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Lipid and Gene Interactions during Differentiation of Human Subcutaneous Adipose Tissue Stromal Vascular Cells

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Abstract

Examination of the changes in cellular lipid composition, allows for an understanding in their contribution to adipocyte differentiation in health and disease. In the future the analysis of body lipid composition could offer an additional tool to diagnosing disease with the predictive capacity for prognosis.

Exposition of cells to excess of energy substrates causes activation of evolutionarily conserved adaptive mechanisms in endoplasmic reticulum stress. In preadipocytes, metabolic stress, through the activation of endoplasmic reticulum stress connected with modification of lipid composition and lipid droplet formation leads to differentiation.

The aim of the study was to examine changes in cellular lipid composition in parallel with gene expression and enzyme activity during preadipocyte differentiation using "omics" results.

Differentiation condition lead to activation of lipid droplets related protein expression and lipid droplet formation in preadipocytes. The predominant increase of the phospholipids, plasmalogens and cholesterol amounts during cell differentiation was observed. This was accompanied by stimulation of de novo synthesis of saturated fatty acids incorporated into sphingolipids and with reduction in amount of arachidonic acid. Our results indicated that the changes in lipid composition of differentiating stromal vascular fraction cells changed in parallel with gene expression and matched the functional requirements.

Keywords: SVF; Adipogenesis; ER-stress; Lipidomics; Microarray

Abbreviations: PC: Phosphatidylcholine; LPC: Lysophasphatidylcholine; PE: Phosphatidyetanolamine; PS: Phosphatidylserine; PI: Phosphatidylinositol; PG: Phosphatidylglycerol; PEPL: PE–based plasmalogen; Cer: Ceramide; SPM: Sphingomyelin; GluCer: Glucoceramide; CE: Cholesterol ester; FC: Free cholesterol; SVF cells: Stromal Vascular Fraction cells; ER-stress: Endoplasmic Reticulum stress; Ctrl(-): SVF cells cultured without differentiation factors for 48h or 15d; Ctrl(+) 48h: initial (48h) stimulation of SVF cells for differentiation; Ctrl(+) 15d: time after initial stimulation of SVF cells for differentiation

Introduction

Cellular lipid species differ in their molecular structure, polar head groups, and fatty acid chain length and desaturation. Lipids taken up and synthesized in cells relate to three general functions [1,2]. Phospholipids, sphingolipids and cholesterol are building blocks of cellular membranes allowing compartmentalization within cells [3, 4]. Triacylglycerols and sterol esters are stored in Lipid Droplets (LD) which serves as energy reservoirs and supply fatty acids and cholesterol needed for membrane biogenesis [2]. Specific lipid-species act as ligands for plasma membrane receptors to activate intracellular signaling as well as for nuclear receptors to regulate transcription [5]. In addition, some lipid classes participate in the formation of membrane microdomains which recruit proteins from the cytosol that subsequently generate exosomes that organize signaling or effect or complexes in the target cells, or endosomes derived from caveolae destined to the Endoplasmic Reticulum (ER) and LD [1,4,6]. Lipid biosynthesis takes place in the ER, which produces the main membrane structural components such as glycerophospholipids, cholesterol and ceramides, the precursors for complex sphingolipids [2]. Both the ER lumen and LD related enzymes produce significant levels of triacylglycerols and Cholesteryl Esters (CE) as storage forms of fatty acids and cholesterol [7]. Lipid synthesis also occurs in mitochondria where Lysophosphatidic Acid (LPA) is synthesized of which a substantial part is used for triacylglycerol formation [8]. Additionally, mitochondria also synthesize Phosphatidic Acid (PA) and Phosphatidyl Glycerol (PG) used for the synthesis of the endolysosymal curvature-lipid Bis-monoacylglycerophosphate (BMP) and the electron transfer complex stabilizing mitochondrial inner membrane lipid cardiolipin (CL) [1,4]. Biosynthesis of Sphingomyelin (SM) and Gycosphingolipid (GSL) existing mainly in the plasma membrane occurs in the Golgi apparatus [2,9].

The ER is the major nutrient sensor and signal transducing organelle that senses and responds to changes of energy homeostasis [10,11]. Excess of metabolic substrates, such as glucose and fatty acids, induces generation of free radicals (ROS) in mitochondria [12,13] initiates ER-stress, leading to lipid droplet formation[14-16].

The adipose tissue provides the basis for the adaptation to chronic nutritional overload and food scarcity. Preadipocytes of the Stromal

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Vascular Fraction (SVF) accumulate Triacylogliceroles (TAG) in LDs to protect other cells from lipotoxic effects of ectopic lipid accumulation, thus preventing development of metabolic dysfunction (insulin resistance, glucolipotoxicity, Non-alcoholic Fatty Liver Disease (NAFLD), type 2 diabetes, atherosclerosis) [17-19].

