Effect of Androgen Receptor Activation on Adipogenic and Myogenic Transcription Factor Expression in Mesenchymal Stem Cells

We hypothesize that the direct activation of androgen receptor (AR), bypassing the androgen activation of Wnt pathway signaling, does not inhibit CCAAT/enhancer-binding protein-α (C/EBP-α) gene expression and also does not stimulate MyoD. We tested this hypothesis by transfection of cell line C3H-10T1/2 with plasmid pAct-AR, expressing constitutively activated AR-VP16, which can bind to androgen-receptor elements (ARE) in androgen responsive promoters, up-regulating gene expression. As a positive control for expression of the activated AR-VP16 protein, cells were cotransfected with plasmid p(ARE)4-luc to test for hyperexpression of the Luciferase reporter gene. We observed a 130-fold stimulation of the ARE-Luciferase promoter by cotransfection with pAct-AR encoding activated AR-VP16. Protein extracts from these transfected cells were also analyzed by Western immunoblotting to test if activated AR-VP16 protein stimulates gene expression of the MyoD, while inhibiting C/EBP-α, as was found to be true for androgen-activated AR. While the C/EBP-α protein was undetectable, the expression of the MyoD protein was essentially equal for cells with or without constitutively activated AR-VP16. This finding confirms our hypothesis that direct androgen-independent activation of AR, stimulating ARE-dependent transcription, does not induce MyoD expression to stimulate myogenesis. This finding indicates that androgen stimulation of myogenesis occurs primarily through bypassing of Wnt to activate β-catenin/TCF4 signaling pathways to induce myogenic stem cell differentiation. More work is needed to determine if adipogenesis similarly is not inhibited through direct AR activation, but instead through β-catenin signaling.

Student Researcher: Armando Priego;
Mentor: Wayne E. Taylor, PhD; Rajan Singh, PhD; Charles Drew University of Medicine and Science, Los Angeles, California

Introduction

The use of androgenic steroids in athletes has caused controversy for years. A common steroid of abuse among athletes is testosterone, which increases muscle mass while decreasing fat mass. This inhibition of fat tissue growth and increase in muscle mass could be caused by direct androgen action on the fat or muscle tissue, but also by an effect of testosterone on inhibiting the differentiation of adipose stem cells or stimulating muscle stem cells. Testosterone binding to androgen receptor (AR) protein causes this transcription factor to translocate to the cell nucleus and bind to androgen receptor element (ARE) DNA elements in promoters, inducing transcription of androgen-responsive genes. In model cell lines such as CH3-10T1/2 stem cells, testosterone and dihydrotestosterone (DHT), down-regulates adipocyte mRNA transcription factors peroxisome proliferator-activated receptor-γ2 and CCAAT/enhancer-binding protein-α (C/EBP-α), while stimulating myogenic transcription factor MyoD (Singh et al. *Endocrinology* 2006).

In this investigation, we attempted to determine if androgen receptors had a direct role on C/EBP-α expression through binding to ARE elements in promoters; unlike its role in the Wnt pathway, where androgen receptors serve as a coactivator of beta-catenin, bypassing normal Wnt signaling. In order to determine if constitutively activated AR has a direct effect on adipogenesis or myogenesis, we transfected mouse CH3-10T1/2 messenchymal stem cells with plasmid pAct-AR, encoding the AR protein fused with a strong transcriptional activation domain called VP16. The plasmid pAct-AR, expressing constitutively activated AR-VP16, can bind to ARE in androgen-responsive promoters, upregulating gene expression. As a positive control reporter for expression of AR-VP16 and ARE-dependent gene expression, the cells were also cotransfected with the p(ARE)4-luc plasmid, containing a promoter turned on by the activated AR-VP16 protein. This plasmid contains a reporter gene, the firefly Luciferase gene, which allows us to assay the Luciferase reporter enzyme to indirectly measure activated AR bonded to the ARE of the plasmid's promoter site. However, to see the effect of the activated AR on the expression of the C/EBP-α protein, or MyoD protein, Western immunoblotting was done to directly measure the amount of these proteins in cells.

Methods

Cell Culture

Mouse 10T1/2 cells were grown in Dulbecco minimal essential medium with 10% fetal bovine serum growth medium at 37°C for three days.

