Original article

MOLECULAR METABOLISM

The ω6-fatty acid, arachidonic acid, regulates the conversion of white to brite adipocyte through a prostaglandin/calcium mediated pathway

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ABSTRACT

Objective: Brite adipocytes are inducible energy-dissipating cells expressing UCP1 which appear within white adipose tissue of healthy adult individuals. Recruitment of these cells represents a potential strategy to fight obesity and associated diseases.

Methods/Results: Using human Multipotent Adipose-Derived Stem cells, able to convert into brite adipocytes, we show that arachidonic acid strongly inhibits brite adipocyte formation *via* a cyclooxygenase pathway leading to secretion of PGE2 and PGF2α. Both prostaglandins induce an oscillatory Ca^{++} signaling coupled to ERK pathway and trigger a decrease in UCP1 expression and in oxygen consumption without altering mitochondriogenesis. In mice fed a standard diet supplemented with ω6 arachidonic acid, PGF2α and PGE2 amounts are increased in subcutaneous white adipose tissue and associated with a decrease in the recruitment of brite adipocytes.

Conclusion: Our results suggest that dietary excess of ω6 polyunsaturated fatty acids present in Western diets, may also favor obesity by preventing the "browning" process to take place.

Keywords Polyunsaturated fatty acids; PGE2; PGF2α; PGI2; Calcium oscillation; UCP1

1. INTRODUCTION

Both overweight and obesity result from an imbalance between energy intake and energy expenditure. So far, regulation of energy intake by dietary and pharmacological treatments has met limited success. In the last few years, the characterization of functional brown adipose tissue (BAT) in adult humans has opened new perspectives for regulating energy expenditure. In contrast to white adipose tissue (WAT) involved in energy storage, BAT is endowed with a thermogenic activity and regulates body temperature by dissipating energy through heat production [1]. This process of non-shivering thermogenesis is due to the occurrence of the Uncoupling Protein 1 (UCP1) localized in BAT mitochondria and is induced in rodents in response to cold *via* β-adrenergic stimulation. The energy-dissipating properties of UCP1 lead to an increased oxidation of fatty acids and are important for body weight regulation [2]. Interestingly, another population of thermogenic adipocytes is present in rodent WAT and termed brite for "brown in white" or beige adipocytes [3,4]. These brown-like adipocytes appear in response to cold exposure or high-fat diets and stem either from progenitors or by direct conversion of mature white adipocytes [5–7], and have been recently found in adult humans [8–12]. Induction of their activity appears as an interesting strategy to fight obesity by enhancing body energy expenditure as increased oxidation of fatty acids within these cells limits their release into the general circulation. We isolated human Multipotent Adipose-Derived Stem (hMADS) cells as the first model of cells undergoing the conversion from white to brite functional phenotype [13,14]. This cell model appears suitable for studies aimed at delineating the role of key components of diets in this process, among which the possible involvement of essential ω6 polyunsaturated fatty acids (PUFAs).

Dietary fats are the source of the essential PUFAs, both ω6 linoleic acid (LA), a precursor of ω6 arachidonic acid (ARA), and ω3 α-linolenic acid,

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Received August 13, 2014 • Revision received September 3, 2014 • Accepted September 4, 2014 • Available online 16 September 2014

http://dx.doi.org/10.1016/j.molmet.2014.09.003

a precursor of ω3 eicosapentaenoic (EPA) and docosahexaenoic acid (DHA). These very long-chain PUFAs trigger a variety of biological responses and are required for a healthy development. Prostacyclin (PGI2) and other prostaglandins of the 2 series (PGD2, PGE2 and PGF2α) are prostanoids synthesized from ARA and are involved in the differentiation, maturation and function of white adipocytes. PGI2 triggers adipocyte differentiation *in vitro* [15] and *in vivo* [16,17], while PGF2α behaves as a strong inhibitor [18,19]. The role of PGE2 is more controversial, as it has been described to inhibit or to promote adipogenesis [20,21], and is likely due to the diversity of its receptors able to modulate differently both Ca^{++} and cAMP-dependent pathways [22].

During the last decade, dietary recommendations have taken into account the insufficient intake of ω3 PUFAs and the excess of ω6 PUFAs which is correlated with overweight/obesity [23,24]. Interestingly, high ω6/ω3 ratios are positively associated with adiposity of infants at 3 and 4 years of age [25,26]. ARA levels correlate positively with body mass index (BMI) and the associated metabolic syndrome [27–30]. Diets with higher ω6/ω3 ratio result in higher arachidonic acid and lower EPA + DHA levels in plasma and adipose tissue and enhance ARA availability to synthesize PGs of the 2 series in adipose tissue.

Since brite/brown adipocytes appear absent from obese patients [8], we therefore sought to analyze the possible role of ω6 ARA and its metabolites in human brown/brite adipocyte development and functions. Herein, we show a potent inhibitory effect of ARA on white to brown adipocyte conversion of hMADS cells. ARA inhibits the expression of UCP1 and leads to a decrease in the thermogenic capacity of hMADS adipocytes characterized by a lower mitochondrial activity and basal oxygen consumption. The effect of ARA is mediated *via* cyclooxygenase activities leading to increased synthesis and release of PGE2 and PGF2α. Thorough analysis of the role of PGE2 and PGF2α demonstrate that the ARA/prostaglandin/calcium pathway is responsible of impairing the browning process. Finally, we show that supplementing mice with an ARA-enriched diet leads to increased synthesis of both prostaglandins while lowering the occurrence of inducible brite adipocytes upon stimulation of the recruitment by β3-adrenergic receptor agonist.