This study used high throughput methods to identify parallel changes in lipid composition and gene expression and their interactions during the differentiation of subcutaneous human SVF preadipocytes in a standardized *in vitro* tissue culture model. In the future the analysis of body lipid composition could offer an additional tool to diagnosing disease states with the predictive capacity for prognosis.

Materials and Methods

Chemicals

All reagents for tissue culture were purchased from Sigma-Aldrich (Poznan, Poland), except FBS which was purchased from Life Technologies (Carlsbad CA, USA). HPLC grade chemicals for ESI-MS/MS as described [20]. Reagents for RNA purification were from Life Technologies (Carlsbad CA, USA). Microarrays and reagents for microarrays were from Agilent technologies (Santa Clara, CA). All used dyes were purchased from PROGEN Biotechnik (Heidelberg, Germany).

Cell culture

All work was performed with the permission of the Polish Ethics Commission (no KBET/117/B/2008). Adipose Tissue (AT) was obtained by liposuction from ten healthy women's aged 20-60 years. For SVF cells isolation, adipose tissue was cleaned carefully from remaining connective tissue and blood vessels and resulting adipose tissue incubated with collagenase solution (200 U/ml) for approx 60-90 min in a shaking water bath at 37°C, separated by centrifugation (600xg for 10 min at room temperature) and washed twice with DMEM/F12 medium containing 10% of FBS.

The supernatant was discarded and the cellular pellet was resuspended in DMEM/Ham's: F12 medium (50:50, v:v) supplemented with gentamicin (50 µl/ml) and 10% fetal calf serum (FBS 10%). For cell selection cell strainers (ø70 µm; optionally 25 or 40 µm) (BD Biosciences Discovery Labware, Bedford, MA, USA) were used [21]. SVF cells were cultured in medium containing 10% FBS. Twenty-four hours after isolation of the SVF cells from adipose tissue, inoculation medium containing FBS was changed to fresh adaptation medium and SVF cells were propagated in medium without FBS supplemented with human transferrin (10 µg/mL), human insulin (66 nM) and hydrocortisone (100 nM).Cells from every donor were cultured for up to two passages and pooled to obtain pool of SVF cells used for experiments.

The "adipogenic" cocktail that mimics the enriched nutritional/hormonal conditions sufficient for cAMP signaling activation at an initial stage of fat tissue expansion was used for preadipocyte differentiation [22,23]. SVF cells were incubated in adipogenic MDI medium (0.5 nM *isobutylmethylxanthine* (IBMX), 0.25 nM dexamethasone, 66 nM insulin) for 48 hours (Ctrl (+) 48 h). Then cells were incubated for 15 days in DMEM/ Ham's: F12 medium (50:50, v:v) supplemented with gentamicin (50 µl/ml), human transferrin–10 µg/mL, human insulin–66 nMm, hydrocortisone–100 nM, triiodothyronine–1 nM without FBS (Ctrl (+)).The negative control cells (Ctrl (-)) were incubated in the same composition of medium but without differentiation factors (dexamethasone, IBMX, insulin). All experiments were performed at 2 time-points – first after 48 hours (initial period of differentiation (Ctrl (+) 48 h) and second after 15 days of differentiation (Ctrl (+) 15d)).

Oil-Red O staining

To measure lipid accumulation during SVF differentiation the Oil-Red O staining method was performed [24]. Cells were fixed with 3.7% paraformaldehyde and stained with a 0.5% solution of Oil-Red O in isopropanol. Cells were then washed with distilled water and lipid droplets were visualized using a light microscope (Olympus CK40; 10x magnification).To determine the amount of accumulated dye [24], Oil Red-O was eluted with isopropranol and the absorbance of obtained supernatant was measured (OD at 500 nm using 100% isopropanol as blank). The protein content of each probe was determined according to the Lowry method.

The lipid droplet PAT protein expression and BODIPY quantification (Bioimager^{BD})

For visualization of perilipin, adipophilin and TIP47 (PAT) protein expression [25] and lipid droplets formation before and during cell differentiation, the cells in culture were fixed with paraformaldehyde (3.7%). PAT proteins were stained with the following goat monoclonal primary antibodies: adipohilin (1:2000 dilution), perilipin (1:500 dilution), TIP47 (1:1000 dilution) and Hoechst (1:5000 dilution) and visualized with secondary polyclonal anti-goat antibody with Cy3. Additionally BODIPY 492/503 staining (1:5000 dilutions) was performed. All the imaging studies were performed in 96-well plates using the BD Pathway 855 Bioimager (BD Biosciences Discovery Labware, Bedford, MA, USA). The imaging data were collected and analyzed using the Attovision software package. The results are presented as ratios of total fluorescent intensity generated in the cytoplasmic rings around nuclei to the mean fluorescent intensity of the negative control (n=3).

