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MOLECULAR METABOLISM

Epoxygenase inactivation exacerbates diet and aging-associated metabolic dysfunction resulting from impaired adipogenesis

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ABSTRACT

Objective: When molecular drivers of healthy adipogenesis are perturbed, this can cause hepatic steatosis. The role of arachidonic acid (AA) and its downstream enzymatic cascades, such as cyclooxygenase, in adipogenesis is well established. The exact contribution of the P450 epoxygenase pathway, however, remains to be established. Enzymes belonging to this pathway are mainly encoded by the *CYP2J* locus, but the latter shows extensive allelic expansion in mice, an obstacle for adipogenesis-related studies. The human CYP2J locus contains a single gene (CYP2J2) whereas mice and rats have 8 and 3 paralogues, respectively.

Methods: We took advantage of the simpler genetic architecture of the *Cyp2j* locus in the rat and generated a *Cyp2j4* (orthologue of human *CYP2J2*) knockout rat. We used *Cyp2j4*$^{-/-}$ rats in two models of metabolic dysfunction: physiological aging and cafeteria diet (CAF). The phenotyping of *Cyp2j4*$^{-/-}$ rats under CAF was integrated with proteomics (LC-MS/MS) and lipidomics (LC-MS) analyses in the liver and the adipose tissue.

Results: We report that *Cyp2j4* deletion causes adipocyte dysfunction under metabolic challenges. This is characterized by (i) down-regulation of white adipose tissue (WAT) PPARγ and C/EBPα, (ii) adipocyte hypertrophy, (iii) extracellular matrix remodeling, and (iv) alternative usage of AA pathway. Specifically, in *Cyp2j4*$^{-/-}$ rats treated with a cafeteria diet, the dysfunctional adipogenesis is accompanied by exacerbated weight gain, hepatic lipid accumulation, and dysregulated gluconeogenesis.

Conclusion: These results suggest that AA epoxygenases are essential regulators of healthy adipogenesis. Our results uncover their synergistic role in fine-tuning AA pathway in obesity-mediated hepatic steatosis.

Keywords Adipogenesis; Cytochrome P450 2j4; Cafeteria diet; Aging; Steatosis; Arachidonic acid

1. INTRODUCTION

Obesity is a complex metabolic disorder with complications such as insulin resistance, chronic inflammation, and hepatic steatosis, all of which under the influence of white adipose tissue (WAT), a highly dynamic, master-regulatory endocrine organ crucial for metabolic homeostasis [1,2]. It is argued that the key mediators of obesity-mediated metabolic disease (i.e. insulin resistance and inflammation) are evolutionarily conserved but could display pathological properties under modern obesogenic environment, which is characterized by excess nutrient consumption [3].

At the heart of WAT homeostasis, adipogenesis is the process of differentiation of pre-adipocytes to become mature under a core transcriptional program driven by nuclear hormone receptor peroxisome proliferator-activated receptor-γ (PPAR-γ) and CCAAT/enhancer binding protein-α (C/EBPα) [4,5]. In addition to the role of PPAR-γ in embryonic adipogenesis, C/EBPα and PPAR-γ are actively involved in adult WAT expansion following high dietary fat exposure [6]. During

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Abbreviations: WAT, white adipose tissue; PPAR-γ, peroxisome proliferator-activated receptor-γ; C/EBPα, CCAAT/enhancer binding protein-α; ECM, Extracellular Matrix; NAFLD, Non-alcoholic fatty liver diseases; Cyp, cytochrome P450; sEH, epoxide hydrolase; CAF, Cafeteria diet; EET, Epoxyeicosatrienoic acid; COX, Cyclooxygenase; LOX, Lipoxygenase; MSC, Mesenchymal Stromal Cells; SVF, Stromal Vascular Fraction; *FASN*, Fatty acid synthase gene

