biophysical and physiologic character of a cellular membrane. In this thesis different influences on FA composition of PLs and the possibility to refer specific FA ratios to their metabolizing enzymes were investigated.

MUFA and SAFAs content in PLs - In this studies the MUFA (18:1n9, 16:1n7) and SAFA (18:0, 16:0) content in PLs was mainly influenced by alterations of gene expression of rate limiting enzymes SCD1 and ELOVL6. MUFAs and SAFAs derived from diet revealed less impact on PL fatty acid composition. Comparing diet interventions MUFAs, in particular OA, were not found to alter gene expression of SCD1. However a significantly lower hepatic gene expression of ELOVL6 and SCD1 after fish oil diet compared to olive oil was seen in this studies which has been observed for SCD1 in adipocytes comparing OA to n3-PUFA treatment. N3-PUFAs like found in fish oil or n6-PUFAs contained in sunflower oil were reported in several studies to be responsible for this suppression. For sunflower oil diet the suppression could be confirmed only in dose dependent manner (study2) and only partially in comparison to other diets (study1). Further n3-PUFAs have a higher potential to suppress gene expression of SCD1 compared to n6-PUFAs which increases with length of the carbon chain. As it has been found that n6-PUFA AA supplementation suppresses SCD1 gene expression stronger than supplementation with n6-PUFA LA. On the contrary, hepatic gene expression of SCD1 and ELOVL6 were increased by SAFA enriched coconut fat diet as found in this but also reported in previous studies. Surprisingly the highest induction of SCD1 gene expression in liver could be detected after margarine diet. The FA profile of margarine revealed a mixture of SAFA, PUFA and MUFA which would expect to suppress hepatic gene expression of SCD1 compared to coconut fat diet due to the high content of PUFAs. However hepatic gene expression of SCD1 as well as metabolic products of this enzyme incorporated into PLs were highest in margarine diet group. Margarine is a highly processed dietary fat which has not been investigated before for its potential to regulate the gene expression of SCD1. Margarine could be interesting new target for further studies.

Other regulators of SCD1 and ELOVL6 gene expressions are dietary and non-dietary activators of NHRs. Vitamin A, a natural ligand for NHRs, was reported to induce the hepatic

gene expression of SCD1. However, no induction by dietary vitamin A for hepatic gene expression of SCD1 and additionally for ELOVL6 could be proven within studies of this thesis if diet was synergistically applied with sunflower oil. Possibly the dose dependent suppressive effect of sunflower oil which has been shown also in previous studies abolished the inductive effect of vitamin A. Therefore the role of vitamin A in the regulation of SCD1 expression remains controversial and reveals further the question of how several different nutrients interact with each other on gene expression regulation. Dietary supplement studies investigated so far mainly single isolated nutrients while interaction of nutrients have been studied rarely so far.

Further in this thesis in study3 an induction of gene expression of SCD1 and ELOVL6 was found by application of synthetic ligands to activate LXR and RXR. The induction of SCD1 by RXR and LXR has been previously reported. For ELOVL6 only the induction by LXR was reported so far. Other NHRs like PPAR α,β/δ,γ did not show any regulatory tendencies towards the hepatic gene expression of SCD1 or ELOVL6 although the induction of hepatic gene expression of SCD1 and ELOVL6 by PPARα and for SCD1 also by PPARγ in obese Zucker fa/fa rats and in HepG2-cells was proposed.

SCD1 specific FAs ratios 18:1/18:0 and 16:1/16:0 are proposed as "SCD1" or "desaturation" index reflecting SCD1 enzymatic activity. However, comparing dietary FAs to FAs incorporated into PLs the ratio of 18:1n9/18:0 reflected well SCD1 gene expression in all studies conducted within this thesis. Suppression of SCD1 by fish oil diet for example lead to a reduction of this FA ratio in PLs. This could be observed in PL subgroups of PE, PC and LPC (study1). The suppressive effect of n3-PUFA supplementation was shown by others in reducing the ratio of 16:1n7/16:0 in ceramides and 18:1n9/18:0 in PLs which was also seen after a cod diet rich in n3-PUFAs in healthy subjects corresponding to lower triglycerides. Dose dependent suppression of SCD1 by sunflower oil was also followed by reduction of these specific fatty acid ratios in PLs, in PE, PC and in LPC accordingly similar to suppressed hepatic gene expression (study1). On the other hand SAFA rich diet induced the SCD1 gene expression which confirmed the higher ratio of substrate and products in PLs (study1). Similar, the induction of SCD1 by LXR and RXR activation increased SCD1 specific FA ratios in PLs (study3). Dietary influences on SCD1 specific FA ratios in PLs remained marginal. Dietary 18:1n9 like derived from olive oil diet was not associated with an increased 18:1n9/18:0 in PLs although reported by others in skeletal muscle in humans. 16:1n7,

however, high in fish oil diet led to an increase of 16:1n7/16:0 ratio in our study. Therefore the ratio of 16:1n7/16:0 could be considered to be less reliable to represent SCD1 gene expression.

