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Weight gain and inflammation regulate aromatase expression in male adipose tissue, as evidenced by reporter gene activity

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ABSTRACT

Obesity and white adipose tissue (WAT) inflammation are associated with enhanced aromatization in women, but little is known about the regulation of aromatase (*CYP19A1*) gene expression in male WAT. We investigated the impact of weight gain and WAT inflammation on the regulation of *CYP19A1* in males, by utilizing the hARO-Luc aromatase reporter mouse model containing a >100-kb 5′-region of the human *CYP19A1* gene. We show that hARO-Luc reporter activity is enhanced in WAT of mice with increased adiposity and inflammation. Dexamethasone and TNFα, as well as forskolin and phorbol 12-myristate 13-acetate, upregulate hARO-Luc activity, suggesting the involvement of promoters I.4 and I.3/II. Furthermore, we show that diet enriched with antioxidative plant polyphenols attenuates WAT inflammation and hARO-Luc activity in obese males. In conclusion, our data suggest that obesity-associated WAT inflammation leads to increased peripheral *CYP19A1* expression in males, and that polyphenol-enriched diet may have the potential to attenuate excessive aromatization in WAT of obese men.

1. Introduction

The final step in the biosynthesis of estrogens is catalyzed by the cytochrome P450 aromatase (*CYP19A1*) enzyme, which converts androstenedione and testosterone (T) to estrone and estradiol (E_2), respectively. In humans, white adipose tissue (WAT) is an important site for extragonadal aromatization and estrogen production, and it has been estimated that 80% of E_2 in men is produced in extragonadal tissues (MacDonald et al., 1979). Increase in WAT mass, thus, leads into increased peripheral conversion of androgens to estrogens. Moreover, obesity-associated inflammatory factors upregulate aromatase gene expression in WAT of women (Morris et al., 2011; Subbaramaiah et al., 2012), indicating that low-grade inflammation further contributes to increased estrogen biosynthesis in WAT of obese individuals. Very little, however, is known about the regulation of *CYP19A1* gene expression, or the effects of locally produced estrogens in male WAT.

Excessive estrogen production in WAT of obese men can be postulated to play a role in the development of various disorders, such as male obesity-associated secondary hypogonadism (Tajar et al., 2012) and breast cancer (Liukkonen et al., 2010). Obesity is associated with elevated serum E_2/T ratio in men (Dobs et al., 2001; Foresta et al., 2009), and aromatase inhibitors normalize T levels in hypogonadal obese men (de Ronde and de Jong, 2011). In order to understand the pathophysiology of obesity-related endocrine dysregulation in men, and to develop effective interventions, it is necessary to define the mechanisms by which adiposity, inflammation and dietary factors regulate the expression of *CYP19A1* in male WAT. These mechanisms are difficult to study in human subjects, and experimental studies *in vivo* have been hampered by the lack of appropriate rodent models, due to the critical differences in the regulatory regions of human and rodent *CYP19A1* genes (Zhao et al., 2009). Human *CYP19A1* gene is widely expressed in extragonadal tissues, and consists of at least 11 noncoding first exons, alternatively expressed in different tissues and each controlled by distinct sets of cytokines and hormones (Demura et al., 2008; Simpson et al., 2002). In rodent aromatase gene, the regulatory region is less complex, and lacks several of the first exons that drive extragonadal aromatase expression in humans (Golovine et al., 2003).

Abbreviations: CLS, crown like structure; DEX, dexamethasone; E_2, 17β-estradiol; FSK, forskolin; HFD, high fat diet; GF, gonadal fat tissue; LFD, low fat diet; Luc, luciferase; MSC, mesenchymal stromal cell; PKE, pine knot extract; PMA, phorbol 12-myristate 13-acetate; SCF, subcutaneous fat tissue; T, testosterone; WAT, white adipose tissue.

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For example, exons I.4 and I.3/II that are expressed in human WAT have not been detected in mouse WAT (Zhao et al., 2009).

In order to study the expression pattern and regulation of human *CYP19A1* gene *in vivo*, we have generated a transgenic reporter mouse model (hARO-Luc mouse) with >100-kb 5′-region of human *CYP19A1* gene attached to a luciferase (Luc) reporter gene (Strauss et al., 2013). The tissue distribution of Luc reporter in hARO-Luc mice closely resembles that described for *CYP19A1* gene in human tissues (Strauss et al., 2013). The objective of this study was to investigate the regulation of human *CYP19A1* gene in different WAT depots in obese male hARO-Luc mice, and to identify exogenous factors that can modulate *CYP19A1* expression in WAT of obese individuals. As an intervention, we chose to use a diet supplemented with mixture of antioxidative plant polyphenols (pine knot extract, PKE) (Yatkin et al., 2014), expected to alleviate WAT inflammation (Jeon et al., 2012; Subbaramaiah et al., 2013; Tomé-Carneiro et al., 2013).

2. Materials and methods

2.1. Animals and diets

Animal care and studies were conducted in accordance with the Finnish Act on Animal Experimentation and EU laws, guidelines, and recommendations. The National Animal Experiment Board in Finland approved all studies.

Male hARO-Luc reporter mice were housed (1–4 mice per cage) in 12-hour light/dark cycle at 22–24 °C and 50% relative humidity, and fed with soy-free RM3 chow (Special Diets Services, Witham, UK) and tap water *ad libitum*. At the age of 6 weeks hARO-Luc males were allocated into the dietary intervention groups with similar body weights, body adiposity and littermate distributions: (i) LFD group (n = 17) fed with a purified low fat diet providing 10% of calories from fat (LFD, D12450; Research Diets Ltd, New Brunswick, NJ); (ii) HFD-group (n = 17) fed with a purified high fat diet (HFD, D12492; Research Diets Ltd), providing 60% of calories from fat, and (iii) HFD-PKE-group (n = 10) fed with HFD supplemented with polyphenol-rich extract (PKE, 1600 mg/kg/diet). Food intake per cage was recorded twice a week.