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healthy WAT expansion, hypoxia and inflammation caused by activated macrophages lead to extracellular matrix (ECM) remodeling, which enables adipocyte hypertrophy [7]. However, in the case of chronic over-nutrition, this state of homeostasis is perturbed and causes unresolved, low grade WAT inflammation and fibrosis. The fibrotic and unrestrained WAT expansion, often promoted by pro-inflammatory macrophage activity, can eventually progress into adipose tissue dysfunction and ectopic lipid accumulation, in particular in the liver, one of the major contributors of obesity-mediated type 2 diabetes [1]. Non-alcoholic fatty liver disease (NAFLD) is characterized by hepatic lipid accumulation, which could lead to inflammation and fibrosis in the liver. The central role of adipose tissue in the development of NAFLD was established in humans [8,9] and animal models of diet-induced obesity [10]. Aging is also considered as a risk factor for insulin resistance and adipose tissue plays a central role in longevity. Aging and diet-induced obesity share pathways including WAT-mediated lipotoxicity [11], which suggests that common genes orchestrate WAT homeostasis and its regulatory role on NAFLD.

Oxylipins are endogenous, bioactive lipid mediators derived from arachidonic acid (AA) and related polyunsaturated fatty acids. Prostaglandins and leukotrienes are eicosanoids generated by well-defined enzymatic cascades initiated by cyclooxygenase and lipoxygenase [12]. A third pathway involves cytochromes P450 (CYPs). In humans, cytochrome P450 2J2 (CYP2J2), CYP2C8, and CYP2C9 are considered to be largely responsible for metabolizing AA into four regioisomeric epoxyeicosatrienoic acids (5,6-, 8,9-, 11,12-, and 14,15-EET) [13]. EETs are metabolized by soluble epoxide hydrolase (sEH) to the corresponding dihydroxyeicosatrienoic acids and sEH inhibition is a commonly used pharmacological approach aimed to increase intracellular EET pools. The previously reported biological effects of EETs are remarkably pleiotropic, ranging from anti-inflammatory and cardioprotective actions [14–16] to a regulatory role in cancer [17], organ/tissue regeneration [18], and embryonic haematopoiesis [19]. EETs are PPARγ ligands [20] and activators of PPARα [21]. Given the central role exerted by PPARγ in regulating adipogenesis, the link between epoxygenase-mediated EET production and obesity-associated syndromes was explored in transgenic animal models over-expressing human endothelial CYP2J2 or by inactivation of sEH (either by pharmacological inhibition or its targeted gene deletion [22–25]). These studies, which aimed to increase endogenous EET levels, achieved amelioration of obesity-associated metabolic dysfunction (i.e. dyslipidemia, prevention of hyperglycemia, improved insulin signaling and sensitivity, reduced AT inflammation). However the exact mechanisms through which the main endogenous epoxygenase regulate metabolic dysfunction remain poorly understood, mainly because of the technical obstacle encountered in gene targeting approaches in mice. The *Cyp2j* locus in mice contains eight potentially functional genes as it underwent allelic expansion [26]. The synthenic rat *Cyp2j* locus contains three genes and offers a relatively simplified genetic architecture for studying epoxygenase-related mechanisms. Thus, we have generated a rat deficient in *Cyp2j4*, the orthologue of human *CYP2J2* [27]. *Cyp2j4* is the main rat macrophage epoxygenase, which also shows wide-tissue expression including brain, left ventricle, kidney, lung, and spleen [27]. Although *Cyp2j3* (LOC100912642, cytochrome P450 2J3-like) maps to rat chromosome 5 and was initially reported as the rat orthologue of human *CYP2J2* [28], both genes were found to be expressed in major rat organs and share 79% homology. We did not find any expression of *Cyp2j16* in the rat, suggesting that the main Cyp2j-derived epoxygenase activity in the rat is determined by Cyp2j3 and Cyp2j4.

