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Use of artificial androgen receptor coactivators to alter myoblast proliferation

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Abstract

Skeletal muscle has long been thought to be a target tissue for androgens, eliciting their effect through the androgen receptor. In order to better understand androgen receptor action, a series of mutated androgen receptors were developed and their degree of specificity and cellular responses determined. Specificity, as measured by a reporter assay using HeLa cells, indicated that mutation of the ligand-binding domain or the AR (mutation H865Y), in combination with the p65 transactivating domain, resulted in an increased response to androgens as well as decreased specificity. Transfection of the mutant AR into mouse and rat myoblast cell lines resulted in an increase in expression of the reporter gene consistent with the data from HeLa cells. Overexpression of the wild type or mutant AR into myoblasts and treatment with testosterone induced both greater proliferation and faster differentiation of the cells compared to those expressing endogenous AR. Additionally, when treated with estrogen, these cells were able to proliferate and differentiate to similar levels as cells treated with testosterone. The ability of the mutated AR to act as an artificial coactivator to up-regulate androgen responsive genes is a useful tool for understanding the interaction of androgens and muscle growth.

Keywords: Androgen receptor; Muscle growth; Coactivators

1. Introduction

Steroid hormone receptors constitute a family of inducible transcription factors that mediate the multifold effects of steroids on development, reproduction, proliferation, and cellular homeostasis [1,2]. Activation through the binding of the cognate hormone enables the receptors to bind with high affinity to specific response elements in the promoters of target genes [3], resulting in stimulation or repression of transcription [4]. The androgen receptor is a member of this steroid receptor super-family that mediates androgen-induced physiological responses, which are essential to maintenance of the male phenotype [5].

Coactivators are recruited by the receptor dimers on the androgen response element of DNA to facilitate assembly of basal transcription factors into a stable preinitiation complex (PIC) and can remodel chromatin by acetylating histones [6]. Coactivators require two domains to be able to bridge the receptor to the PIC: a receptor-interacting domain, and a transcription activation domain that contacts and stabilizes assembly of the PIC [7].

Sui et al. [8] created an androgen receptor specific artificial coactivator by fusing the ligand-binding domain (LBD) of the AR to the transactivation domain from a viral protein. Both the VP16 and the p65/RelA have strong activation domains and have been shown to directly contact proteins of the PIC and associate with additional coactivators [9]. The AR LBD can interact with the full length AR, but not with the progesterone or glucocorticoid receptor [8]. It was also shown that the fusion of VP16 or p65 did not impair the specificity of the AR LBD dimerization to the AR or the folding, ligand binding, and nuclear import [8].

Mutations in the LBD of the AR have been shown to alter the specificity of the AR as seen in some prostate cancer cells [10]. Various mutations allow the AR to activate with non-androgenic steroid hormones such as estrogen and progesterone. Based on previous studies of the functional importance of various areas of the LBD, the AR amino acids 893 to 897 in the AF-2 domain are essential for interaction with coregulators as well as with the N-terminal domain [11]. In the LNCaP cells, a single point mutation was detected (A–G) in codon 868 resulting in a transition of a

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threonine to an alanine. This transition resulted in an AR with a similar affinity for androgenic compounds, and also increased affinity to non-androgenic compounds such as progestins and estradiol. The mutated AR also showed slightly increased affinity for antiandrogens (except casodex) [10]. Various alterations in the structure or function of the AR have provided unique insights to the underlying mechanisms of the AR signaling pathways. It has also proved to be very complex. Recent advances in the ability to generate artificial coactivators for the AR have led to methods of altering AR function.

The effect of androgens promoting muscle growth has been demonstrated as early as 1970 [12], but the molecular pathway for its action has not yet been established. It has been suggested that the androgens work on skeletal muscle by an indirect method through changes in the somatomedin levels or the glucocorticoid levels. Finding of a cytosolic androgen receptor in skeletal muscle provided further support for a direct effect of androgens on muscle [13]. Additionally, muscle wasting is noted in patients undergoing androgen deprivation therapy due to prostate cancer [14] and age-related sarcopenia may be attributed to the decline in circulating growth hormone and testosterone [15]. Therefore, androgens and their cognitive receptors may provide a mechanism to manipulate skeletal muscle growth properties.