The rate limiting enzyme of elongation of MUFA and SAFA ELOVL6 showed a repressed hepatic gene expression by n3-PUFAs containing fish oil diet which confirmed other previous studies. Further this repression led to reduced incorporation of metabolic products into PLs in liver. An increased hepatic gene expression of ELOVL6 was revealed in this thesis after consumption of coconut fat supplemented diet as well as by activation of RXR and LXR by synthetic ligands elevating ELOVL6 specific FA ratios in PLs in liver and in plasma. According to results in this thesis the FA ratio of 18:1n7/16:1n7 is more reliable to represent ELOVL6 hepatic gene expression than the suggested ratio of 18:0/16:0.

PUFA content in PLs - PUFAs in PLs are derived more likely from diet. For example, n6-PUFA 18:2n6 was measured highest for sunflower oil feeding group in diet and in PLs and n3-PUFAs of 20:5n3 (EPA), 22:5n3 (DPA) and 22:6n3 (DHA) highest in fish oil diet were measured also highest in PLs. However, also hepatic gene expression of PUFA metabolizing enzymes FADS1, FADS2 and ELOVL5 displayed partially influence on FA composition in PLs (study1). An increase of FADS1, FADS2 and ELOVL5 gene expression could be observed in margarine diet group compared to fish oil, which would explain the elevated level of downstream metabolic products of these enzymes incorporated into PLs. These PUFA downstream products might not derive necessarily from diet but could derive from metabolism by desaturation and elongation by PUFA metabolizing enzymes. This result suggests that only 18:3n3 and 18:2n6 are absolutely essential to be provided by diet and can be converted to gain the desired higher n3-PUFAs like 20:5n3, 22:5n3 and 20:4n6, respectively. Supplementation with 18:3n3 enriched diets instate of consumption of fish is therefore recommended to avoid n3-PUFA shortage. However, investigation of health benefits associated to non-fish oil n3-PUFA enriched substitutes revealed controversial results and are not fully proven. Further hepatic gene expression of PUFA metabolizing enzymes FADS1, FADS2 and ELOVL5 was suppressed in liver and other tissues by fish oil supplemented diets or diets high n3-PUFAs. Therefore PUFAs like 20:5n3, 22:5n3 and 22:6n3 incorporated in PLs of fish oil diet group derived more likely from diet than from enzymatic metabolism. This suppression diminishes the generation further downstream products derived from 18:2n6 and is suggested to reduce the pro-inflammatory metabolites derived from n6-PUFAs like 20:4n6. The results of studies in this thesis and previous studies

in healthy humans suggest that the endogenous AA (20:4n6) content is limited by the metabolizing enzymes FADS1 and 2, the rate limiting enzyme in GLA (20:3n6) conversion to AA (20:4n6). Differently than previously reported studies results of study1 of this thesis revealed that availability of metabolic n6-PUFA substrates 18:2n6 like from margarine and from sunflower oil increased AA levels in plasma PLs. A high hepatic gene expression of FADS1 might contribute to an increased conversion of the main substrate 18:2n6 in sunflower oil to the metabolic product 20:4n6.

Discussing specific fatty acid ratios for PUFA metabolizing enzyme FADS1, FADS2 and ELOVL5 remains controversial because their reliability is not fully proven. Enzymatic activity of FADS1 was investigated by specific FA ratio of 20:4n6/20:3n6 (study1). This FA ratio was found lowest in PLs and diet of fish oil supplemented diet group assuming a minor influence by hepatic gene expression of FADS1. On the contrary however, in human this particular ratio was reported to increase with consumption of n3-PUFA enriched diet. FADS2 ratio of 22:6n3/22:5n3 in PLs reflects the hepatic gene expression of FADS2 ELOVL5 ratio of 22:5n3/20:5n3 lowest in fish oil diet group in PLs reflected the reduced hepatic gene expression. Other proposed enzyme specific FA ratios followed the dietary pattern in PLs rather than the hepatic gene expression of the metabolic enzyme. The specific ratio of 18:3n6/18:2n6 is mainly proposed to indicate the enzymatic activity of FADS2 and was reported to be increased in PLs with n3-PUFA enriched diet in healthy subjects. This result could be confirmed by study1 although the hepatic gene expression of FASD2 was significantly reduced in fish oil supplemented diet group.

Regulatory link between fat and vitamin A metabolism - Although research progresses on the regulatory potentials of several dietary fats, it remains unclear for almost 40 years why PUFAs and especially n3-PUFAs suppress so efficiently SREBP-1c target genes like SCD1and ELOVL6. One explanation suggested the ability of n3-PUFAs especially DHA to reduce trans-activation of LXRα, a SREBP1c regulator. This question was followed studying if the suppressive effect could be connected to the RXR activation and vitamin A metabolism. The administration of fish oil supplemented diet induced the hepatic gene expression of CYP26A1, a target gene of RAR and therefore of natural dietary vitamin A. CYP26A1 is the rate limiting enzyme for the availability of RXR natural ligands derived from dietary vitamin A. RXR is activated by vitamin A derived metabolites 9-cis RA which is found to be catabolized by Cyp26a1. Less available natural ligands capable to activate RXR could be an explanation for a reduced expression level of RXR target genes. RXR target

genes involve the TF SREBP1c which controls targets like fat metabolizing enzymes such as SCD1 and ELOVL6 as well as FADS1, FADS2 and ELOVL5. Gene expressions of all of these enzymes were suppressed in liver after fish oil diet leading to reduced metabolic products of these enzymes incorporated into PLs. However, this hypothesis requires further investigation.