Isolation of total RNA

Following incubation with the studied compounds, total RNA was isolated from cells using TRIzol® Plus RNA Purification System according to the manufacturer's instructions. RNA quality was assessed using an Agilent Bioanalyzer 2100 and quantified by spectrophotometry using a NanoDrop ND-1000 spectrophotometer.

Microarray hybridization -gene expression

Microarray experiments were performed using Agilent Single Color human oligonucleotide arrays. Each separate RNA sample was hybridized to a single array and all expression changes were detected through the comparison to control (ctrl (-)) of appropriate chips using the same software package.

Labeling of total RNA was done using the Quick Amp labeling kit as per the manufacturer's protocol. Briefly, using T7 promoter element coupled oligoT primer, cDNA was generated. Subsequently from cDNA, labeled cRNA was synthesized using T7 RNA polymerase and dyes. Labeled cRNA was used for hybridization. Signal intensity of the labeled cRNA was measured by hybridization to human gene microarray. Hybridization was performed for 17 h, rotating at a speed of 10 rpm at 65°C in a hybridization oven. The arrays were washed according to the manufacturer's recommendations and detection of the fluorescent signal performed by the GeneChip® Scanner 3000 System with the GeneChip® Operating Software Version 1.0.

Microarray data analysis was done using Gene Spring version 10

(Agilent Technologies, Santa Clara, CA). To identify differentially expressed genes and reduce noise, each data set was filtered according to several criteria. For any probe to be included within each data set, the signal intensity should be >100 in either test or control samples. Genes in which expressions were altered >1.3-fold were included for analysis.

Lipid profile

Analysis of the lipid profile of SVF cells at different stages of differentiation was performed in cooperation with the center at Regensburg by mass spectrometry as previously described [20,26,27]. The resulting data set as the amount of lipid/mg protein from three experiments were averaged and analyzed as the absolute content of individual classes of lipids in cells and relative content of fatty acids with different carbon chain lengths in the various lipid fractions.

Statistical analysis

All data are expressed as mean ± SD and mean with range in parentheses, respectively. Statistical significance was determined by an ANOVA and post-hoc Turkey test for normally distributed data using the STATISTICA software. A p-value <0.05 was considered statistically significant.

Results

Incubation of SVF cells in serum-free adipogenic MDI medium for 48 h promoted cell differentiation and lipid droplet formation. Some LD formation was seen soon after 48 hours, but the maximal accumulation of lipids and PAT protein expression in SVFs was observed after 15 days of culture (Figures 1 and 2). Quantitative analysis of cellular lipid accumulation revealed a 3-fold increase of lipids accumulated in lipid droplets measured by the Oil-Red-O staining after 15 days (Figure 1).

The same effect was observed by analysis of BODIPY 492/503 staining after 15 days of SVF differentiation (Figures 1 and 2).

In order to get an overview of the changes in the lipidome during differentiation of SVF cells, cell homogenates corresponding to 100 µg protein were subjected to analysis by ESI-MS/MS for the following lipid classes: Phosphatidylcholine (PC), Lysophosphatidylcholine (LPC), Phosphatidylethanolamine (PE), PE-based Plasmalogen (PEPL), Phosphatidylglycerol (PG), Phosphatidylinositol (PI), Sphingomyelin (SPM), Ceramide (Cer), Free Cholesterol (FC) and Cholesteryl Ester (CE).

Long-term SVF cell culture without the presence of differentiating factors resulted in an insignificant increase of individual classes of lipids, (analysis Ctrl (-) 48 h vs Ctrl (-) 15d) data not shown). SVF cell differentiation, caused by MDI medium, resulted in a statistically significant change in lipid profile and increased the content of all lipid classes analyzed soon after 48 hours (analysis Ctrl (+) 48 h vs Ctrl (-) 48 h). The longest SVF incubation period (15 days), after an initial stimulation of differentiation (48 h), resulted in the formation of microscopically visible lipid droplets. Concomitantly changes of lipid species (as described above) were observed but these changes did not reach significance levels (analysis Ctrl (+) 15d vs Ctrl (-) 15d) (Table 1 and Figure 3).

Differentiation factors significantly changed the amount of lipid species classes in SVF cells and changed the absolute amount of lipid content (nmol/mg protein) (Figure 3 and Table1).

