Testosterone induces cell proliferation and cell cycle gene overexpression in human visceral preadipocytes

Anna Barbosa-Desongles, Cristina Hernández, Rafael Simó, and David M. Selva

Diabetes and Metabolism Research Unit, Vall Hebron Institut de Recerca (VHIR), Universitat Autònoma de Barcelona and CIBER de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM; ISCIII), Barcelona, Spain

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Barbosa-Desongles A, Hernández C, Simó R, Selva DM. Testosterone induces cell proliferation and cell cycle gene overexpression in human visceral preadipocytes. Am J Physiol Cell Physiol 305: C355–C359, 2013.—Evidence from the literature suggests that testosterone plays an important role in visceral fat accumulation since both men and women with hyperandrogenism accumulate more adipose tissue in the abdominal cavity than healthy women. However, the underlying mechanisms remain to be elucidated. To shed light on this issue, we have used an in vitro approach to examine the effect of testosterone on human visceral preadipocyte proliferation. Our results showed that testosterone treatment significantly increased proliferation of human visceral preadipocytes in proliferation assays using flow cytometric analysis. We next performed a microarray gene expression analysis of human visceral preadipocytes treated with testosterone or vehicle to identify which genes were involved in the testosterone-induced increase in preadipocyte proliferation. The results showed a total of 140 genes differentially expressed between testosterone vs. vehicle. Among the top 10 upregulated genes, 5 were involved in cellular cycle and proliferation, and 3 (APOBEC3b, CCNA2, and PRC1) were significantly overexpressed by testosterone treatment when analyzed by real-time PCR. We conclude that testosterone exerts a proliferative effect on preadipocytes that may participate in the sex differences in fat distribution and that it may explain visceral fat accumulation in women with hyperandrogenism.

testosterone; human preadipocytes; cell cycle; proliferation; gene expression

ADIPOSE TISSUE IS A COMPLEX organ composed of adipocytes, a connective tissue matrix, nerve tissue, stromovascular cells, and immune cells (15). Apart from its major function as an energy store lipid form, adipose tissue is well known to be an active endocrine organ with a pivotal role in the control of energy homeostasis (13).

An important sex difference in body fat distribution is generally observed (22). Men are usually characterized by the android type of obesity, with accumulation of fat in the abdominal region, whereas women often display the gynoid type of obesity, with a greater proportion of their body fat in the gluteal-femoral region. Accordingly, the amount of fat located inside the abdominal cavity (intra-abdominal or visceral adipose tissue) is twice as high in men compared with women (4). This sex difference has been shown to explain a major proportion of differing metabolic profiles and cardiovascular disease risk in men and women (24). Given that men accumulate more fat in the intra-abdominal depot than women, it seems reasonable to postulate that testosterone plays an essential role in this type of body fat distribution. In support of this concept, postmenopausal women accumulate more visceral fat than premenopausal women, and studies performed with women at different stages of the menopausal transition have found that bioavailable testosterone is associated with an increase of visceral fat (12).

By contrast, there is clinical evidence that testosterone administration induces a reduction of fat content mainly due to a decrease in visceral adipose tissue (19, 20). However, it should be noted that this effect has been observed in those patients with a testosterone deficit or, in other words, when testosterone was administered as a replacement treatment (1, 3, 8, 10, 11, 14, 18).

The mechanism by which testosterone regulates adipose tissue mass in a depot-specific manner remains to be elucidated. White adipose tissue distribution in different anatomical regions not only depends on the lipid synthesis and/or lipolysis in mature adipocytes but also the site-specific modulation of preadipocyte proliferation and/or differentiation (5). There is significant in vitro information regarding the effects of testosterone in preadipocyte differentiation (4, 9), but little is known about testosterone effects on human preadipocyte proliferation. To shed light on this issue, we have studied the effect of testosterone on human visceral preadipocyte proliferation. In addition, differential expressed genes in preadipocytes treated with testosterone or vehicle were explored by microarray analysis.