SUMMARY

Dietary fat provides FAs incorporated into PLs forming cellular membranes. These membranes are constantly renewed and the composition of FAs aligns according to the provided dietary fat. Influences on membrane PL FA composition were investigated in this thesis. *In vivo* studies in mice have been performed investigating the effect of different types of dietary fat, different amounts of sunflower oil combined with vitamin A and regulation by activated NHRs on PLs and cellular membrane FA profile.

Most important findings of this thesis were that MUFA and SAFA content in PLs is strictly controlled by their metabolizing enzymes SCD1 and ELOVL6. Especially alteration of SCD1 hepatic gene expression by dietary fat altered the PLs profile of its metabolic products accordingly. The SCD1 FA ratio 18:1n9/18:0 was found to be more reliable as "desaturase index" within all conducted studies. Most surprisingly margarine diet was the strongest inducer of SCD1, it was even more effective than coconut fat diet high in SAFA. Furthermore the inductive effect of dietary vitamin A on hepatic gene expression of SCD1 and ELOVL6 was abolished if diet was combined with n6-PUFA rich sunflower oil. Sunflower oil supplemented diet suppressed SCD1 and ELOVL6 gene expression dose dependently. This assumes that nutrients might interfere with each other with unexpected outcome. ELOVL6 activity was most reliable represented by the FA ratio 18:0/16:0. On the contrary to MUFA and SAFA profile in PLs PUFAs in PLs were more but not totally dependent on available dietary PUFAs. Fish oil enriched diet suppresses the hepatic gene expression of main PUFA metabolizing enzymes FADS1, FADS2 and ELOVL5 which was reflected in reduced metabolic activity. This resulted partially in less metabolic products measured in FA profile of PLs. Repressed gene expression could be associated with reliable FA ratios representing enzymatic activity for 22:6n3/22:5n3 FADS2. PUFAs in PLs profiles are more likely to derive directly from diet, but might be adjustable by PUFA enzyme metabolism. The results of this thesis suggest further that inhibition of hepatic gene expression of fat metabolizing enzymes by n3-PUFA enriched diet is caused by reduces activity of RXR. RXR is a heterodimer forming NHR which can be activated by isoforms of vitamin A. CYP26A1 catabolizes active vitamin A derived RXR ligands into inactive metabolites. Induction of CYP26a1 by fish oil supplemented diet decreases the availability of vitamin A and less RXR is activated.

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List of publications related to the dissertation

1. **Weiss, K.**, Mihály, J., Liebisch, G., Marosvölgyi, T., Garcia, A. L., Schmitz, G., Decsi, T., Rühl, R.: Effect of high versus low doses of fat and vitamin A dietary supplementation on fatty acid composition of phospholipids in mice.
Genes Nutr. 9 (1), 368, 2014.
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IF: 2.794

2. **Weiss, K.**, Mihály, J., Liebisch, G., Marosvölgyi, T., Schmitz, G., Decsi, T., Rühl, R.: Effect of synthetic ligands of PPAR alfa, béta/delta, gamma, RAR, RXR and LXR on the fatty acid composition of phospholipids in mice.
Lipids. 46 (11), 1013-1020, 2011.
DOI: http://dx.doi.org/10.1007/s11745-011-3593-6
IF: 2.129

List of other publications

3. Landrier, J. F., Kasiri, E., Karkeni, E., Mihály, J., Béke, G., **Weiss, K.**, Lucas, R., Aydemir, G., Salles, J., Walrand, S., de Lera, Á. R., Rühl, R.: Reduced adiponectin expression after high-fat diet is associated with selective up-regulation of ALDH1A1 and further retinoic acid receptor signaling in adipose tissue.
Faseb J. [Epub ahead of print], 2016.
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4. Mihály, J., Gericke, J., Aydemir, G., **Weiss, K.**, Carlsen, H., Blomhoff, R., Garcia, J., Rühl, R.: Reduced retinoid signaling in the skin after systemic retinoid-X receptor ligand treatment in mice with potential relevance for skin disorders.
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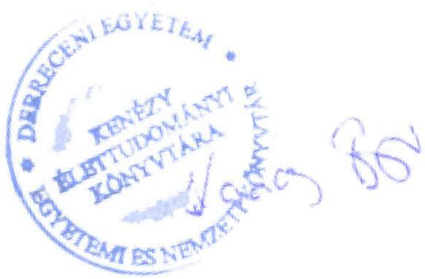

KEYWORDS

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