In previous experiments, it was found that a mutation (H865Y) just upstream of the T868A transition could mimic the effects seen in the LNCaP cell line (unpublished data). This mutation was chosen because it is with the first loop of the binding region and it changes from a basic amino acid to one that has an aromatic side chain which results in an altered protein conformation. Therefore, the aim of this study was to investigate the ability of mutated AR with or without artificial coactivators to induce expression of AR response genes and to determine the cellular responses to the mutated androgen receptors on the proliferation and differentiation of mouse and rat myoblast cells. We report here, the ability of mutated androgen receptors to have an enhanced response and reduced specificity in both HeLa and myoblast cells.

2. Materials and methods

2.1. Hormones

R1881 (17α-methyltrienolone) and R5020 (promegestone) were purchased from New England Nuclear (Boston, MA). Estradiol (4.5% with 2-hydroxypropyl-β-cyclodextrin balance, #E4389), testosterone (10.3% with 2-hydroxypropyl-β-cyclodextrin balance, #T5035), hydroxyflutamide (>99%, #F9397), cyproterone acetate (>98%, #C3412), and dihydrotestosterone (>97.5%, #A8380) were purchased from Sigma (St. Louis, MO). When preparing the ligands, the purity of the compound was taken into consideration and resuspended to account for differences. Casodex (bicalutamide, ICI-176, 334) was obtained as a gift from AstraZeneca Pharmaceuticals (Newark, DE).

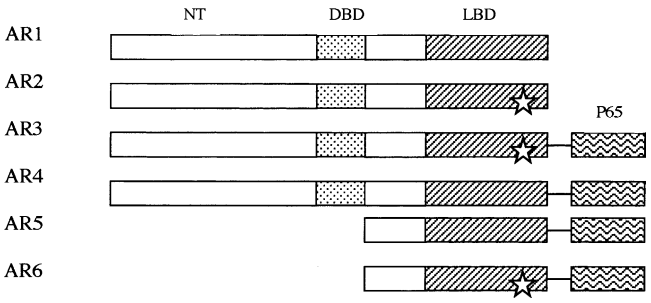

Fig. 1. Schematic representation of constructs. The abbreviations for the basic components of the constructs are: NT, N-terminal domain; DBD, DNA binding domain (dotted area); LBD, ligand-binding domain (striped area); and p65, artificial coactivator (wavy lines). The star signifies the mutation at amino acid 865 changing the histidine to a tyrosine. The fusion proteins are AR(3–910).

2.2. Plasmids

All plasmids (Fig. 1) were designed to contain the open reading frame of the AR constructs flanked by PmeI sites and they were cloned into the mammalian expression vector pcDNA3.1His (Invitrogen). AR1 is the wild type AR cloned from human cDNA as described previously [16] containing the human AR aa 3–910, while AR5 and AR6 comprised of the AR LBD are derived from human AR aa 605–910. AR2, AR3, and AR6 were constructed by site-directed mutagenesis using the primer 5′-CCGTGCAGCCTATTGCGAGAGAGC-3′. AR3, AR4, AR5, and AR6 containing the p65 activation domain were designed as described in Sui et al., in which a 1.3 kb fragment Asp718-Klenow/*Sal*I fragment of p65/RelA encoding amino acids 286–550 was ligated to the *Xho*I/*Hpa*I-digested vector. Resulting fusion protein was AR(3–910)-SVDFPEIYFHTQ-p65(286–550). The $(ARE)_2$TATA-Luc plasmid was described previously [17]. All constructs were sequenced to verify correct nucleotide sequence and correct reading frame.

