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Testosterone regulates 3T3-L1 pre-adipocyte differentiation and epididymal fat accumulation in mice through modulating macrophage polarization

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Formaldehyde (PubChem CID: 712)
Hydroxyflutamide (PubChem CID: 91649)
Insulin (Bovine) (PubChem CID: 16131099)
Isopropanol (PubChem CID: 3776)
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ABSTRACT

Low testosterone levels are strongly related to obesity in males. The balance between the classically M1 and alternatively M2 polarized macrophages also plays a critical role in obesity. It is not clear whether testosterone regulates macrophage polarization and then affects adipocyte differentiation. In this report, we demonstrate that testosterone strengthens interleukin (IL) -4-induced M2 polarization and inhibits lipopolysaccharide (LPS)-induced M1 polarization, but has no direct effect on adipocyte differentiation. Cellular signaling studies indicate that testosterone regulates macrophage polarization through the inhibitory regulative G-protein (Gαi) mainly, rather than via androgen receptors, and phosphorylation of Akt. Moreover, testosterone inhibits pre-adipocyte differentiation induced by M1 macrophage medium. Lowering of serum testosterone in mice by injecting a luteinizing hormone receptor (LHR) peptide increases epididymal white adipose tissue. Testosterone supplementation reverses this effect. Therefore, our findings indicate that testosterone inhibits pre-adipocyte differentiation by switching macrophages to M2 polarization through the Gαi and Akt signaling pathways.

1. Introduction

Obesity represents an important public health issue that is associated with the metabolic syndrome and can involve insulin resistance and inflammation [1,2]. In recent years, androgen deficiency in males has also become a topic of increasing interest [3]. Clinical evidence shows that, in males, obesity is frequently associated with hypogonadism and that low testosterone levels are considered a hallmark of the metabolic syndrome. Furthermore, circulating levels of testosterone and estradiol affect adipocyte proliferation and differentiation [4]. However, the effect of androgen or testosterone deficiency has not been clearly established in early onset obesity.

Abbreviations: Adipoq, adiponectin; AP2-α, transcription factor activating protein-2-α; ARG1, arginase 1; C/EBP-β, CCAAT/enhancer binding protein-β; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPCR, G protein-coupled receptor; HF, hydroxyflutamide; IL-1β, interleukin-1β; IL-4, interleukin-4; IL-6, interleukin-6; IL-10, interleukin-10; Lep, leptin; LHR, luteinizing hormone receptor; LPS, lipopolysaccharide; M1, classically activated macrophage; M2, alternatively activated macrophage; MGL2, macrophage galactose N-acetyl-galactosamine specific lectin 2; MRC1, mannose receptor, C type 1; NOS2, nitric oxide synthase 2; PPAR-α, peroxisome proliferator activated receptor-α; PTX, pertussis toxin; T, testosterone; TNF-α, tumor necrosis factor-α; TPI, testosterone propionate injection.

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The relationship between obesity and androgen deficiency is multifactorial and complex. In males, testosterone is produced mainly by Leydig cells in the testes and constitutes the primary androgen [5]. Testosterone deficiency is a clinical syndrome characterized by a set of symptoms in combination with low serum testosterone concentrations [6]. Andrisse et al. developed a mouse model that displayed a low serum androgen levels albeit normal body mass, which was thought to be a direct consequence of obesity [7]. Numerous epidemiologic studies have established that low testosterone levels are commonly associated with inflammatory diseases, such as arthritis, type 2 diabetes mellitus, cardiovascular disease, chronic kidney disease, aging, and obesity [8–10]. Moreover, it has been shown that castration induced hyperglycemia and hyperlipidemia in mice and androgen deficiency in islet β-cells of male mice caused altered inflammatory responses [11]. However, their mechanisms of these effects are not clear.

Recent studies suggest that macrophages play a critical role in obesity [12]. The accumulation of macrophages in adipose tissue serves as a primary characteristic of obesity-associated chronic inflammation and is critical in regulating the development of obesity [13]. Macrophages are a major component of adipose tissue and represent an important factor in obesity-associated pathologies [14]. Based on their biochemical characteristics and biological functions, activated macrophages can be divided into classically (M1) and alternatively (M2) activated subsets [15,16]; which is termed macrophage polarization. The M1 phenotype responds to interferon (IFN) -γ or lipopolysaccharide (LPS), whereas the alternative activation M2 phenotype responds to interleukin (IL)-4 [17]. The M1 and M2 phenotypes are also characterized by the upregulated expression of specific molecular markers. In particular, nitric oxide synthase (NOS) 2, IL-6, IL-1β, and tumor necrosis factor (TNF)-α are associated with the M1 phenotype, whereas the M2 anti-inflammatory phenotype is characterized by an upregulation of arginase (ARG) 1, IL-10, macrophage galactose-N-acetyl-galactosamine specific lectin (MGL) 2, and mannose receptors C type 1 (MRC1, CD206) [18]. An imbalance of active macrophage phenotypes plays an important role in the pathogenesis of metabolic disorders [19].