MATERIALS AND METHODS

Cell culture experiments. Human male preadipocytes obtained from Tebu-Bio (cat. no. 088OP-F-3; Tebu-bio, Barcelona, Spain) were maintained in a humidified incubator with 5% CO2 at 37°C. Cells were cultured in triplicates in preadipocyte commercial media OM-PM (Tebu-bio) for 3 days in the presence of testosterone (100 nM) or vehicle (ethanol) to 80% confluence.

Proliferation and cell-cycle analyses. To evaluate the cell cycle in human preadipocytes, we used the APC BrdU Flow Kit (BD Biosciences PharMingen, San Diego, CA) according to the supplier’s instructions. Briefly, cells were seeded on 150-mm plates with DMEM 15 mM glucose supplemented with 10% FBS and antibiotics (100 U penicillin/ml and 100 μg streptomycin/ml). After 24 h, the medium was changed to DMEM 15 mM glucose with testosterone 100 nM or with ethanol, and it was changed every day for 3 days. During the last 3 or 6 h of the experiment, cells were labeled with bromodeoxyuridine (BrdU) 10 μM in DPBS buffer. After that, cells were washed in DPBS and were fixed with the Cytofix/Cytopermed provided buffer, treated with DNase for 1 h at 37°C, and incubated with the APC labeled-BrdU antibody. The DNA was subsequently stained with 7-amino-actinomycin D (7-AAD), and cells were resuspended in staining buffer for flow cytometric analysis. For cell cycle analysis, 7-AAD and BrdU were measured simultaneously using a BD FACSCalibur.
Flow cytometry. Acquired multiparameter data were analyzed using FCS Express version 3 software (De Novo Software, Los Angeles, CA). Flow cytometric analysis of cells stained with the reagents allowed the discrimination of cell subsets that were in S phase, G0/G1 phase, G2 phase, and apoptotic. Fluorescence cut off was chosen at 1% positive events in a negative control for APC.

RNA isolation. Preadipocyte cells were scraped at the end of the treatments and total RNA was isolated using an RNeasy Mini Kit from Qiagen following the manufacturer’s instructions. Integrity and concentration of RNA were analyzed by Bioanalizador 2100 (Agilent) in the Scientific and Technical Support Unit of our Research Institute. RNAs with RIN score above 7 were used in microarrays.

Microarray hybridization and analysis. The goal of the study was to compare gene expression patterns between preadipocytes treated with testosterone or vehicle. Microarrays were carried out using the Affymetrix microarray platform and the Genechip Human Gene 1.0 ST Array (an array with 14,500 well-characterized human genes used to explore human biology and disease processes). The arrays were performed at the Scientific and Technical Support Unit of our Research Institute as described elsewhere (www.affymetrix.com). Data obtained from the microarrays were analyzed by the Statistics and Bioinformatics Unit of our research institute. To select differentially expressed genes, they used a method described by Smyth GK (X20). All the statistical analysis was done using the free statistical language R and the libraries developed for microarray data analysis by the Bioconductor Project (www.bioconductor.org).

Real-time PCR analysis. Reverse transcription (RT) was performed at 42°C, for 50 min using 3 µg of total RNA and 200 U of Superscript II together with an oligo-dT primer and reagents provided by Invitrogen. An aliquot of the RT product was amplified in a 25-µl reaction using SYBRGreen (Invitrogen SA) with appropriate oligonucleotide primer pairs for hRRM2 (forward 5’-GAGCAAGGATGCATAGT, reverse 5’-GGGCTTCTGAACTTC), hCCNA2 (forward 5’-AGCTGCTTTTCACTTAGCTAC, reverse 5’-TAAGACCTTTTCCAGGAATATCCAGTC), hCDC20 (forward 5’-GCCCCAGGAGAAGAAC, reverse 5’-TTCTCCACTGAGCCGAAG), hAPOBEC3B (forward 5’-CTATGGTCAGGACATCTTGG, reverse 5’-GGAAACACTTGATAGCAG), and 18S (forward 5’-TACGAGACTGACCTCTGGA, reverse 5’-GGGAGTCAAAGTCAATCA). Amplification was done as follows: one step at 95°C 10 min; one step at 95°C 15 s; 40 cycles at 95°C 15 s, 60°C 1 min followed by dissociation curve (95°C 15 s, 60°C 20 s, and 95°C 15 s). For each condition, all reactions were run in triplicate. Results were analyzed using the 7000 SDS program and we used the 2−ΔΔCt method.