2.3. Cell culture and transient transfections

HeLa cells (human epithelial cervix carcinoma, American Type Culture Collection), C_2C_{12} mouse myoblasts (ATCC), and L6 rat myoblasts (ATCC) were maintained in Dulbecco Modified Eagle Medium (DMEM)—F12, 10% fetal bovine serum, and L-glutamine. Cells were plated at 10^5 cells/cm^2 in each well of 12-well dishes, with medium containing dextran-coated charcoal-stripped serum, 24 h before transfection. Cells were cotransfected with 0.3 μg of $(ARE)_2$TATA-Luc, 0.03 μg of appropriate AR plasmid, and 2.5 μg of LipofectAMINE^{TM} (Invitrogen) according to the manufacturer's protocols. After 12–14 h incubation, the medium was replaced with DMEM-F12 contain-

ing 10% charcoal-stripped FBS and appropriate hormone treatment. (All hormone treatments were performed in the same experiment, but separated into androgenic and non-androgenic groups in the results for simplicity.) Cell monolayers were incubated with hormone treatments for 48 h, then harvested, and assayed for luciferase activity using the Luciferase Assay System (Promega). Luciferase values were corrected for total protein content and the data is presented as the mean ± S.D. of the experiments performed in triplicate. While AR protein expression was not measured, all electroporation conditions, DNA preparations, and cell and DNA concentrations were the same for all treatments. Additionally, we performed all experiments in triplicate to ensure transfections differences, if any, could be accounted for.

2.4. Fusion

Cells were transfected as previously described in 12-well plates and treated with 1 nM testosterone then harvested at 0, 12, 24, 48, 60, 72, 120, 155, and 171 h. At each time point, cytoplasmic enzymes were removed using digitonin extraction buffer (17 mM 3-(*N*-morpholino)-propanesulfonate acid (MOPS), pH 7; 250 mM sucrose, 2.5 mM EDTA, 1.0 mg/ml digitonin (50% purity), 2 mg/ml aprotinin). Creatine kinase (CK) was measured spectrophotometrically at 340 nm in the presence of p^1p^5-di(adenosine-5′)pentaphosphate (Ap5A, Sigma).

2.5. Proliferation

C_2C_{12} myoblasts were seeded at 0.05×10^4 cells per well in a 96-well flat-bottom white plate, transfected with appropriate AR construct and hormone treatment as described previously. After 24 h of treatment, cells were treated with 5-bromo-2′-deoxyuridine (BrdU) labeling solution from the Cell Proliferation ELISA kit (Roche Diagnostics) and incubated at 37 °C for 2 h before measuring chemiluminescence as directed by the manufacturer. All values are expressed as the mean of triplicate samples.

2.6. Statistical analysis

Data was analyzed by one-way analysis of variance and means separated by Fisher's LSD. Significance was set at $P < 0.05$, unless otherwise specified.

3. Results

3.1. HeLa cell transfection

To test the ability of the AR plasmids to induce expression in HeLa cells of an androgen response element, plasmids were cotransfected with the $(ARE)_2$TATA-Luc reporter construct. In addition to individual AR plasmid transfections, AR2 plus AR5 as well as AR2 plus AR6 were transfected together with the response element reporter construct. The combination of the full length androgen receptor carrying the point mutation at amino acid 865 can interact with the artificial coactivators created with the AR LBD-p65 activation domain to generate 'super activation' in the presence of androgens as well as progesterone and estradiol. Expression of the reporter gene element (measured as relative light units (RLU)) was significantly greater in those cells expressing the AR3 ($P < 0.01$), AR4 ($P < 0.05$), AR2 + 5 ($P < 0.01$), and AR2 + 6 ($P < 0.01$) than the AR1 control when treated with androgens (Fig. 2). The expression driven by AR2 was not different than that by AR1 and the expression driven by AR5 plus AR6 was significantly lower than the AR1 for all hormone treatments.