2. MATERIALS AND METHODS

2.1. Animals

The experiments were conducted in accordance with the French and European regulations (directive 2010/63/EU) for the care and use of research animals and were approved by local experimentation committees (Nice University and Ciepal Azur: protocol NCE-2012-57). Ten-week-old C57Bl/6JRccHsd female mice were from Harlan and maintained at constant temperature (21 ± 2 °C) and 12:12-hour light–dark cycles, with *ad libitum* access to diet and water. Mice were fed for 4 weeks with ARA- or oleic acid (OA)-supplemented diet. Chronic β-adrenergic receptor stimulation was carried the last week of the diet by daily intra-peritoneal injections of CL316,243 (1 mg/kg/day in saline solution). Control mice were injected with vehicle only. Standard chow diets (ref. 2016, Harlan Lab., WI, USA) were enriched with 11 g/Kg of oleate–ethyl–ester or arachidonate–ethyl–ester (Harlan Lab., WI, USA, Nu-Chek-Prep, MIN, US) and 5 g/Kg of Safflower Oil to favor dispersion of ethyl esters in the diet. Blood, interscapular BAT (iBAT) and inguinal subcutaneous WAT (scWAT) were sampled and used for different analysis. Histology, protein and RNA extracts as well as further analysis are described in Supplemental procedures.

2.2. hMADS cell culture

The establishment and characterization of hMADS cells have been described in Refs. [13,31–33]. In the experiments reported herein hMADS-3 cells were used and came originally from the prepubic fat pad of a 4-month-old male. Cells were used between passages 14 and 25, and all experiments have been performed at least 3 times using different cultures. Cells were seeded at a density of 5000 cells/cm^2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 15 mM Hepes, 2.5 ng/ml hFGF2, 60 μg/ml penicillin, and 50 μg/ml streptomycin. hFGF2 was removed when cells reached confluence. Cells were triggered for differentiation at day 2 post-confluence (designated as day 0) in DMEM/Ham's F12 media supplemented with 10 μg/ml transferrin, 10 nM insulin, 0.2 nM triiodothyronine, 1 μM dexamethasone and 500 μM isobutyl-methylxanthine. Three days later, the medium was changed (dexamethasone and isobutyl-methylxanthine omitted) and 100 nM rosiglitazone were added. At day 9 rosiglitazone was withdrawn to enable white adipocyte differentiation (R3–9) but again included between days 14 and 17 to promote white to brite adipocyte conversion (R3–9/14–17). Media were changed every other day and cells used at day 17. Fatty acids and prostaglandins were bound to BSA (0.04% for 15 min at 37 °C) prior to inclusion to culture media. Analysis of secreted prostanoids was performed at day 17 after incubation of the cells for 10 min in fresh culture media. PGE2, PGF2α and 6-keto-PGF1α were quantified by EIA following manufacturer's instructions (Cayman, BertinPharma, Montigny le Bretonneux, France). Glycerol-3-phosphate dehydrogenase (GPDH) activity measurements and Oil Red O staining were performed as described previously in Ref. [34]. Immunostaining, *Cytochrome c oxidase activity* measurements, protein and RNA extracts preparation and further analyses are described in Supplemental procedures.

2.3. Prostanoid quantification *in vivo* by mass spectrometry analysis

All tissues were snap-frozen with liquid nitrogen immediately after collection and stored at −80 °C until extraction. For extraction, each frozen adipose tissue was crushed with a FastPrep®-24 Instrument (MP Biomedical) in 500 μL of HBSS (Invitrogen) and 5 μL of internal standard (Deuterium labeled compounds). After 2 crush cycles (6.5 m/s, 30 s), 20 μL were withdrawn for protein quantification and 300 μL of cold methanol (MeOH) were added. After centrifugation at 900 g for 15 min at 4 °C, supernatants were transferred into 2 ml 96-well deep plates and diluted in H_2O to 2 ml. Samples were then submitted to solid phase extraction (SPE) using HRX 96-well plate (50 mg/well, Macherey Nagel) pretreated with MeOH (2 ml) and equilibrated with 10% MeOH (2 ml). After sample application, extraction plate was washed with 10% MeOH (2 ml). After drying under aspiration, lipid mediators were eluted with 2 ml of MeOH. Prior to LC-MS/MS analysis, samples were evaporated under nitrogen gas and reconstituted in 10 μL of MeOH.

LC-MS/MS analyses of prostanoids were performed as previously described in Ref. [35]. Briefly, lipid mediators were separated on a ZorBAX SB-C18 column (2.1 mm, 50 mm, 1.8 μm) using Agilent 1290 Infinity HPLC system coupled to an ESI-triple quadruple G6460 mass spectrometer (Agilent Technologies). Data were acquired in Multiple Reaction Monitoring (MRM) mode with optimized conditions (ion optics and collision energy). Peak detection, integration and quantitative analysis were done using Mass Hunter Quantitative analysis software (Agilent Technologies) based on calibration lines built with commercially available prostanoid standards (Cayman Chemicals).

2.4. Measurement of oxygen consumption

Oxygen consumption was measured using polarometric technique. Briefly, differentiated cells were introduced in a closed chamber