It is generally noted that obesity affects the M1/M2 macrophage ratio in various tissues [2]. More M2 macrophages can be observed in lean animals, whereas there is a significant increase in M1 macrophages in adipose tissue during obesity [20]. In the current study, we examined the effects of testosterone on macrophage polarization. The results indicate that testosterone inhibits pre-adipocyte differentiation by modulating macrophage polarization through the inhibitory regulative G-proteins (Gαi), instead of the classical androgen receptors, and through the phosphorylation of Akt.

2. Materials and methods

2.1. Culture and treatment of RAW264.7 cells

RAW 264.7 cells (ATCC, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium with high glucose (DMEM/ high glucose; Hyclone, Logan, UT, USA) supplemented with 10% foetal bovine serum (FBS; Sijiqing, Hangzhou, Zhejiang, China), maintained in 5% CO_2 at 37 °C until they are 80% confluent, and sub-cultured (1: 5 split ratio) every 2–3 days.

For this study, cells incubated in serum-free medium for 12 h, then in the presence of testosterone (0–10^{-5} mol/L; Aladdin, Shanghai, China) for 12 h to evaluate the effect of testosterone on macrophages. Cells were incubated with lipopolysaccharide (LPS, 1 μg/ml [21]; Sigma-Aldrich, St. Louis, MO, USA) or interleukin (IL)-4 (20 ng/ml [22]; PeproTech, Rocky Hill, NJ, USA) and 0–10^{-5} mol/L of testosterone for 12 h to assess the effect of testosterone on macrophage polarization. The cells were then harvested to analyse the expression of marker genes of macrophage polarization using reverse transcription-qPCR (RT-qPCR). To assess the role of androgen receptors, cells were pre-treated with an androgen antagonist Hydroflutamide (HF, 10 μmol/L [23]; Adooq Bioscience, Irvine, CA, USA) or a selective enhancer of androgen receptor degradation ASC-J9 (5 μmol/L [24]; MedChem Express, Monmouth Junction, NJ, USA) for 30 min. This was followed by co-incubation with LPS or IL-4 and testosterone for 12 h prior to the analysis of polarization marker genes. Some cells were pre-treated with pertussis toxin (PTX, 1 μg/ml [25]; Sigma-Aldrich) for 30 min prior to co-incubation with LPS or IL-4 and testosterone for 12 h to assess the impact of Gαi.

2.2. 3T3-L1 cells culture and differentiation

3T3-L1 cells (ATCC) were cultured in DMEM/ high glucose with 10% bovine calf serum (Sijiqing) at 37 °C in 5% CO_2. At 2 days post-confluency (day 0), the medium was replaced with DMEM/ high glucose containing 10% FBS, 500 μmol/L 3-isobutyl-1-methylxanthine (Sigma-Aldrich), 1 μmol/L dexamethasone (Sigma-Aldrich), and 1 μg/ml bovine insulin (Biosharp, Hefei, Anhui, China). After 2 days (day 2), the medium was changed to insulin medium (10% FBS in DMEM/ high glucose containing 1 μg/ml insulin). After another 2 days (day 4), the medium was changed to 10% FBS in DMEM/ high glucose, which was then replaced every 2 days. Full differentiation was usually achieved by day 12. During differentiation, different concentrations of testosterone (0–10^{-5} mol/L) were added. In addition, conditioned medium of RAW264.7 cells incubated with LPS or IL-4 and testosterone (10^{-7} mol/L) was added.

2.3. Silencing of Akt1 and Akt2 expression via siRNA

The expression of Akt1 and Akt2 was silenced by using commercially available siRNAs following the manufacturer's protocol (GenePharma, Shanghai, China). The oligonucleotides used were: Akt1 (5′-GCU AUU GUG AAG GAG GGU UTT-3′; 5′-AAC CCU CCU UCA CAA UAG CTT-3′); and Akt2 (5′-GAU CUU UCA UUG GGU AUA ATT-3′; 5′-UUA UAC CCA AUG AAA GAU CTT-3′). Control cells were treated with the GenePharma Negative Control (5′-UUC UCC GAA CGU GUC ACG UTT-3′; 5′-ACG UGA CAC GUU CGG AGA ATT-3′) provided in the kit. Transfection was performed using a Lipofectamine™ 2000 Transfection Kit (Invitrogen, Carlsbad, CA, USA) according to manufacturer's protocol. Transfection efficiency was estimated according to the relative mRNA expression of Akt1 and Akt2 as determined using the primers shown in Table 1.

2.4. Analysis of mRNA expression by real-time qPCR

Total RNA from RAW264.7 and 3T3-L1 cells was extracted using the RNAiso Plus reagent (TaKaRa, Tokyo, Japan) according to the manufacturer's instructions. RNA concentration and quality were analysed by detecting with NanoDrop™ 2000 spectrophotometers (Thermo Scientific, Waltham, MA, USA). The complementary DNA (cDNA) was synthesized using the PrimeScript™ RT reagent kit with gDNA Eraser (TaKaRa). To detect the expression of the target genes, the cDNA was amplified by RT-qPCR using the SYBR® *Premix Ex Taq*™ II (Tli RNaseH Plus) (TaKaRa) with specific primers (10 μmol/L each; Table 1) and CFX384 Real-Time PCR Detection System (Bio-Rad, Hercules, CAL, USA). Data were normalized to *Gapdh* expression for each sample. The relative expression was calculated using the $2^{-\Delta\Delta Cq}$ method. MIQE guidelines were followed.