The androgens (T, R1881, and DHT) activated expression of all the constructs except AR5 and AR6. Constructs AR5 and AR6 only contain the LBD of the AR fused to the p65 transactivation domain. Estradiol induced expression to levels that were not significantly different from the androgens in cells expressing the AR3, but displayed elevated levels with the AR2 + 6 over the AR1 wild type control. R5020 was unable to induce significant expression under any condition. Antiandrogen treatments of casodex, flutamide, and CA produced minimal levels of expression. To test the ability of the antiandrogen casodex to block the effects of T, samples were treated simultaneously with a combination of casodex and T (C/T). In cells expressing AR3, AR4, AR2 + 5, and AR2 + 6, the combination treatment induced high expression of the reporter gene.

3.2. Myoblast transfection

To test the ability of the AR plasmids to induce expression of an androgen response element in skeletal muscle cells, plasmids were cotransfected using the same treatment groups as the HeLa cell experiment. Overexpression of the AR1 had increased expression with the addition of the androgens T and R1881. The AR2 (containing the H865Y mutation) did not increase expression of the target gene with androgens or other steroid hormones over the levels of the wild type AR. When the H865Y mutation was fused to the p65 transactivation domain (AR3), the expression of T ($P < 0.01$), R1881 ($P < 0.05$), and estradiol ($P < 0.01$) was significantly higher than expression with the AR1 plasmid. Fig. 3 displays the expression levels of the target gene with the various treatments. Transfection of AR4 resulted in expression levels of reporter plasmid that were increased with testosterone and estradiol treatments. AR5 and AR6 did not produce expression levels that were much higher than the background expression. This is consistent with the data that was generated from the HeLa cell transfection study described previously. When AR2 was cotransfected with AR5, expression was significantly higher when treated with T ($P < 0.01$), but not with R1881, DHT, and estradiol treatments.

