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Influence of dietary fatty acids on differentiation of human stromal vascular fraction preadipocytes

Anna Polus [a,*], Beata Kiec-Wilk [b], Urszula Razny [a], Anna Gielicz [d], Gerd Schmitz [c], Aldona Dembinska-Kiec [a]

[a] Department of Clinical Biochemistry Collegium Medicum, Jagiellonian University, Cracow, Poland
[b] Department of Metabolic Diseases Collegium Medicum, Jagiellonian University, Cracow, Poland
[c] Institute of Clinical Chemistry and Laboratory Medicine, University of Regensburg, Regensburg, Germany
[d] Department of Molecular Biology and Clinical Genetics Laboratory Collegium Medicum, Jagiellonian University, Cracow, Poland

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ABSTRACT

Mediators such as cytokines, eicosanoids, nitric oxide and growth factors may regulate adipogenesis as well as inflammation. It is well documented that production of some form of eicosanoids activates lipid synthesis during adipogenesis but also contributes to the formation of factors maintaining low-level systemic inflammation. Developing nutrients for reduction of adipogenesis and inflammation can enhance preventive efficacy of daily diet. This study examined the effects of free fatty acid influence on changes in lipid biosynthesis and corresponding gene expression during differentiation of human subcutaneous adipose tissue stromal vascular fraction (SVF) cells.
Proadipogenic conditions promoted SVF cell differentiation and lipid droplet (LD) formation up to 15 days. This correlated with gene expression of adipocyte differentiation markers as well as inflammatory cytokines and their receptors.
Addition of free fatty acids to differentiation medium increased their incorporation during the first period of differentiation (48 h). Presence of eicosanoid acid (EPA) during the initial period of differentiation by elevation of Perilipin 3 protein (TIP47), may be responsible for smaller LD formation. Presence of arachidonic acid (AA) tends to deposit lipids in large form of LDs. Prolongation of differentiation up to 15 days decreased AA or EPA in cellular lipids. PUFA through up-regulation of both phospholipase 2 and enzymes related to eicosanoid production influenced type and quantity of eicosanoids which regulated the extent of SVF cell differentiation. Formation of small LDs and reduction of pro-inflammatory mediators in adipose tissue are the consequence of eicosanoid production with anti-inflammatory potential from EPA.

1. Introduction

Excess energy supplied with the daily diet results in accumulation of triglycerides and sterol esters in adipocyte lipid droplets (LDs). This event plays an important role in protecting other cells from lipotoxic effects of ectopic lipid accumulation and prevents development of metabolic diseases (insulin resistance, non-alcoholic fatty liver (NAFLD), diabetes type 2 with its cardiovascular complications) [1–3].

Differentiation of preadipocytes undergoes a subsequent multi-step program activation of a transcription factor network resulting in the adipocyte phenotype [4–6]. Proadipogenic conditions in *in vitro* cell culture experiments mimic excess of metabolic substrates, such as glucose and fatty acids, and lead to the generation of free radicals (reactive oxygen species–ROS) due to the initiation of ER-stress resulting in LD formation [7,8].

The molecular basis of the co-dependence of adipogenesis and low-grade inflammation has been the focus of research in recent years [4,6]. Mediators such as eicosanoids, cytokines, nitric oxide or growth factors regulate adipogenesis as well as inflammation with associating angiogenesis [5,9]. Certain eicosanoids activate fatty acid biosynthesis and lipid turnover during adipogenesis and contribute in maintaining low-level systemic inflammation [9–12]. On the other hand the same factors that initially induce inflammatory responses may also participate in stimulation of biosynthesis of pro-resolving mediators [13].

The aim of this study was to establish the impact of free fatty acids on LD formation during human preadipocyte (stromal vascular fraction cells (SVF)) differentiation using high throughput methods.