Statistical analyses. Data regarding cell proliferation and real-time PCR were analyzed using a nonparametric test (U-Mann-Whitney). Values of statistical significance were set at P < 0.05 and 0.01. The statistical analyses corresponding to microarrays were performed using the free statistical language R and the libraries developed for microarray data analysis by the Bioconductor Project (www.bioconductor.org).

RESULTS

Cell cycle and proliferation analysis. Asynchronous cultured human visceral preadipocytes were treated with ethanol or testosterone (100 nM). After treatment, cells were stained with 7-AAD to study cell DNA content and the cell cycle. After flow cytometric analysis, our results showed that both ethanol- and testosterone-treated preadipocytes had divided correctly, without any cell cycle arrest being produced by the treatment, and with the correct peaks corresponding to the proportion of DNA amount in each cell cycle phase (Fig. 1, A and B).

We next quantified and analyzed the amount of BrdU incorporation to check if testosterone treated cells proliferated more than ethanol-treated cells (Fig. 2, A and B). To confirm this, the number of BrdU-positive cells in the S phase was used to

Fig. 1. Cell cycle and bromodeoxyuridine (BrdU) incorporation analysis. Human preadipocytes were analyzed using flow cytometry to study the cell cycle and cell proliferation through BrdU incorporation. A: 3-day ethanol-treated cells were incubated with 7-amino-actinomycin D (7-AAD) and their DNA content was measured by flow cytometry. B: 3-day testosterone-treated cells were incubated with 7-AAD and their DNA content was measured by flow cytometry.

Fig. 2. Analysis of BrdU incorporation. Graphics of the analysis of BrdU incorporation in ethanol-treated cells (A) and testosterone-treated cells (B). C: both ethanol and testosterone 3-day-treated preadipocytes were incubated with BrdU during 3 and 6 h and later the percentage of cells in S phase incorporating BrdU was calculated. Experiment was performed 3 times, and data points are means ± SD. *P < 0.05, compared with vehicle. **P < 0.01, compared with vehicle.
quantify the proliferating index in human visceral preadipocytes treated with ethanol or testosterone. We observed that treatment with testosterone induced an increase in cell proliferation (at 3 and 6 h of BrdU incorporation) since the percentage of cells in the S phase was significantly increased when compared with ethanol-treated preadipocytes (Fig. 2C).

**Validation of the top five cell cycle genes of microarray data.** Among the top 10 upregulated genes from the testosterone vs. ethanol microarray data comparison, we found five genes involved in cell cycle regulation: CDC20, APOBEC3b, CCNA2, PRC1, and RRM2 (Fig. 4A). We next analyzed and validated the upregulated cell cycle genes by real-time PCR. Although all five genes were upregulated, three out of five genes (APOBEC3b, CCNA2, and PRC1) were significantly overexpressed by testosterone treatment when compared with vehicle-treated preadipocytes (Fig. 4B).

**DISCUSSION**

Due to sex differences in body fat distribution, it can be postulated that testosterone regulates adipose tissue mass in a depot-specific manner. White adipose tissue distribution in different anatomical regions not only depends on lipid synthesis and/or lipolysis in mature adipocytes but also on the site-specific modulation of preadipocyte proliferation and/or differentiation.

Fig. 3. Microarrays results and differentially expressed genes in the top biology functions between ethanol and testosterone-treated preadipocytes. A: heat map illustrating differentially expressed genes generated from DNA microarray data, reflecting gene expression values in testosterone or ethanol treatment conditions of the human visceral preadipocytes. Every condition was done in triplicate. B: number of molecules differentially expressed from the comparison of testosterone and ethanol preadipocyte treatments in the top biology functions.