Plasmid Transfection

Transfection of cells with plasmid DNA was done by lipofection using plasmids pAct-AR and p(ARE4)-luc as a control. Renella plasmid pRL-TK was used also in all samples as a positive control for transfection efficiency.

Luciferase Assay

Luciferase assay was done with the TD-20/20 Single-tube Luminometer (Turner). Cells were dissolved. Lysates were then mixed with stock Luciferin

Assay Reagent II. Results were then assayed in the luminometer. Stock Stop and Glo was then mixed with the sample, and it was assayed again. Results were averaged and normalized by dividing Luciferase luminescence activity by Renella activity.

Western Immunoblot

For the Western blot, protein samples were transferred to polyvinylidine fluoride membranes and left to soak overnight. The next day, membranes were soaked in diluted rabbit C/EBP-α or MyoD antibodies, washed, and then soaked in donkey anti-rabbit antibody horseradish peroxidase. We focused primarily on the proteins from the cells that were transfected with the p(ARE)4-luc plasmid and both the p(ARE)4-luc and pAct-AR plasmids but in medium without testosterone.

RESULTS

From the Luciferase activity in our transfected cell lysates, we observed >130-fold stimulation of the ARE-Luciferase promoter by cotransfection with pAct-AR encoding activated AR-VP16. Cells transfected with the p(ARE)4-luc plasmid alone gave very low Luciferase activities; because pAct-AR did not have the Luciferase gene attached, it produced no measurable results in the Luciferase assay when transfected alone. We also found that treatment of the transfected cells for one day with 100 nmol/L testosterone increased Luciferase approximately two-fold in cells transfected with p(ARE)4-luc, either with or without activated AR-VP16. Protein extracts from these transfected cells were also analyzed by Western immunoblotting to test if activated AR-VP16 protein stimulates gene expression of MyoD, while inhibiting C/EBP-α, as was found to be true for androgen-activated AR. From the Western immunoblotting, we determined that the activated AR-VP16 does not directly affect myogenesis by increasing expression of MyoD, since the level of MyoD protein (44 kDa) was equal in control cells (with p[ARE]4-luc alone) and in cells transfected with pAct-AR. We were unable to detect the proteins of the C/EBP-α, since this protein is composed of two subunits of 42 kDa and 30 kDa, which were not visible, while our immunoblot had only one protein visible for a 75-kDa cross-reacting peptide. The molecular weights of these proteins were determined by comparing their electrophoretic mobilities in the gel to that of the known protein markers (Kaleidascope Protein Markers, Bio-Rad).

DISCUSSION

Transfection of the 10T1/2 mesenchymal stem cells with the pAct-AR plasmid caused overexpression of the activated AR-VP16 protein, since this plasmid was necessary and sufficient for >100-fold activation of the AR-dependent promoter on the cotransfected pARE4-Luciferase plasmid. In the protein extracts from cells with constitutive hyperactivated AR-VP16, versus controls with no AR-VP16, we used Western blotting to measure the amounts of C/EBP-α and MyoD proteins. While C/EBP-α protein was undetectable, the expression of MyoD protein (44 kDa) was essentially equal for cells with or without constitutively activated AR-VP16. This finding confirms our hypothesis that direct androgen-independent activation of AR, stimulating ARE-dependent transcription, does not induce MyoD expression to stimulate myogenesis. More experimentation will be needed to test for levels of C/EBP-α protein and mRNA. In conclusion, the constitutively activated AR-VP16 protein, even without testosterone, binds to the ARE DNA elements in the ARE-Luciferase promoter to activate promoter gene expression. However, since this binding did not result in upregulation of MyoD, we can conclude that activated AR protein, without stimulation by testosterone, cannot induce MyoD gene expression. Normal AR, bound to testosterone, may have a unique effect on regulation of adipogenic and myogenic transcription factors C/EBP-α and MyoD, respectively, through activation of the β-catenin and TCF4 proteins in the distal Wnt signaling pathway of differentiating stem cells.

ACKNOWLEDGMENTS

We thank and acknowledge Dr. Xin Yuan (Harvard Medical School) for providing plasmids pAct-AR and p(ARE)-4-Iuc used in this research. The author also thanks Wayne E. Taylor, PhD, for his brilliance and patience and Charles McNeal, Rajan Singh, PhD, and others of the Division of Endocrinology, Metabolism, and Molecular Medicine at Charles Drew University for their help.