The predominant lipid classes in differentiated SVFs were

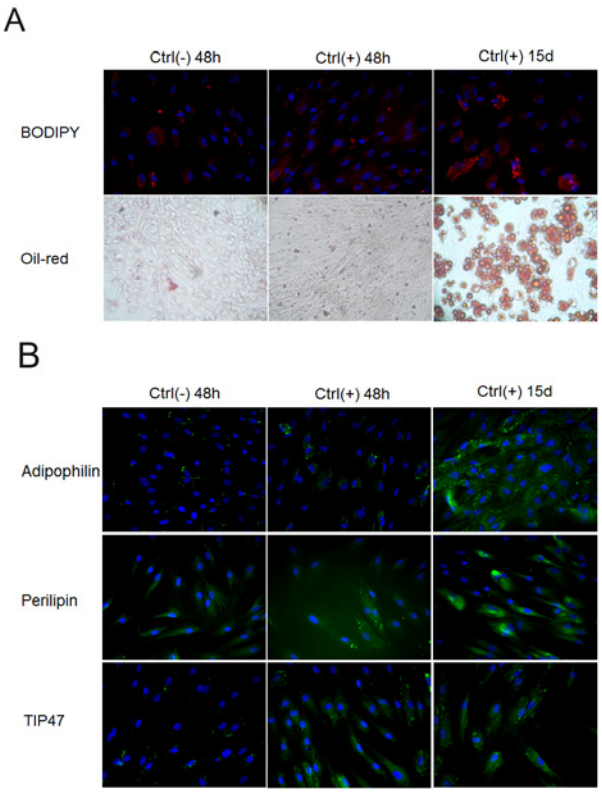


Figure 1: Visualization of lipid droplets and PAT proteins during the SVF differentiation using BD Pathway Bioimager 855 (20x objective magnification). SVF cells were incubated for 48 hours in adipogenic MDI medium for initiation of differentiation (Ctrl (+) 48 h) and then without differentiation factors for 15 days (Ctrl (+) 15d). The negative control cells (Ctrl (-)) were incubated without differentiation factors. A. Lipid droplets stained using BODIPY 492/503 and Oil-Red-O (10x objective magnification). B The visualization of PAT protein during differentiation (stained using antibody with Cy3). (red- BODIPY, blue-Hoechst, green-PAT proteins).

phosphatidylcholine (PC) and Free Cholesterol (FC). Calculated cell type specific PC/FC ratios as marker of membrane fluidity [28] was low in non-differentiated SVF cells and increased after 48 h of stimulated differentiation and continued to increasing during time of differentiation (15d). The predominant increase of the amount of phospholipids and cholesterol during cell differentiation indicates the demand of synthesis of lipid fractions needed as building blocks of the membranes necessary to form extracellular vesicles [1,2] (Figure 3).

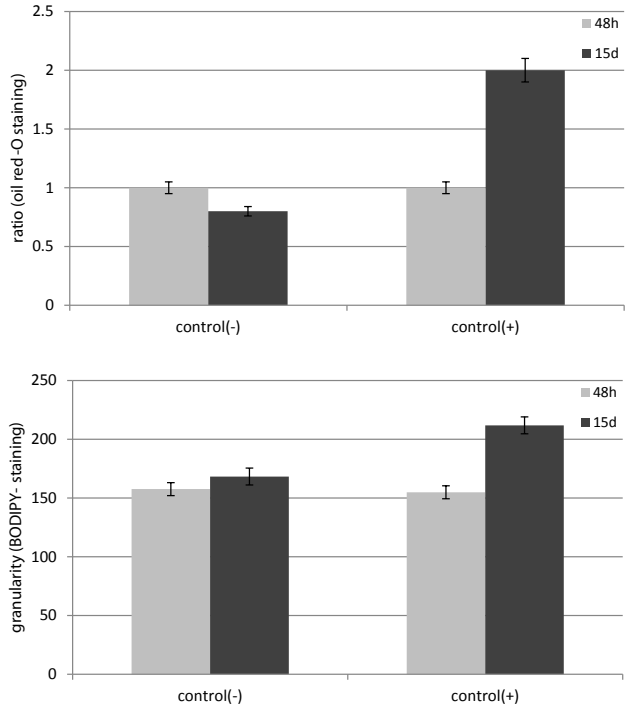
Analysis of changes in the amount of lipid classes, containing fatty acids of different lengths with different degrees of desaturation, revealed that long-term culturing without stimulation of differentiation (Ctrl (-) 48 h vs Ctrl (-) 15d) significantly changed the content of PEPL 18:0/16:0, CE 18:0, CE 22:1, SPM 20:0, SPM 22:1.