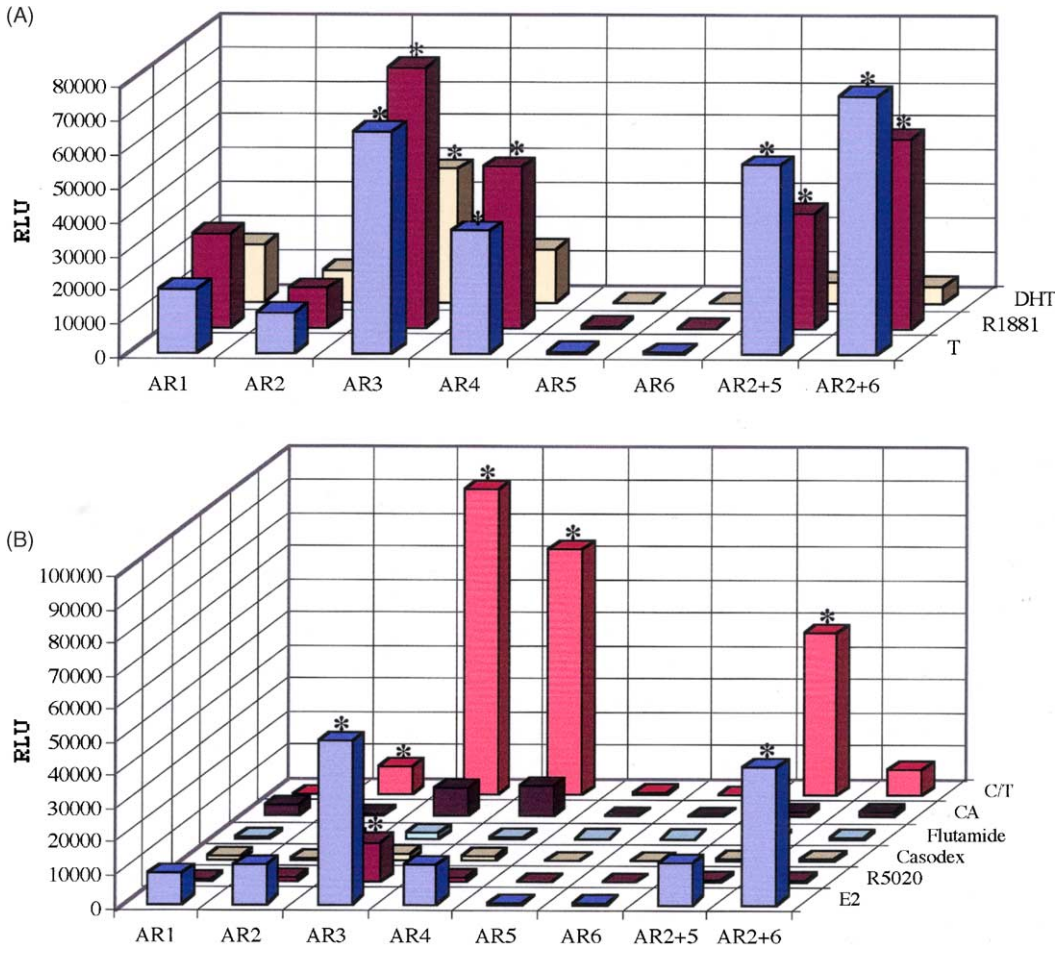

Fig. 2. Transactivation in HeLa cells of $(ARE)_2$TATA-Luc reporter gene construct by various hormone treatments. The luciferase activity (RLU) was measured after treatment with 1 nM androgen (A) or 100 nM for other steroid hormone or antiandrogen (B). Activities were corrected for total protein and shown as RLU over empty plasmid control. Columns with asterisk (*) are significantly different ($P < 0.05$) than AR1 control.

Although R1881, DHT, and estradiol produced greater luciferase expression than control cells, the values were not significantly higher ($P > 0.05$). AR2 + 6 induced significant levels of expression for all the androgens ($P < 0.01$) and for estradiol ($P < 0.01$).

For the L_6 myoblast experiment, all the transfection combinations previously studied were performed with T, estradiol, and R5020. Additionally, AR2 and AR2 + 6 were treated with R1881 and DHT (Fig. 4). AR1 treated with T displayed significant increase in expression over estradiol ($P < 0.05$) and R5020 ($P < 0.01$) as expected. The AR2 expressed luciferase at levels that were not significantly different from AR1 ($P > 0.05$) when treated with androgens and estradiol. Testosterone ($P < 0.01$), estradiol ($P < 0.01$), and R5020 ($P < 0.05$) activation of the AR3 resulted in significantly higher levels of expression ($P < 0.01$) than AR1 treated cells similar to the HeLa and C_2C_{12} experiments. Cotransfections of AR2 + 5 and AR2 + 6 displayed elevated levels of expression with the androgens and estradiol.

3.3. Myoblast proliferation

Wild type or mutant androgen receptors were overexpressed in C_2C_{12} myoblast cells. Cells overexpressing the wild type AR were then treated with T, R1881, or E2 for 120 h, and cell numbers counted at various time points. As expected, samples overexpressing the AR1 and treated with T showed a trend toward an increase in cell number (Fig. 5). A temporal analysis of changes in cell numbers indicated they were not significantly different ($P > 0.05$) at 0 or 12 h; but at 24 h, the cells overexpressing AR1 were significantly greater ($P < 0.01$) than normal cells (Fig. 5). At 48 h, the difference between samples is even greater ($P < 0.01$); but at 72 h, the normal cell numbers increase slightly and the AR1 cell numbers decreased so that they are no longer significantly different ($P > 0.05$). To demonstrate that the differences in cell numbers were due, at least, in part due to proliferation, DNA synthesis was measured by incorporation of BrdU. Since cellular proliferation requires the replication of cellular DNA, the monitoring of DNA synthesis is an