The preparation, composition and bioavailability of pine knot extract (PKE), obtained from *Pinus sylvestris*, have been described earlier (Yatkin et al., 2014). Mice were weighed weekly and their body adiposity was measured with EchoMRI body composition analysis device (EchoMRI LCC, Houston, TX) at the start of the diet intervention, after 4 weeks and at week 8 (*i.e.*, at the end of the intervention period). For the analysis of PKE-derived polyphenols, 24-h urine samples were collected in metabolic cages and concentrations of polyphenols pinosylvin monomethylether, pinosylvin, nortrachelogenin, and matairesinol were determined as previously described (Yatkin et al., 2014).

Prior to sacrifice, animals were fasted for 4 hours, and blood glucose was measured from tail vein samples. For serum, blood was collected by intracardiac puncture and the serum samples were stored at –70 °C. Inguino-abdominal subcutaneous and gonadal fat depots (SCF and GF, respectively) were weighed and samples of tissues were snap frozen to liquid nitrogen and stored at –70 °C for Luc activity measurements. Fat samples were collected for *ex vivo* cultures and for histological analyses which will be described later.

2.2. Determination of average adipocyte volume and CLS density

Adipose tissue samples were taken from all animals in the intervention studies. Formalin fixed paraffin embedded tissues were cut into 5 µm thick sections, de-paraffinized, rehydrated, and stained with hematoxylin and eosin (HE). HE-stained sections were scanned with an Olympus BH2 virtual microscope (Digital Virtual Microscope, Soft Imaging System, Olympus, Germany) and the number of adipocytes and crown like structures (CLS), *i.e.* necrotic adipocytes surrounded by macrophages (Cinti et al., 2005), were calculated from four randomly selected 1 mm^2 areas per each sample. CLS density indicates the average number of CLS per 1 mm^2 area. The average adipocyte cross-sectional area was calculated as follows: 1/(number of adipocytes per 1 mm^2). The average volume of adipocytes was calculated by assuming that the shape of cells is globular.

2.3. Luciferase activity measurements

Luc activity was analyzed as previously described (Strauss et al., 2013). All reagents were obtained from Life Technologies (Life Technologies Ltd, Carlsbad, CA), unless stated otherwise. Shortly, tissues were homogenized in 25 mM Tris acetate buffer (pH 7.8) supplemented with 1.5 mM EDTA, 1% Triton X-100, 2 mM dithiotreitol (Sigma Aldrich Ltd, St. Louis, MO), 10% glycerol and Complete Miniproteinase inhibitor tablets (Roche Diagnostics, Penzberg, Germany). Homogenates were centrifuged at 800 *g* for 30 minutes at 4 °C and supernatants measured for luciferase activity with commercial luciferase assay kit (BioThema AB, Handen, Sweden) by using the Victor^2 Multilabel Counter (PerkinElmer, Turku, Finland). Results were related to the total protein concentration of samples measured with the Pierce BCA^2 Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL).

2.4. Ex vivo *adipose tissue cultures*

Method modified from McGillicuddy et al. (2011) was used. GF and SCF tissues were divided into approximately 100 mg explants, weighed, and placed on 24-well plates (1 explant per well) in 1 ml of phenol red-free Dulbecco's modified Eagle medium (#11880, Life Technologies Ltd) containing 100 IU/ml penicillin and 100 µg/ml streptomycin (both from Life Technologies). Explants were equally distributed to treatment groups, and in every experiment each group included explants from 3–6 mice. Samples were incubated for 24 h at 37 °C in a humidified atmosphere with 5% CO_2 and media were collected and frozen at –70 °C until analyzed for cytokines.

To study the modulation of the hARO-Luc reporter expression in GF and SCF explants, the culture medium was supplemented with 10–250 nM dexamethasone (DEX, SERVA Electrophoresis GmbH, Heidelberg, Germany) and 100–1000 ng/ml lipopolysaccharides (LPS) from *Escherichia coli* 0111:B4 (Sigma Aldrich Ltd). After 24 h incubation, tissues were collected and analyzed for the Luc activity as described earlier.

2.5. Isolation and culture of bone marrow derived mesenchymal stromal cells (MSCs)

MSCs were isolated from the bone marrow of tibiae and femora of HFD-fed hARO-Luc male mice. Eight mice in total were used to isolate the MSCs. Each MSC batch used in experiments consisted of a pool of cells collected from 2–4 mice. Dissected bones cleaned from soft tissues were cut from metaphyses from both ends and bone marrow cells were collected by flushing the diaphysis with MSC-medium (all components from Life Technologies Ltd), *i.e.*, Minimum Essential Medium alpha (#41061-029) supplemented with 15% inactivated fetal bovine serum (USA origin), 10 mM HEPES, 100 IU/ml penicillin, 100 µg/ml streptomycin, 2.5 mM L-glutamine, and 0.25 µg/ml amphotericin B (Fungizone). MSCs were cultured at 37 °C humidified atmosphere with 5% CO_2. Cells were allowed to attach to the plastic culture dishes in MSC-medium supplemented with 10 nM DEX (SERVA Electrophoresis GmbH). After 2 days medium was replaced with fresh and the cells cultured for 4–7 days until 60–90% confluent, detached by using 0.25% trypsin–EDTA and split in the ratio of 1:3.

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