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Interleukin 4 affects lipid metabolism and the expression of pro-inflammatory factors in mature rat adipocytes

Dawid Szczepankiewicz[a,*], Marek Skrzypski[a], Ewa Pruszyńska-Oszmałek[a], Paweł A. Kołodziejski[a], Maciej Sassek[a], Barbara Stefańska[b], Krzysztof W. Nowak[a], Aleksandra Szczepankiewicz[c]

[a] Department of Animal Physiology and Biochemistry, Poznań University of Life Sciences, Wołyńska 35 St., 60-637 Poznań, Poland
[b] Department of Animal Nutrition and Feed Management, Poznań University of Life Sciences, Wołyńska 33 St., 60-637 Poznań, Poland
[c] Laboratory of Molecular and Cell Biology, Department of Pediatric Pulmonology, Allergy and Clinical Immunology, IIIrd Department of Pediatrics, Poznan University of Medical Sciences, Szpitalna 27/33 60-572 Poznań, Poland

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ABSTRACT

Chronic low-grade inflammation contributes to diseases associated with fat tissue metabolism such as obesity and diabetes by the disturbed production of adipose tissue proteins, both pro- and anti-inflammatory. Interleukin-4 (IL-4) is one of the main inflammatory cytokines that activates Th2-dependent immune response and its increased expression was observed in the course of diseases characterized by chronic low-grade systemic inflammation such as obesity and asthma. We aimed to investigate if IL-4 may influence lipid metabolism and inflammatory responses in primary mature rat adipocytes.

Mature adipocytes were isolated from male Wistar rats in incubated with IL-4 at three concentrations. We measured lipogenesis and lipolysis as well as the expression of selected genes using ddCt method was used to calculate relative gene expression. Protein level in tissue was analyzed using Western blot. Protein concentration in cell medium was analyzed using ELISA. Statistical analysis was done using GraphPad Prism 5 software.

In primary cell model, we found that IL-4 stimulated lipogenesis and inhibited lipolysis in mature rat adipocytes. It also stimulated the expression of pro-inflammatory cytokines produced by adipocytes and decreased the expression of anti-inflammatory protein, adiponectin. Moreover, we found increased expression of inflammatory cytokines and transcription factors associated with Th2 response.

Our observations suggest that low-grade inflammation enhances fat accumulation and significantly alters adipocyte metabolism.

1. Introduction

Low-grade inflammation is associated with obesity and promotes insulin resistance (Wlazlo et al., 2012; Llaurado et al., 2012; Makki et al., 2013), but was also suggested to play a role in other chronic inflammatory diseases such as asthma (Chinkwo and Bwititi, 2016). Chronic low-grade inflammation contributes to diseases associated with fat tissue metabolism such as obesity and diabetes by the disturbed production of adipose tissue proteins (Mokdad et al., 2001; Guerre-Millo, 2004; McLachlan et al., 2007). Previous studies indicated mainly the alterations in pro-inflammatory cytokines profile produced in adipocytes such as leptin, resistin, interleukin 6 (IL-6) and tumor necrosis factor alpha (TNFα) (Lugogo et al., 2010; Rajkovic et al., 2014; Jung and Choi, 2014; Mirza et al., 2012). Similar effects were observed in obese asthmatic patients who showed higher concentrations of inflammatory factors produced in fat cells as compared to non-obese asthma including C-reactive protein (CRP), tumor necrosis factor-alpha (TNFα), interleukin (IL)-6, leptin and resistin (Lessard et al., 2011; Shore, 2010; Lugogo et al., 2011). Therefore, the clinical studies indicated that increased amount of fat tissue contributes to enhanced pro-inflammatory mediators production and that low-grade systemic inflammation may modulate the course of chronic diseases such as obesity, diabetes type 2 or asthma (Hotamisligil et al., 1993; Lessard et al., 2008).

Interleukin-4 (IL-4) is one of the main inflammatory cytokines that activates Th2 immune response by controlling the production of pro-inflammatory mediators from macrophages (Paul, 1997; Garcia-Zepeda et al., 1996; Kang et al., 2008), mainly via inducing M2-like

* Corresponding author.
E-mail address: dawszczepankiewicz@gmail.com (D. Szczepankiewicz).

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macrophages polarization (Mauer et al., 2014). Increased IL-4 production was observed in the course of diseases characterized by chronic low-grade systemic inflammation such as diabetes mellitus 2, obesity and asthma (Paul and Seder, 1994; El-Wakkad et al., 2013; Gruchala-Niedoszytko et al., 2013).

Recent studies showed that IL-4 decreased lipid content in 3T3-L1 adipocytes (Tsao et al., 2014; Shiau et al., 2015). Similar results were obtained in another study showing that IL-4 inhibited triglyceride accumulation in mouse fat tissue, leading to decreased weight gain and reduced fat accumulation in 3T3-L1 adipocytes (Chang et al., 2012). However, these data were obtained in a cell line model that might not have reflected well the effects observed *in vivo* thus limiting translation into clinical context. In addition, it has been shown that 3T3-L1 cell line has characteristics of other adipocytes cell types. It was found that 3T3-L1 may represent features specific not only for white but also brown adipocytes that show different expression profile. This suggests that the results obtained in 3T3-L1 cell line may differ from primary cells e.g. isolated mature adipocytes (Morrison and McGee, 2015).

Taking into account the conflicting data from clinical studies and *in vitro* observations in a cell line model, we hypothesized that IL-4 may increase deposition of lipids in adipocytes and enhance secretion of inflammatory factors that underlie low grade inflammation in adipose tissue. The aim of this study was to investigate the effect of IL-4 exposure on the lipid metabolism and the expression of inflammatory factors of rat adipocytes in primary cell culture model.