2. Materials and methods

2.1. 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 healthy donors aged 20–60 years. All reagents for

* Corresponding author at: Department of Clinical Biochemistry, Medical College, Jagiellonian University, Kopernika 15a, 31-501 Krakow, Poland. Tel.: +48 12 421 40 06; fax: +48 12 421 40 73.
E-mail address: apolus@cm-uj.krakow.pl (A. Polus).

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tissue culture were purchased from Sigma-Aldrich (Poznan, Poland), except FBS which was purchased from Life Technologies (Carlsbad CA, USA).

For SVF cell isolation, adipose tissue was carefully cleaned from the remaining connective tissue and blood vessels and resulting adipose tissue was incubated with collagenase solution in PBS (200 U/mL) in a shaking water bath at 37 °C for approximately 60–90 min, separated by centrifugation (600 × *g* for 10 min at room temperature) and washed twice with DMEM/F12 medium containing 10% of fetal bovine serum (FBS). Supernatant was discarded and the cellular pellet was resuspended in DMEM/Ham's:F12 medium (50:50, v:v) supplemented with gentamicin (50 μg/mL) and 10% FBS. For cell selection cell strainers (ø70 μm; optionally 25 or 40 μm) (BD Biosciences Discovery Labware, Bedford, MA, USA) were used [15]. SVF cells were cultured in medium containing FBS. Twenty-four h after isolation of the SVF cells from adipose tissue, inoculation medium containing FBS was changed to fresh adaptation medium. SVF cells were propagated in FBS free medium supplemented with human transferrin (10 μg/mL), human insulin (66 nM) and hydrocortisone (100 nM).

For preadipocyte differentiation the proadipogenic medium (MDI: 0.5 nM isobuthylmetyloxantine (IBMX), 0.25 nM dexamethasone, 66 nM insulin), necessary for cAMP signaling activation at an initial stage of fat tissue expansion, was used [16,17]. SVF cells were incubated in proadipogenic medium for 48 h (Ctrl(+)48h) then followed by 15 days of incubation in DMEM/Ham's:F12 medium (50:50, v:v) supplemented with gentamicin (50 μg/mL), human transferrin – 10 μg/mL, human insulin – 66 nM, hydrocortisone – 100 nM, triiodothyronine – 1 nM without FBS (Ctrl(+)15d) and differentiation stimulating factors. For the measurement of free fatty acid (FFA) effect, palmitic acid (PA), oleic acid (OA), arachidonic acid (AA) or eicosapentaenoic acid (EPA) at concentrations of 30 μM were added separately for the last 24 h of incubation with proadipogenic medium only during the initial period of differentiation. The negative control cells (Ctrl(−)) were incubated with the same regime with the exclusion of adipogenic factors (dexamethasone, IBMX, insulin). Analyses were performed at 2 time-points – first after 48 h (initial period of differentiation) and the second after 15 days of differentiation.

2.2. Lipid accumulation. Oil-Red O staining

To measure lipid accumulation the Oil-Red O staining method was used [18]. Cells were fixed with 3.7% paraformaldehyde and stained with a 0.5% solution of Oil-Red O (Sigma, Poznan, Poland) in isopropanol (POCH, Gliwice, Poland). Then cells were washed with distilled water and lipid droplets were visualized using a light microscope (Olympus CK40). For quantitative measurement Oil Red-O was eluted with isopropranol and the absorbance of the obtained supernatant was measured (OD at 500 nm) using 100% isopropanol as blank and recalculated for protein content of each probe determined according to the Lowry method. Data are presented as percentage of appropriate Ctrl(+) in two time points ($n = 5$) [18].

