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Androgens inhibit adipogenesis during human adipose stem cell commitment to preadipocyte formation

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ABSTRACT

Androgens play a pivotal role in the regulation of body fat distribution. Adipogenesis is a process whereby multipotent adipose stem cells (ASCs) initially become preadipocytes (ASC commitment to preadipocytes) before differentiating into adipocytes. Androgens inhibit human (h) subcutaneous (SC) abdominal preadipocyte differentiation in both sexes, but their effects on hASC commitment to preadipocyte formation is unknown. We therefore examined whether androgen exposure to human (h) ASCs, isolated from SC abdominal adipose of nonobese women, impairs their commitment to preadipocyte formation and/or subsequent differentiation into adipocytes. For this, isolated hASCs from SC abdominal lipoaspirate were cultured in adipogenesis-inducing medium for 0.5–14 days in the presence of testosterone (T, 0–100 nM) or dihydrotestosterone (DHT, 0–50 nM). Adipogenesis was determined by immunofluorescence microscopy and by quantification of adipogenically relevant transcriptional factors, PPARγ, C/EBPα and C/EBPβ. We found that a 3-day exposure of hASCs to T (50 nM) or DHT (5 nM) in adipogenesis-inducing medium impaired lipid acquisition and decreased PPARγ, C/EBPα and C/EBPβ gene expression. The inhibitory effects of T and DHT at this early-stage of adipocyte differentiation, were partially and completely reversed by flutamide (F, 100 nM), respectively. The effect of androgens on hASC commitment to a preadipocyte phenotype was examined via activation of Bone Morphogenic Protein 4 (BMP4) signaling. T (50 nM) and DHT (5 nM) significantly inhibited the stimulatory effect of BMP4-induced ASC commitment to the preadipocyte phenotype, as regards PPARγ and C/EBPα gene expression. Our findings indicate that androgens, in part through androgen receptor action, impair BMP4-induced commitment of SC hASCs to preadipocytes and also reduce early-stage adipocyte differentiation, perhaps limiting adipocyte numbers and fat storage in SC abdominal adipose.

1. Introduction

Adipocytes are derived from multipotent adipose stem cells (ASCs), which have the capacity to differentiate into not only adipocytes but also other mesenchymal cell lineages including myocytes, chondrocytes, and osteocytes [1]. Under appropriate stimuli, ASCs are restricted to produce adipocyte progenitors or preadipocytes (i.e., ASC commitment to preadipocytes) [2–4]. These preadipocytes then differentiate into adipocytes (i.e., cell differentiation) [5–9]. Sex steroids influence this adipogenic progression, as evidenced by a sexual dimorphism of body fat distribution [10–12] beginning at puberty and promoting preferential accumulation of subcutaneous (SC) abdominal and visceral adipose in women and men, respectively [10,13–15]. Many of these sexually dimorphic changes in body fat appear mediated through androgens, since physiological androgen administration to women down-regulates SC abdominal adipose hormone-sensitive lipase, the final step in lipolysis; and diminishes *in vivo* lipolysis [16,17]. In addition, androgen regulates insulin-mediated glucose uptake in SC adipose tissue [18].

Earlier in adipogenesis, moreover, androgens also inhibit SC abdominal preadipocyte differentiation into adipocytes in both men and women, implying that this particular androgen action differs by sex in magnitude of androgen exposure [19]. The effect of androgens on antecedent hASC commitment to preadipocytes, however, is unknown.

The present study, therefore, examines whether androgen exposure of hASCs from SC abdominal adipose of nonobese women impairs their commitment to the preadipocyte phenotype *in vitro,* with subsequent effects on preadipocyte differentiation into adipocytes. Our utilization of hASCs from SC abdominal adipose

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retrieved from nonobese women through elective liposuction [1], unlike other studies [19,20–21], eliminates the confounding effects of obesity on these cells during *in vivo* adipogenesis.

Our data show that androgens, partially acting through the androgen receptor (AR), impair BMP4-induced commitment of SC abdominal hASCs to the preadipocyte phenotype and also reduce early-stage preadipocyte differentiation to adipocytes. These results support the hypothesis that excess of androgens in women may diminish lipid storage capacity within SC abdominal adipose, promoting ectopic lipid deposition and lipotoxicity.

2. Experimental

2.1. Subcutaneous abdominal hASC isolation

Lipoaspirates (100–200 g per aspirate) were obtained from SC abdominal adipose of 10 nonobese Caucasian women (mean BMI, 23.4 ± 4.2 [SD] kg/m^2; mean age, 52 ± 11 [SD] years) undergoing elective liposuction. None of the medications used by these individuals were known to affect adipogenesis. All studies were approved by the University of California Los Angeles, Institutional Review Board. Lipoaspirates were obtained from the lower SC abdomen using standard procedures and aspirated fat was washed with phosphate buffered saline (PBS) and digested at 37 °C in PBS containing 0.075% collagenase for 30 min on a shaker. Adipocytes (floating cells) were separated from pellets by centrifugation (800*g*, 10 min). Pellets containing stromal-vascular cells were resuspended in DMEM/5% FCS, filtered through 150-mm mesh and washed with DMEM/5% FCS. Cells were plated in 6 well clusters containing DMEM/10% FCS, 0.05 U/ml penicillin, 0.05 mg/ml streptomycin, 1.25 mg/ml fungizone (regular DMEM medium) and cultured at 37 °C until cells reached confluency. hASCs from each patient were cultured and processed in a uniform manner for the purpose of three independent experiments, as described below.