2. Materials and methods

2.1. Animals

Twelve adult male Wistar rats (weight of 230 ± 20 g) were used (Laboratory Animals, Brwinów, Poland) (n = 12). Rats were kept under standard conditions (12 h dark/l2h light; 21 °C ± 1 °C). Rats were given *ad libitum* access to food (Labofed B, Kcynia, Poland) and water. Rats were sacrificed by decapitation and epididymal fat pad tissue was immediately collected. All procedures were approved for the study and were performed in accordance with the "Act on the protection of animals used for scientific purpose" of the Republic of Poland, which complies with the EU directive (no. 2010/63/EU) for the protection of animals used for scientific purposes (decision no. 21/2014).

The experimental design has been presented on Supplementary Fig. 1.

2.2. Adipocytes isolation

Mature rat adipocytes were isolated according to the method by Rodbell et al. (Rodbell, 1964) with modifications by Szkudelska et al. (Szkudelska et al., 2000). The epididymal fat pad were washed in 0.85% NaCl cut into pieces and transferred to plastic flask with Krebs-Ringer buffer pH = 7.4 (118 mM NaCl, 4.8 mM KCl, 1.3 mM CaCl 2, 1,2 mM KH2PO4, 1.2 mM MgSO4, 248 mM NaHCO3, 10 mM Hepes, 3% BSA fraction V, 5 mM glucose) containing 2 mg/ml collagenase type II (EC 3.4.24.3). After 90 min incubation at 37 °C with shaking steps, cells were washed 4 times in Krebs-Ringer buffer without collagenase and filtered through nylon mesh (250 μM pore size). The viability of the cells was about 90% and was determined by trypan blue staining. Adipocytes quantity and purity was determined by use microscope and Bürker-Türk counting chamber.

2.3. Lipolysis and lipogenesis

Both procedures were performed according to the method described previously by our research group (Pruszynska-Oszmalek et al., 2013). For stimulation of lipolysis and lipogenesis upon insulin the dose 10 nM of insulin was used. In this study, instead for 1 μM of adrenalin during lipolysis stimulation, isoproterenol (1 μM) was used. Rat IL-4 (Sigma-Aldrich Co, Poland, cat. no I3650) in three different doses, based on our previous optimization experiments (1, 10, 100 nM) was used. Control group was incubated with PBS (phosphate buffered saline). For lipolysis and lipogenesis experiments, isolated adipocytes were incubated with IL-4 for two hours whilst for gene and protein expression analysis, the calculation of hormones concentration in medium was done 8 h post-incubation. Cells cultured without IL-4 (PBS) were a control group of the experiment. Each procedure was performed three times. Four rats were used for one procedure.

2.4. Non-esterified fatty acids (NEFA)

Quantitative determination of NEFA in cell medium was performed by use of NEFA-HR(2) kit (cat. no. 434 91795 NEFA-HR(2) R1, 436 91955 NEFA-HR(2) R2, 270 77000 NEFA Standard, WAKO Chemicals GmbH, Germany) according to manufactured protocol. Optical density of samples was analyzed using microplate reader (Synergy 2, Biotek, USA).

2.5. RNA isolation and reverse transcription

Total RNA was isolated from adipocytes with TriPure Reagent (Roche Diagnostic, Penzberg, Germany) according to the manufacturer's protocol. The amount and quality of isolated RNA was measured on spectrophotometer NanoDrop 1000 (Thermo Scientific, Wilmington, DE, USA). Total RNA (1 μg) was used for reverse transcription with Transcriptor first strand cDNA synthesis kit (Roche Diagnostic, Penzberg, Germany). A negative control (RT-) lacking reverse transcriptase was also prepared.

2.6. Real-time PCR

The expression of selected genes encoding pro-inflammatory cytokines produced by macrophages in adipose tissue (*Tnfα, Il-6, Crp*) or involved in Th2 stimulation (*Il-13*) were analyzed. To confirm the biological action of IL-4, the two main transcription factor genes inducing Th2-dependent response upon IL-4 (*Gata3, Stat6*) were analyzed. Obtained cDNA was diluted 1:10 and used for quantitative RT-PCR (qPCR). The reaction was performed using SYBR® Green PCR Master Mix with ROX Passive Reference Dye and Quant Studio 12 K Flex system (Life Technologies (ThermoFisher Scientific, USA) and analyzed using the comparative ΔΔCt method. Melting curve analysis was done to verify the specificity of PCR product. For each sample, the expression levels of the target genes were normalized to the reference gene (*Gapdh*). Negative and positive control of reverse transcription and qPCR reactions were also performed. Gene-specific primers sequences that span exon-exon junction (Roche Assay Designed Center) are presented in Supplementary table 1. Gene expression analyses are present as relative quantification. Each sample was performed in duplicate.

2.7. Western blot analysis

Protein isolation from adipocytes and Western blot detection of GLUT4 (the main glucose transporter in adipocytes) (Abcam cat. ab654), lipase (phospoHSL Ser660, Cell Signaling, Netherlands cat. 4126), perilipin (PLIN, Cell Signaling, Netherlands cat. 9349), the main protector of lipid droplets in adipose tissue, and GAPDH as a standard was carried out as described previously (Skrzypski et al., 2014). In brief, after the experiment adipocytes were collected and placed in 100 μl of RIPA buffer containing 50 mM Tris–HCl, pH 8.0 with 150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS and protease inhibitor cocktail (Roche Diagnostics, Penzberg Germany. Then, cells were lysed on ice for 10 min and centrifuged for 10 min at 12,000 *g*. Obtained supernatants were collected and kept at −80 °C for Western blot analyses. Antibodies and concentrations used in the experiments are presented in Supplementary table 2. Analysis was made by use of