Long-term culture after prior stimulation of differentiation (Ctrl (+) 15d vs Ctrl (-) 15d) significantly changed the content of: SPM 14:0, SPM 24:2, PS 32:1, PI 32:0, LPC 22:0, the ether lipids PC O 32:1, PC O 34:2, PE O 38:7 and plasmalogen fractions containing mainly acids of 16 and 18 carbon atoms (PEPL16:0/16:1, PEPL 16:0/18:3, PEPL 16:0/18:2, PEPL 16:0/18:1, PEPL 16:0/20:3, PEPL 18:1/16:1, PEPL 18:1/18:2, PEPL 18:1/18:1, PEPL 18:1/20:3, PEPL 18:0/16:1, PEPL 18:0/18:3, PEPL 18:0/18:2, PEPL 18:0/18:1, PEPL 18:0/20:5, PEPL 18:0/20:3, PEPL 18:0/22:3).

Activation of SVF cell differentiation in a short period of time (48 h) generally led to biosynthesis of lipid species in the various lipid classes with unsaturated fatty acids, especially monounsaturated fatty acids (MUFA) including SPM, PS, PI, PE, LPC, CE, PG, polyunsaturated (PUFA) fatty acids in the SPM, PS, PI, PE, LPC, CE. The amount of saturated fatty acids incorporated in SPM and Cer were significantly increased. This may suggest that the commonly used activation of differentiation conditions [21,29,30] induce(ER)-stress resulting in stimulation of de novo synthesis of fatty acids which were incorporated into the structure of ceramides and other sphingo lipids. Longer activation of SVF by the differentiating factors lead to a statistically significant increase in PEPL 18:0/18:3 and a decrease in LPC 22:5.

Based on the quantities of the various classes of lipids and incorporated fatty acids the change in the relative content of fatty acids during SVF cell differentiation was analyzed (Figure 4).

The study of fatty acid composition in cellular lipid species was used as an indirect method of analysis of enzyme activity involved in the elongation and desaturation of fatty acids [31]. The product/substrate relation of fatty acids (Table 2) can be indirectly used to assess the activity of individual enzymes [32]. This activity was correlated with



Oil-red staining				BODIPY staining			
48h	control(-)	1	0.05	48h	control(-)	157.52	5.52
	control(-)	1	0.05		control(-)	154.85	7.75
15d	control(+)	0.8	0.04	15d	control(+)	168.24	7.19
	control(+)	2	0.1		control(+)	211.80	11.95

Figure 2: The quantitative analysis of lipid accumulation in lipid droplets during SVF differentiation using Oil Red O (A) and BODIPY 492/503 staining (B). SVF cells were incubated for 48 hours in adipogenic MDI medium for initiation of differentiation (Ctrl (+) 48 h) and then without differentiation factors for 15 days (Ctrl (+) 15d). The negative control cells (Ctrl (-)) were incubated without differentiation factors. Significance: p<0.01; Ctrl (+) vs Ctrl (-) 15d n=5.

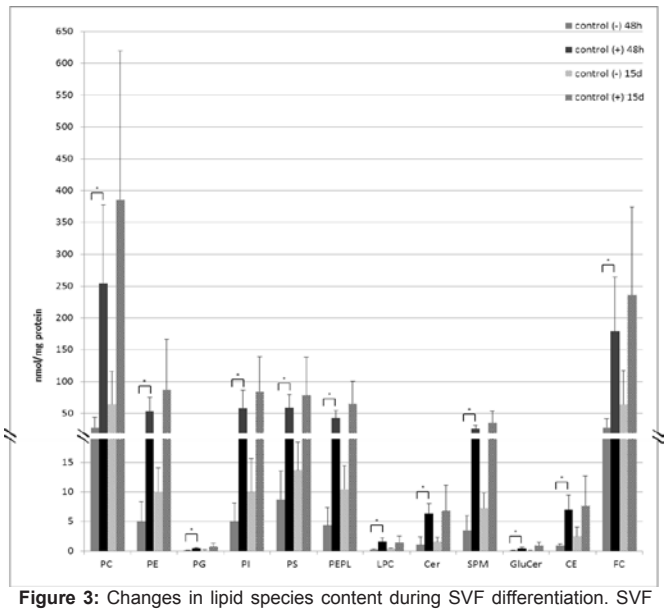


Figure 3: Changes in lipid species content during SVF differentiation. SVF cells were incubated for 48 hours in adipogenic MDI medium for initiation of differentiation (Ctrl (+) 48 h) and then without differentiation factors for 15 days (Ctrl (+) 15d). The negative control cells (Ctrl (-)) were incubated without differentiation factors. The results are presented as average lipid species concentration (nmol/mg protein) detected using mass spectrometry ESI-MS/MS (n=3). Significant differences in relation to the control (-) 48 h are indicated by asterisks, *p<0.05.

gene expression results of enzymes involved in fatty acid metabolism (values derived from analysis of the microarray data) (Table 3).