Fig. 4. Top 10 upregulated genes from the testosterone vs. ethanol microarray data comparison and validation of cell cycle genes overexpression. A: 5 cell cycle genes among the top 10 upregulated genes from the testosterone vs. ethanol microarray data. B: overexpressed cell cycle genes validation by RT-PCR. CDC20, APOBEC3b, CCNA2, PRC1, and RRM2 mRNA abundance was determined in relation to S18 RNA (means ± SD) of vehicle-treated preadipocytes. The experiment was performed 3 times and data points are means ± SD. *P < 0.05, compared with vehicle.
Our results showed for the first time that testosterone exerts a proliferative effect on human visceral preadipocytes. In addition, after analyzing the microarray data, from the top 10 upregulated genes by testosterone when compared with vehicle-treated preadipocytes, five were involved in cell cycle regulation. Moreover, three out of five genes (APOBEC3b, CCNA2, and PRC1) were significantly overexpressed when analyzed by real-time PCR.

There is clinical evidence that testosterone administration induces a reduction of fat content (19, 20). However, it should be noted that whereas the reduction in fat content and the change in body fat distribution has been clearly shown when testosterone is administered as replacement treatment in men with a testosterone deficit (1, 3, 8, 10, 11, 14, 18), there is no evidence of this effect in men with normal testosterone levels. In this regard, Münzer et al. (21) in a randomized, double-masked, placebo-controlled study showed that visceral fat did not decrease significantly after 6 mo administration of testosterone enanthate (100 mg, intramuscular, every 2 wk) in healthy men with age-related reduction of serum testosterone. Taken together, these data suggest that topographical variations in responsiveness of fat to testosterone administration depend on gonadal status, age, and dose. The results observed in the present study do not contravene these clinical observations because instead of mature adipocytes, preadipocytes were used. By contrast our results could help towards understanding the underlying mechanisms involved in the increase of visceral adiposity detected in men compared with women or the increase of visceral fat observed in both postmenopausal woman with hyperandrogenism and in female-to-male transsexuals under testosterone treatment (7).

The effect of androgens on human preadipocyte proliferation has not been extensively explored in the literature. In this regard, there has been only one report analyzing the effects of androgens in human preadipocyte proliferation (9). In this work, dihydrotestosterone treatment did not change cellular proliferation when compared with the untreated cells (9). There is an important aspect of the experimental design that distinguishes our work from this previous study, which is that we used testosterone as an androgenic compound instead of dihydrotestosterone. The main reason why we have used testosterone is because it is the major circulating androgen in blood and although there is 5α-reductase activity in human adipocytes, the conversion to 5α-reduced androgens is low and ranges from 1 to 10% (16).

The effect of testosterone on adipose tissue depends not only on proliferation and differentiation of preadipocytes but also on highly complex enzymatic system modulating steroid action on a local basis. In this regard, recent evidence suggests that enzymes from the aldoketoreductase 1C family are very active and may be important modulators of androgen action in adipose tissue. In addition, the expression of steroidalogenic and steroid-inactivating enzymes by adipose tissue itself is crucial in accounting for body fat distribution (4). Furthermore, the effect of estradiol on preadipocyte proliferation should also be considered. In this regard, experiments performed in vitro in mouse and human preadipocytes showed that estradiol exerts different effects depending on the concentration and the source of preadipocyte used (male or female, and visceral or subcutaneous; Refs. 2, 17). Therefore, the testosterone-induced preadipocyte proliferation herein reported should be contemplated only as a component of a multifactorial event regulating body fat distribution.

In conclusion, testosterone increases preadipocyte number in visceral fat. This finding could be one of the mechanisms by which men exhibit more visceral fat than women and also could be an underlying factor involved in the accumulation of intra-abdominal adipose tissue observed in women with hyperandrogenism. However, further studies exploring the significance of this finding within the complex mechanisms involved in the regulation of body fat distribution are needed.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: A.B.-D. performed experiments; A.B.-D., C.H., R.S., and D.M.S. analyzed data; A.B.-D., C.H., R.S., and D.M.S. interpreted results of experiments; A.B.-D., prepared figures; A.B.-D., and D.M.S. drafted manuscript; A.B.-D., C.H., R.S., and D.M.S. edited and revised manuscript; A.B.-D., C.H., R.S., and D.M.S. approved final version of manuscript; R.S. and D.M.S. conception and design of research.

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