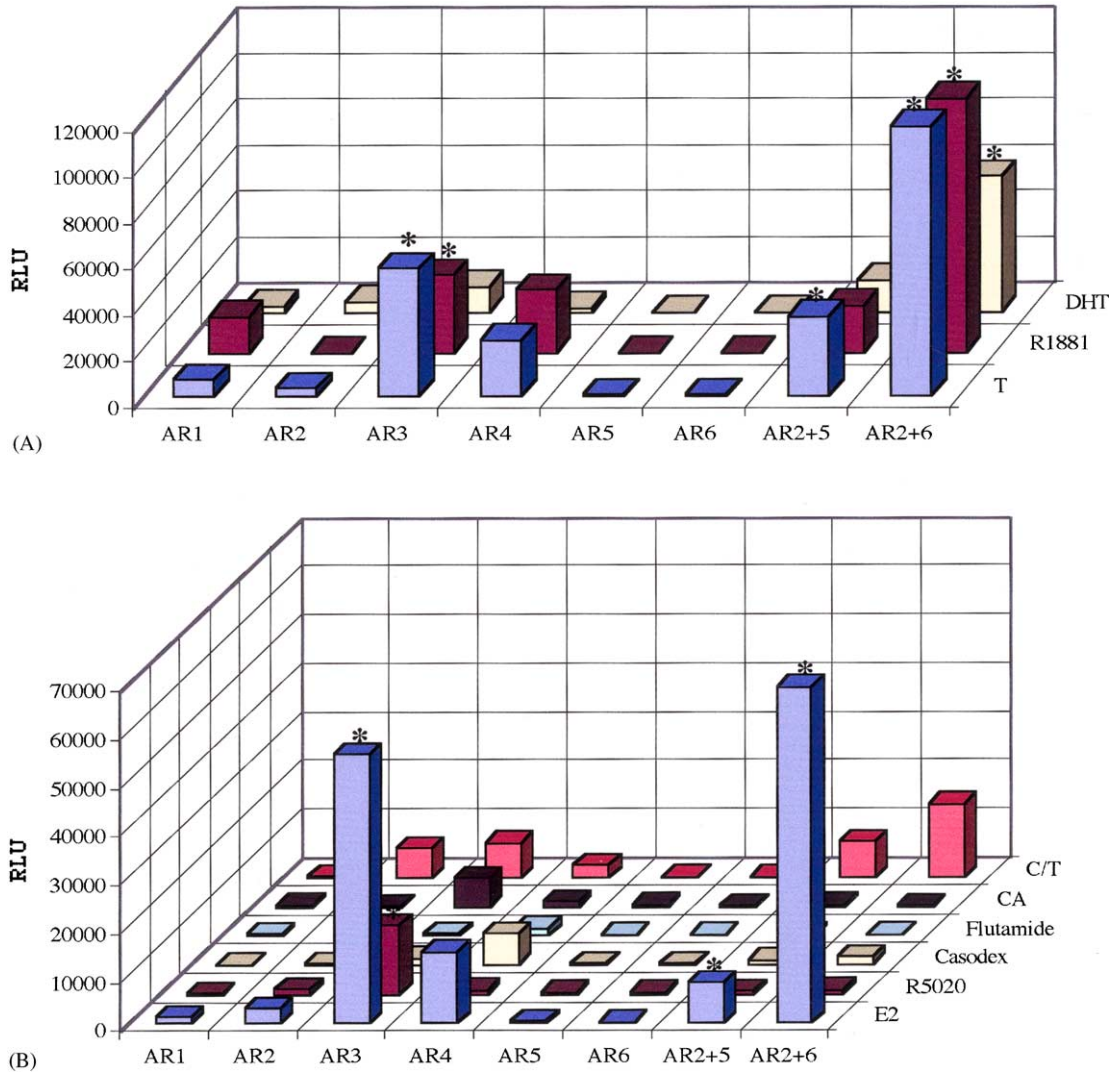

Fig. 3. Transactivation of $(ARE)_2$TATA-Luc reporter gene construct by various hormone treatments to C_2C_{12} myoblasts. The luciferase activity (RLU) was measured after treatment with 1 nM androgen (A) or 100 nM other steroid hormone or antiandrogen (B). Activities were corrected for total protein and presented as RLU exceeding empty plasmid control. Columns with asterisk are significantly different ($P < 0.05$) from cells treated with AR1.