Here we took advantage of the reduced allelic expansion in the rat *Cyp2j* locus and used two distinct models of metabolic dysfunction to study epoxygenase-mediated adipogenesis in the wider context of obesity and NAFLD. In addition to physiological aging, we used a Western diet-induced obesity, previously described as cafeteria diet (CAF), which models hedonic feeding (or voluntary hyperphagia) [29]. We have previously shown strain-specific differences in CAF-induced metabolic dysfunction in the rat [30,31]. Here we found that *Cyp2j4* is essential for maintaining a healthy adipogenesis status, which, under metabolic challenges (e.g. CAF, aging), causes adipocyte dysfunction characterized by down-regulation of WAT PPARγ and C/EBPα and AA pathway shunt towards COX and LOX-derived eicosanoids. This dysfunctional adipogenesis causes hepatic lipid accumulation and *Cyp2j4*$^{-/-}$ treated with CAF show increased *de novo* lipogenesis in the liver, dysregulated gluconeogenesis, and increased hepatic and systemic triglyceride levels. These results determine the role of *Cyp2j4* in physiological (healthy) adipogenesis and show how this 'controlled' phenomenon progresses into adipocyte dysfunction and NAFLD under metabolic stresses such as diet and aging.

2. MATERIALS AND METHODS

2.1. Animals

Male wild type Wistar Kyoto (WKY) rats (Charles River, UK) and *Cyp2j4*$^{-/-}$ rats, previously generated on a WKY genetic background [27] were housed individually at 22 °C with a 12 h light/dark cycle with access to water and a standard diet *ad libitum*. The animals were maintained according to the ethical guidelines of Universitat Rovira i Virgili (URV, Committee on Animal Investigations) or the UK Home Office (United Kingdom Animals Scientific Procedures Act, 1986).

2.2. Cells and reagents

Mesenchymal stromal cells (MSCs) from 12-week old WT and *Cyp2j4*$^{-/-}$ rats were obtained as previously described [32]. MSCs cells were allowed to grow in Supplemented MesenCult™ MSC Medium (STEMCELL Technologies, UK) for 5 days on Petri dishes (Nunc, ThermoFisher Scientific, UK). MSCs from WT and *Cyp2j4*$^{-/-}$ rats were differentiated into mature adipocytes by incubation with an adipogenic induction medium (StemPro®,Gibco, UK) for 14 days.

Antibodies used in western blot were: anti-PPAR-γ (C26 H12, Cell Signaling #2435, 1:1000), anti CEBP-α (Cell Signaling #2295, 1:1000), anti-Phospho-Akt-Ser473 (D9E, Cell Signaling #4060, 1:2000), anti-Phospho-Akt-Thr308 (244F9, Cell Signaling #4056, 1:1000), anti-panAkt (C67E7, Cell Signaling #4691, 1:1000) and anti-β-Actin Antibody (C4, sc-47778, 1:10,000), anti-PPARα (H2, SC-398,394, 1:1000), anti-PPARβ/δ (F-10, SC-74517, 1:1000), anti-FXR (D-3, SC-25309, 1:1000), anti-LXRα (ab2585, 1:1000), and anti-β-Actin Antibody (C4, sc-47778, 1:10,000)

2.3. Cafeteria diet and aging

Eight-week-old WT and *Cyp2j4*$^{-/-}$ rats were randomly distributed into the four different experimental groups to receive either a standard laboratory chow (STD, A-04; Panlab) or a standard laboratory chow together with cafeteria diet (CAF) consisting of 300 ml of sugary milk (220 g/L), 25 g of bacon, 1 sausage, 1/4 carrot and 2 biscuits smeared with paté. Both WT and *Cyp2j4*$^{-/-}$ rats were fed either with standard diet (WT STD, *Cyp2j4*$^{-/-}$ STD) or CAF (WT CAF, *Cyp2j4*$^{-/-}$ CAF). Animals were fed *ad libitum* with fresh food daily for 12 weeks. For the aging protocol, WT and *Cyp2j4*$^{-/-}$ rats received a standard laboratory chow during 15 months. At the end of both CAF and aging protocols,