2.3. The lipid droplet PAT proteins and BODIPY quantification (Bioimager^{BD})

For visualization of Perilipin 1 (PLIN1), Adipophilin (ADFP/PLIN2) and TIP47 (PLIN3) – PAT proteins [19] and LD formation, the cell cultures were fixed with paraformaldehyde (3.7%). PAT proteins were stained with antibodies against: Perilipin 1 (1:500 dilution), Adipophilin (1:2000 dilution), and TIP47 (1:1000 dilution) (PROGEN Biotechnik, Heidelberg, Germany). Lipids and nuclei were imaged by BODIPY 492/503 staining (1:5000 dilution) and Hoechst (1:5000 dilution) respectively. Studies were performed in 96-well plates ((BD Biosciences, Bedford, MA, USA)) using the BD Pathway 855 Bioimager (BD Biosciences). The imaging data was automatically collected and analyzed using the supplemented Attovision software package. Results were presented as ratios of total fluorescence intensity generated in the cytoplasmic rings around the nuclei to the mean fluorescence intensity of the positive control ($n = 5$).

2.4. Changes in gene expression

2.4.1. Isolation of total RNA

Total RNA was isolated using TRIzol® Plus RNA Purification System (Life Technologies Carlsbad CA, USA) according to the manufacturer's instructions. RNA quality was assessed using the RNA 6000 Nano kit on the Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) and quantified by spectrophotometry using a NanoDrop ND-1000 UV-VIS spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Samples with RIN >7.0 were selected for further analysis.

2.4.2. Gene expression—microarray hybridization

Microarrays were performed using Single Color human oligonucleotide arrays and reagents from Agilent (Agilent Technologies Santa Clara, CA, USA). Each separate RNA sample was hybridized to a single array and all expression changes were detected by comparison to the control (Ctrl(−)). Labeling of 100 ng of total RNA was done using the Quick Amp labeling kit according to the manufacturer's guidelines. Briefly, using T7 promoter element coupled oligo-T primer, cDNA was generated. Subsequently labeled cRNA was synthesized from cDNA using T7 RNA polymerase and dyes. RNA concentration and dye incorporation were measured using Nanodrop ND-1000. Labeled cRNA was used for hybridization. Signal intensity of the labeled cRNA was measured by hybridization to a human gene microarray. Hybridization was conducted 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 Agilent SureScan Microarray Scanner using extraction software (Agilent Technologies, Santa Clara, CA, USA).

Microarray data analysis was done using Gene Spring version 10 (Agilent Technologies, Santa Clara, CA, USA).

2.5. Lipidomics

Analysis of the changes in lipid profile during differentiation was performed by gas chromatography and mass spectrometry (GC-MS/MS) in cooperation with the center at Regensburg as previously described [14,20]. The resulting data sets as the amount of lipid/mg protein from three independent experiments were averaged and analyzed as, the relative content of individual classes of lipids in cells, and fatty acids with different carbon chain lengths in the various lipid fractions.

For detection of eicosanoids secreted during SVF differentiation, collected supernatants at 2 time-points were frozen. Subsequently they were thawed on ice and ultracentrifuged to remove cellular debris. Supernatant was spiked with internal chemically identical deuterated standards – LXA_4-d5, 5-HETE-d8, 12-HETE-d8, 15-HETE-d8 (Cayman Chemical Co., Ann Arbor, USA) and pH adjusted to 3.5 using acetic acid. Organic phase extraction was performed twice using ethyl acetate. The combined organic layer was evaporated under nitrogen. The residue was dissolved in methanol and appropriate aliquots were injected into the HPLC column.

Separation of analytes was performed using reverse phase C18 columns (Synergi Fusion-RP, Phenomenex, Torrance, USA; 2 × 100 mm, 2.5-μm particle) stabilized at 37 °C and an HPLC system (UFLC LC-20AD, Shimadzu Scientific Instruments, Columbia, USA). Mobile phases were A): acetonitrile:water:acetic acid (80:20:0.001, v/v/v) and B): acetonitrile:acetic acid (100:0.001, v/v) at constant flow rate of 0.11 μL/min. A gradient elution used phases A/B (92/8, v/v) from 0 to 1 min, then gradient A/B (5/95, v/v) from 1 to 10 min, next unchanged for 0.5 min, subsequently gradient A/B (100/0 v/v) from 10.5 to 11 min, then unchanged during the next 2 min. All reagents