indirect parameter of cell proliferation. To determine the effects of mutant AR, C_2C_{12} myoblasts transfected and culture for 48 h in the presence of different hormones indicated that proliferation of myoblasts was significantly different between cells that were overexpressing AR1 ($P < 0.01$), AR3 ($P < 0.01$), AR2 + 5 ($P < 0.01$), and AR2 + 6 ($P < 0.01$) when treated with T and E2 (Fig. 6). As seen in Fig. 7, there is a rapid rise in proliferation sometime after 24 h in cultures overexpressing AR1 and treated with T as well as E2. This elevation in rate of proliferation is extended through 72 h.

3.4. Myoblast fusion

The parallel increase of creatine kinase and an increase in fusion of myoblasts can be quantified by a coupled enzyme assay [18]. When myoblasts overexpressing AR1 were treated with T, the CK levels were significantly different starting at 24 h after treatment ($P < 0.05$) and continued to be significantly higher through 60 h after start of treatment ($P < 0.01$). At 72 h ($P < 0.05$) and 120 h ($P < 0.01$), the difference between control cells and AR1 cells was significantly different, but the CK levels for AR1 cells had dropped and the control cells had greater CK activity. There was no difference ($P > 0.05$) between cultures at 155 or 171 h. Fig. 8 displays the time study of myoblast fusion. Estrogen treatment had a similar effect on the increase in creatine kinase level, but not to the degree of the testosterone (data not shown).

4. Discussion

Considerable research has been done to evaluate the mechanisms of steroid hormone receptors and their subsequent influence on cellular systems. The amount of information

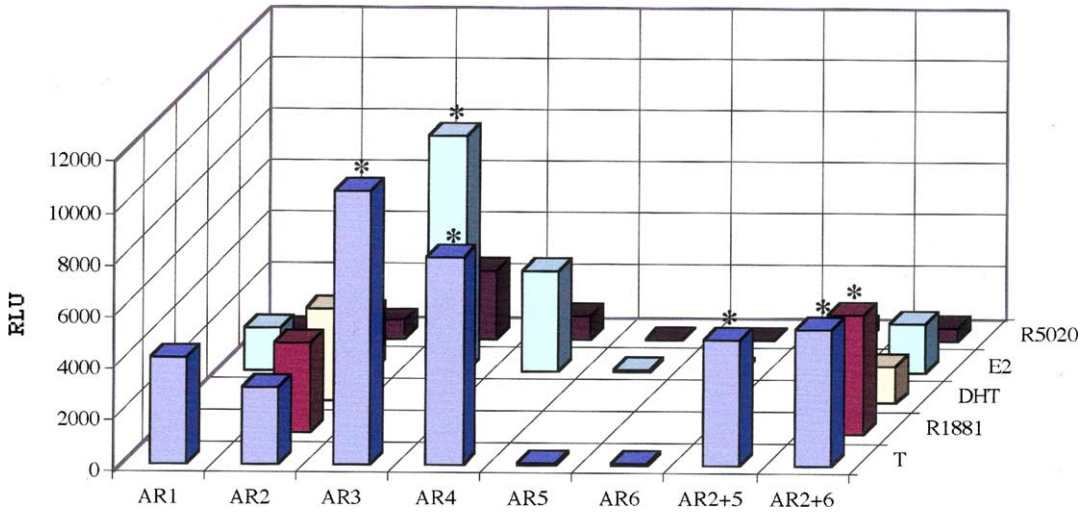

Fig. 4. Transactivation in L_6 myoblasts of $(ARE)_2$TATA-Luc reporter gene construct by various hormone treatments. The luciferase activity (RLU) was measured after treatment with 1 nM androgen or 100 nM E_2 and R5020. Activities were corrected for total protein and presented as RLU exceeding empty plasmid control. Columns with an asterisk are significantly different ($P < 0.05$) than AR1 control.