The stimulation of de novo lipogenesis (DNL), is determined by the ratio of 18:2/16:0 fatty acids [32]. In undifferentiated SVF cells the 18:2 fatty acid predominates and differentiation increases the amount of this fatty acid which confirms this concept. This correlates with the observed up-regulation expression of genes involved in the synthesis of malonyl-CoA (ACACA, ACACB) and the transcription factor SREBF1 regulating lipogenesis during differentiation of SVFs (Tables 2 and 3).

The amount of fatty acids with different carbon chain lengths and different degree of unsaturation depends on the activity of elongase and desaturase enzymes present in the cytosolic leaflet of the ER membrane [33,34]. Desaturation and elongation processes occur alternately in all cells during initial synthesis of palmitate 16:0 and consecutive elongation and desaturation of fatty acids with 20 or more carbon atoms [33-35] with 2-6 double bonds are formed. We observed that SVF cells, both during lengthy culturing and differentiation increased the amount of monounsaturated fatty acids (MUFA) with a length of 16-18 carbon atoms (18:1/18:0). This correlated with the increased expression of the desaturase SCD1 and SCD5 (Tables 2 and 3).

Both long-term cell culture, as well as the used SVF differentiation conditions caused an increase in the incorporation of unsaturated long-chain fatty acids into cellular lipids (Figure 2). These results were consistent with the observed increase in gene expression but did not correlate with the calculated desaturase activity (FADS1, Δ5 desaturase), responsible for the introduction of a fourth unsaturated bond in the structure of fatty acids for the synthesis of e.g. arachidonic acid C20: 4 n-6 from DGLA C18: 3 n-6 fatty acid (dihomo-gamma-linolenic acid) (Tables 2 and 3). The ratio of 20:4/20:3 for FADS1 indicates that the stimulation of differentiation may reduce the amount of arachidonic acid incorporated in lipids. Up-regulated PLA2G6 could remove

	test	SPM	PC	PE	PEPL	PS	PG	PI	LPC	Cer	GluCer	CE	FC
control(+)/control(-) 48h	ANOVA p-value	0.0038	0.0352	0.0206	0.0058	0.0140	0.0075	0.0304	0.0226	0.0133	0.0305	0.0132	0.0379
	post-hoc p-value	0.0040	0.0353	0.0207	0.0050	0.0140	0.0077	0.0305	0.0227	0.0135	0.0307	0.0133	0.0380
control(+)/control(-) 15d	ANOVA p-value	0.0649	0.0817	0.1689	0.0534	0.1364	0.0946	0.0797	0.1600	0.1136	0.0802	0.1802	0.1163

Table 1: Influence of SVF cell differentiation stimulating conditions on the change of individual lipid class contents. SVF cells were incubated for 48 hours in adipogenic MDI medium for initiation of differentiation (Ctrl (+) 48 h) and then without differentiation factors for 15 days (Ctrl (+) 15d). The negative control cells (Ctrl (-)) were incubated without differentiation factors. Statistical Analysis (n=3, ANOVA, post-hoc Turkey test).

arachidonic acid from membrane phospholipids [36]. It was also found that both long-term cell culture conditions of SVF as well as stimulation of differentiation decreased elongation of saturated fatty acids (SFA) (18:0/16:0, 22:0/20:0) when elongation of monounsaturated fatty acids (MUFA) (18:1/16:1, 22:1/20:1) with chain length 16-22 carbon atoms occurred (Table 2). This correlates with the increased expression of appropriate elongases ELOVL3 and ELOVL6, which elongate chains of SFAs and MUFAs with a chain length of 16-22 C-atoms. At the same time the stimulation of differentiation up-regulated the expression of elongase ELOVL5 elongating polyunsaturated fatty acids (PUFA's) and thus increased the amount of long-chain PUFA's incorporated into cellular lipids (20:3/18:3) (Tables 2 and 3).

