Testosterone Inhibits Adipogenic Transcription Factors PPAR-γ2 and C/EBP-α Through TCF4 Transcription Factor

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Our laboratory has shown that testosterone (T) increases skeletal muscle and decreases fat in men. Previous research showed that within cells treated with higher concentrations of T and Dihydrotestosterone (DHT), fat cell formation decreased in parallel with diminishing expression of PPAR-γ2 and C/EBP-α protein and mRNA as shown respectively by Western immunoblotting and Real Time Quantitative Reverse Transcriptase–Polymerase Chain Reaction (PCR). We showed that T and DHT inhibit adipogenic differentiation by signaling through the androgen-receptor (AR). The mechanisms for signaling through AR are still unknown.

It is hypothesized that T inhibits adipogenic differentiation downstream from AR by signaling through the Wnt10b (Wingless) beta-Catenin pathway involving transcription factor TCF4 and inhibiting expression of the key adipogenic transcription factors (PPAR-γ2 and C/EBP-α). As a model adipogenic cell line, mouse 3T3-L1 preadipocytes were transfected with plasmids overexpressing TCF4 or a dominant negative TCF4 (Dn-Tcf4), then grown in adipogenic media (AM) with or without T or lithium for 0–12 days. PPAR-γ2 and C/EBP-α mRNA expression were measured by real-time reverse transcription polymerase chain reaction (RT-PCR).

As hypothesized, high levels of TCF4 decrease (but Dn-Tcf4 increases) the expression of PPAR-γ2 and C/EBP-α mRNA levels. We also showed that treatment with lithium, known to activate the beta-Catenin pathway, down-regulates fat cell differentiation by inhibiting PPAR-γ2 and C/EBP-α. Therefore, T inhibition of adipogenic differentiation in the 3T3-L1-cell line is mediated through TCF4. Though this study clarifies one of the mechanisms by which T and DHT operate to inhibit fat mass, further research is needed.

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Introduction

Regardless of the prevalent abuse of androgenic steroids by professional and amateur athletes, the effects of these agents on athletic performance and physical function have been a source of intense controversy for over three score years. Previous investigations demonstrate that T and DHT down-regulate the expression of PPAR-γ2, and C/EBP-αmRNA expression and inhibit adipogenesis in a 3T3-L1 preadipocyte cell line. The effect of T and DHT on a preadipocyte cell line is mediated through an AR-dependent mechanism, as preliminary data showed the blocking of the AR by bicalutamide, an AR antagonist. After clarifying AR as one step in adipogenic differentiation, there was careful examination in the other downstream effects. Our laboratory observed a nearly simultaneous signaling through AR that transduced to beta-catenin, which then enters the nucleus and forms a complex with TCF to activate/deactivate certain transcription factors. Transcription factors included those specific for fat cell and muscle formation. Previous investigations identified Wnt10b as a potent inhibitor of adipogenesis that must be suppressed for preadipocytes to differentiate in vitro. Further research showed Wnt10b-beta-Catenin to be the initiating effect in a cascade of events that lead to the activation of transcription factor TCF4. We hypothesize that TCF4 will down regulate adipogenic transcription factors and promote myogenic transcription factors. In this investigation, we also used lithium to confirm the Wnt10b-beta-Catenin pathway as functioning properly. It is hypothesized that T inhibits adipogenic differentiation downstream from AR by signaling through the Wnt10b (Wingless)-beta-Catenin pathway involving transcription factor TCF4 and inhibiting expression of the key adipogenic transcription factors (PPAR-γ2 and C/EBP-α) and later transcription factors (AP2).

Methods

Mouse 3T3-L1 cells were allowed to grow in DMEM with 10% fetal bovine serum growth medium at 37° C, were treated with 20 μm 5-azacytidine for three days, split to a one to two ratio, allowed to recover for two days, and seeded at 70% confluence in six-well plates or chamber slides with test agents T and DHT for 0–12 days. The only androgen used was T (100nM); mouse 3T3-L1 preadipocytes were transfected with plasmids overexpressing TCF4 or a dominant negative TCF4 (Dn-Tcf4). Androgens were placed with both the regular growth media (GM) as well as the adipogenic media (AM), and each cell plate or chamber slide was given equal opportunity to differentiate within GM and AM. Cells were fixed with a specific antibody for recognizing PPAR-γ2, C/EBP-α, and AP2.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) and Real Time Quantitative Polymerase Chain Reaction (PCR)

After the twelfth day, total RNA was extracted from cells using the Trizol-Reagent (Invitrogen, Carlsbad, Calif.). Reverse transcription was accomplished in the RT-PCR with 2 μg total RNA and Moloney murine leukemia virus reverse transcriptase at 42 °C for 20 min (Per-

kin-Elmer, Norwalk, Conn.). 4 cDNA samples were amplified by using the PCR method with different primer pairs for 36 cycles in a Gene Mate thermocycler (Intermountain Science, UT) at 94 °C for 30 s, primer annealing at 58 °C for 30 s, and extension at 72 °C for 1 min.

The i-Cycler PCR thermocycler, fluorescent light detector lid (Bio-Rad, Hercules, Calif.), and Qiagen Sybergreen RT-PCR kit with HotStar Taq DNA polymerase (Qiagen, Valencia, Calif.) was all required for the real time RT-PCR analysis of mRNA. Samples were analyzed for PPAR-γ2, C/EBP-α, and AP2 in quadruplicate wells of 250 ng total RNA templates in comparison with GAPDH control in quadruplicate wells of 25 ng of identical RNA template. Standard curves for the respective primers were acquired by log dilutions of pPPAR-γ2, pCEBP-α, pGAPDH, and pAP2 plasmids, from 1 ng decreasing to 1 fg of plasmid DNA.

Data Analysis

Data analysis was done by use of the i-cycler (iQ, Bio-Rad, Hercules, Calf.) software to calculate a standard curve (cycle threshold vs pg DNA standard, respectively) for PPAR-γ2, C/EBP-α, AP2, and GAPDH. Plasmids were used to calculate starting quantities (SQ) for the mRNA levels in each sample. Quadruplicate samples were averaged, standard deviations calculated, and the ratio for PPAR-γ2, C/EBP-α, and AP2 respectively to GAPDH were averaged and values normalized to 100% for the control (0 nM).

Results and Discussion

These data demonstrate that TcF4 down-regulate the expression of key adipogenic transcription factors PPAR-γ2, and C/EBP-α and also the late cursor AP2. TCF4 inhibits adipogenesis in a 3T3-L1 cell line and promotes myogensis. The effect of T and DHT on a preadipocyte cell line is mediated through an AR-dependent mechanism, which is combined with beta-Catenin to enter the nucleus. This cascade of effects lead toward the promotion of the transcription factor TCF4, which attributes to cell differentiation. Though our data were consistent with our original analysis, mRNA levels of C/EBP-α were unusually high. These results demonstrate that C/EBP-α alone cannot increase adipogenic differentiation, but further research is needed. The results of this experiment prove our hypothesis correct and make our investigation successful.

Implications

Although the results of this investigation were successful, the data should be tested three or more times to accurately test the validity of this newfound information. Also, since the results of this investigation are contrived on the effects of testosterone in a preadipocyte, the next question to ask is whether T and DHT regulate adipocyte cell differentiation *in vivo* and in a different cell line. We suggest that clinical trials be conducted to fully understand such a "controversial" topic as testosterone and its effects.

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