2.2. Cell culture

The first set of *in vitro* experiments examined the entire process of adipogenesis, including (1) hASC commitment to preadipocytes and (2) their subsequent progression through early and late stages of preadipocyte differentiation. Confluent hASCs were incubated in an established adipogenesis-inducing medium (containing 0.5 mM isobutylmethylxanthine, 1 μM dexamethasone, 10 μM insulin, 200 μM indomethacin and peroxisome proliferator-activated receptor γ [PPARγ] agonist, according with standard protocol [ZenBio, Inc, Research Triangle Park, NC]) [19,20,22] at 37 °C in 5% CO_2 for 0.5, 3, 7 or 14 days in the presence of vehicle alone (control) (see below), testosterone (T, 50 nM) or dihydrotestosterone (DHT, 5 nM). In some experiments, hASCs were incubated under similar conditions with T (50 nM) or DHT (5 nM) and/or the antiandrogen flutamide (F, 100 nM). These concentrations were chosen to elicit androgen effects at the lowest T and DHT concentrations and reversal of such effects with a maximal flutamide concentration, based upon previous dose–response studies (0–100 nM T), (0–50 nM DHT) and (0–100 nM F) (data not shown). T, DHT and F were initially diluted in ethanol to prepare a 10 mM stock solution and stored at 4 °C. Further dilutions of these reagents were performed in PBS and culture medium leading to a final ethanol concentration of 0.0001% in 20 μl PBS and culture medium. Twenty microlitre of the same concentration of ethanol-containing PBS and culture medium was added to hASCs as vehicle control. Culture medium was changed every two days throughout the entire incubation period.

The second set of experiments examined *in vitro* commitment of hASCs to the preadipocyte phenotype. Confluent hASCs were preincubated for 2 h in regular DMEM medium (see above) with vehicle alone (control), BMP4 (5 nM) (R & D Systems, Minneapolis, MN), T (50 nM), DHT (5 nM) and/or F (100 nM). This BMP4 concentration was chosen based upon initial dose–response studies (0–25 nM) (data not shown) confirming its ability to stimulate hASC commitment to the preadipocyte phenotype, as previously described [2,3]. Cells were then incubated for an additional 3 days in adipogenesis-inducing medium alone (without BMP4, androgen or flutamide).

2.3. Immunofluorescence studies

Following all experiments, cells were fixed in 4% paraformaldehyde, rinsed twice in PBS, and then incubated with the free fatty acid immunofluorescent marker, BODIPY-C1,12 (Invitrogen, Carslbad, CA); and the nuclear staining marker, 4′,6-diamidino-2-phenoylidole (DAPI) (Invitrogen, Carslbad, CA) following standard conditions. ASCs are typical spindle-shape cells, while preadipocytes are more rounded cells with lipid inclusions in the cytoplasm. ASCs were initially characterized by immunofluorescent markers, CD105 and CD146, specific to multipotent mesenchymal stem cells (MSC) (BD Biosciences, San Diego, CA) (Fig. 1A and B). All immunofluorescent experiments were conducted in a blinded manner and were initially performed as time-course studies over 14 days or 3 days (see below), with androgen-induced differences in cell morphology and lipid content compared using semi-quantitative Likert Scale analysis.

2.4. Quantification of number of cells

Fluorescent images were acquired using an Evos imunofluorescence inverted microscope (Advanced Microscopy, Mill Creek, WA). BODIPY-C1, 12/DAPI positive cells were quantified using ImageJ software (http://rsb.info.nih.gov/ij/). The results were expressed as fold changes (treated vs. vehicle control per total single image).

2.5. RNA isolation and quantitative real-time PCR (qRT-PCR)

Total cellular RNA was isolated using an RNeasy kit (Qiagen, Hilden, Germany) according to manufacturer's protocol. First strand cDNA was synthesized using first strand RT2 kit (Qiagen, Hilden, Germany). mRNA was quantified by qRT-PCR using RT2 qPCR Master Mix according to manufacturer's protocol (Qiagen, Hilden, Germany). qRT-PCR was performed on an ABI 7300 (A&B Applied Biosystems, Foster City, CA) using standard temperature cycling conditions. Human PPARγ, C/EBPα, and C/EBPβ primers were provided by A & B Applied Biosystems, Foster City, CA. Glyceraldehyde 3-phosphate dehydrogenase (GADPH) was used as internal control. The relative expression of target genes was measured using the comparative critical threshold (C_t) method. The results were expressed as fold changes (treated vs. vehicle control) obtained from triplicate values. GADPH expression did not change across treatments or cell progression through adipogenesis.

2.6. Statistical analyses

All results were shown as mean ± S.E.M. hASCs isolated from each patient were subjected to at least three independent experiments performed in triplicates. Data were analyzed by using an ANOVA model (for multiple groups) following Tukey HSD or