Discussion

Both excess as well as insufficient supply of the main metabolic

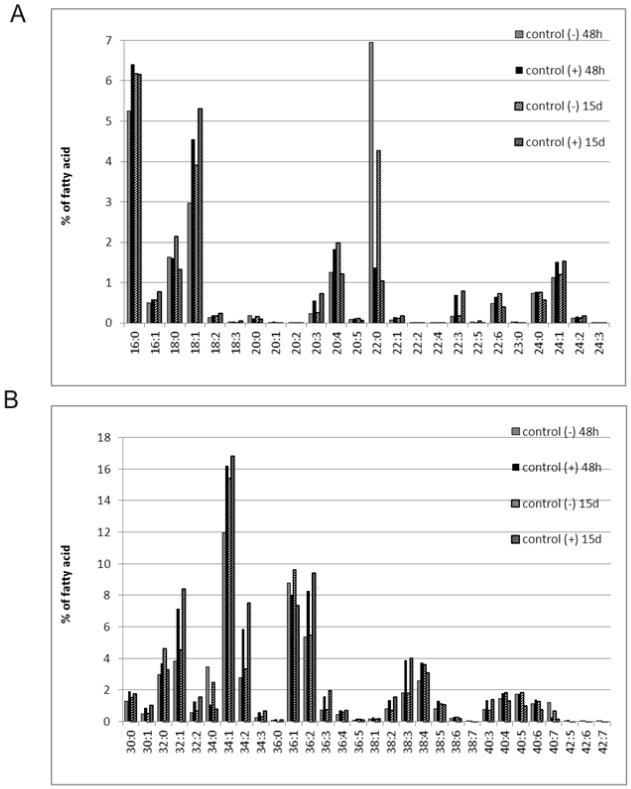


Figure 4: Changes in relative fatty acid content during SVF cells differentiation. SVF cells were incubated for 48 hours in adipogenic MDI medium for initiation of differentiation (Ctrl (+) 48 h) and then without differentiation factors for 15 days (Ctrl (+) 15d). The negative control cells (Ctrl (-)) were incubated without differentiation factors. The results are presented as average (n=3) concentration in mol% of the total lipid/fatty acid species per condition detected using mass spectrometry (ESI-MS/MS, n=3). A change of fatty acids in CE, Cer, LPC, SPM, B-fatty acid content changes in glycerophospholipids.

	control (-) 48h	control (+) 48h	control (-) 15d	control (+) 15d
SCD1/SCD5				
18:1/18:0	1.82	2.83	1.83	4.03
DNL				
18:2/16:0	0.026	0.029	0.028	0.038
ELOVL3/ELOV6				
18:0/16:0	0.31	0.25	0.35	0.21
22:0/20:0	1.53	3.30	2.01	3.24
18:1/16:1	5.88	7.81	7.04	6.92
22:1/20:1	3.81	4.40	3.83	5.50
ELOVL5				
20:3/18:3	8.06	15.90	8.03	14.67
FADS1				
20:4/20:3	5.56	3.25	8.15	1.65
FADS2				
20:3/18:2	1.63	2.99	1.38	3.15
PLAG2G6				
20:5+22:6/20:4	0.452	0.411	0.414	0.374
PC/FC	0.999	1.416	1.008	1.635

Table 2: Changes in enzyme activity during SVF cell differentiation. SVF cells were incubated for 48 hours in adipogenic MDI medium for initiation of differentiation (Ctrl (+) 48 h) and then without differentiation factors for 15 days (Ctrl (+) 15d). The negative control cells (Ctrl (-)) were incubated without differentiation factors. Results calculated as the ratio of product/substrate for enzymes associated with lipid/fatty acid metabolism, determined by ESI-MS/MS, (n=3).

substrates e.g. glucose and fatty acids, induce mitochondrial and ER-stress response [12,37]. It is documented, that metabolic overload (glucolipotoxicity) associated with insulin resistance causes mitochondrial dysfunction, free radical generation and thus leads to lipid droplet formation in cells including hepatocytes, skeletal muscle, endothelium, pancreatic beta cells. Recent reports provide evidence of a strong link between ER stress, synthesis of neutral lipids and LD formation in adipocytes [19,38,39]. In eukaryotes lipid droplets are formed by budding of ER membranes in close association with mitochondria [7,8]. It has also been demonstrated that inhibition of ERAD (Endoplasmic-reticulum-associated protein degradation) components and inhibition of N-linked glycosylation result in increased cellular LD accumulation [14,15]. Our results confirm that the used SVF differentiation stimulation conditions lead to lipid droplet formation, and was accompanied by statistically significant changes of the SVF cells in the lipid profile observed as soon as within the first 48 hours of differentiation. Also the prolongation of SVF incubation time resulted in similar changes of lipid species, however the results were not statistically significant. Analysis of individual lipid classes of fatty acids showed a significant increase in Monounsaturated Fatty Acids (MUFA) SPM, PS, PI, PE, LPC, CE, PG, and polyunsaturated (PUFA) fatty acids SPM, PS, PI, PE, LPC, CE, while the saturated fatty acids were mostly incorporated (statistically significant) into Cer, SPM and CE (border line). Such changes suggest that the adipose tissue SVF cell differentiation may be initiated by stimulation of Endoplasmic

Ensembl Gene ID	Description	ChromLoc	Gene Symbol	control(+)/ control(-) 48h	control(+)/ control(-) 15d
desaturases					
ENSG00000099194	stearoyl-CoA desaturase (delta-9-desaturase)	chr10q24.31	SCD1	1.2	1.5
ENSG00000145284	stearoyl-CoA desaturase 5	chr4q21.3	SCD5	5.6	1.9
ENSG00000149485	fatty acid desaturase 1	chr11q12.2-q13.1	FADS1	1.7	1.6
DNL (de novo lipogenesis)					
ENSG00000132142	acetyl-CoA carboxylase alpha	chr17q21	ACACA	-1.3	1.5
ENSG00000076555	acetyl-CoA carboxylase beta	chr12q24.11	ACACB	4.3	5.0
ENSG00000072310	sterol regulatory element binding transcription factor 1	chr17p11.2	SREBF1	1.3	1.3
ENSG00000198911	sterol regulatory element binding transcription factor 2	chr22q13	SREBF2	-1.3	1.2
elongases					
ENSG00000119915	ELOVL fatty acid elongase 3	chr10q24.32	ELOVL3	3.6	1.7
ENSG00000012660	ELOVL family member 5, elongation of long chain fatty acids (FEN1/Elo2, SUR4/Elo3-like, yeast)	chr6p21.1-p12.1	ELOVL5	2.2	1.5
phospholipase					
ENSG00000184381	phospholipase A2, group VI (cytosolic, calcium-independent)	chr22q13.1	PLA2G6	1.40	1.30

Table 3: Changes in the gene expression of enzymes involved in metabolism of fatty acids during SVF cell differentiation. SVF cells were incubated for 48 hours in adipogenic MDI medium for initiation of differentiation (Ctrl (+) 48 h) and then without differentiation factors for 15 days (Ctrl (+) 15d). The negative control cells (Ctrl (-)) were incubated without differentiation factors (microarray (Agilent), n=3, ANOVA $p<0.05$).

Reticulum Stress (ERS) leading to de novo fatty acid synthesis and its incorporation into ceramides and followed formed sphingolipids.

An increase of Sphingomyelin (SPM) and ceramide (Cer) concentration occurring during differentiation of adipocyte cells has been described [2]. Ceramide generation changes the structure of membrane rafts and caveoli shape and promote membrane permeability [40,41]. The increase of Cer biosynthesis suggests an increase in the curvature of the membrane by stabilizing Cer location by phosphorylation [42]. Sphingomyelin also serves as a structural component of biological membranes in concert with phospholipids, glycolipids, cholesterol and membrane proteins [2].

Stimulation of SVF differentiation significantly changed the plasmalogen fraction content of 16 and 18 carbon atom fatty acids. It has been shown that epithelial morphogenesis and myocytic differentiation are accompanied by an increase in plasmalogen content [43,44]. The other investigations based on the severe phenotypic alterations in mice and cell culture systems with insufficient plasmalogen biosynthesis indicated impaired intracellular cholesterol distribution affecting plasma membrane functions and structural changes of ER and Golgi cisternae [45].

The membrane fluidity of SVFs during differentiation could be also characterized by the molar ratio PC/FC. The observed increase during SVF differentiation of the PC/FC ratio may be related to processes which require more fluidic cellular membranes to enable the cells to perform exocytosis [46]. We observed that SVF differentiation resulted in an increase of the unsaturated long-chain fatty acids content in SVF lipids. These results were consistent with the observed increase in gene expressions and calculated enzyme activity presented as product/substrate ratio.

Activation of SVF cell differentiation caused an increase of long-chain unsaturated fatty acids. Calculated activity ratio (20:4/20:3) for FADS1 indicates that the stimulation of differentiation reduces the amount of arachidonic acid incorporated in lipids, possibly through stimulation of the expression of phospholipase. Up-regulated PLA2G6 shutoff arachidonic acid from membrane phospholipids [36]. Released by phospholipase arachidonic acid could serve as a substrate for eicosanoid synthesis required for stimulation of preadipocyte differentiation by PPAR-gamma activation [47]. The low ratio of EPA+DHA/AA (20:5+22:6/20:4<0) also indicates that membrane lipid released arachidonic acid does not serve as a substrate for the synthesis of DHA or EPA, but undergoes other metabolic pathways.

Thus adipose tissue differentiation is a process that involves cellular membrane remodeling accompanied by ER stress, leading to increase of stress related lipids within corporated saturated FA and LD formation. The parallel increase in plasmalogens, phosphatidylethanolamine, and cholesterol content, argue for remodeling of the cellular membranes necessary for stimulation of exocytosis and regulation of adipogenesis by adiposomes [46,48]. Our results indicated that the changes in lipid composition of differentiating SVF cells changed in parallel with gene expression and matched the functional requirements.

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