gathered and the level of understanding of this system has been exponential, but the complexities of the androgen action are still not fully understood. In vitro experimentation has provided a method to isolate some of the factors involved and manipulate them to focus on certain aspects of the interaction. The experiments presented here focused on alterations in the LBD of the AR that ultimately affect the ability of the receptor to interact and stabilize the preinitiation complex. Functionality is determined by the ability to induce expression of the luciferase gene that is fused to the $(ARE)_2$TATA.

The ability of the AR2 and AR3 to induce expression of an androgen responsive gene suggests that the mutation at amino acid 865 does not interfere with the ability of the receptor to bind a ligand, interact with the N-terminal domain, dimerize, recruit cofactors and ultimately stabilize the PIC. Additionally, the addition of p65 to the AR3 enhances the induction of the target gene. This is consistency with previously published data [8] in which the addition of the p65 transactivating region did not inhibit the ability of the plasmid to induce expression of the response element reporter gene in both HeLa and LNCaP cells.

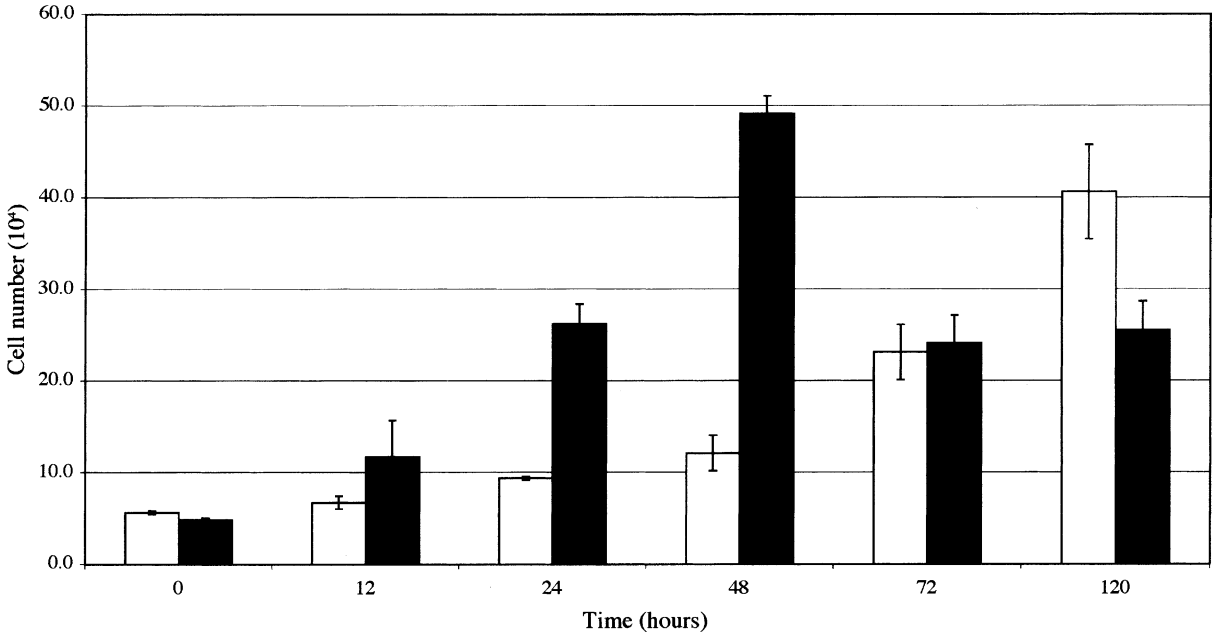

Fig. 5. C_2C_{12} myoblast cell numbers with transfected AR1 receptor (black columns) or empty plasmid control (white columns) after treatment with 1 nM T from 0 to 120 h. Data is presented as the mean of triplicate samples ± S.E.M.

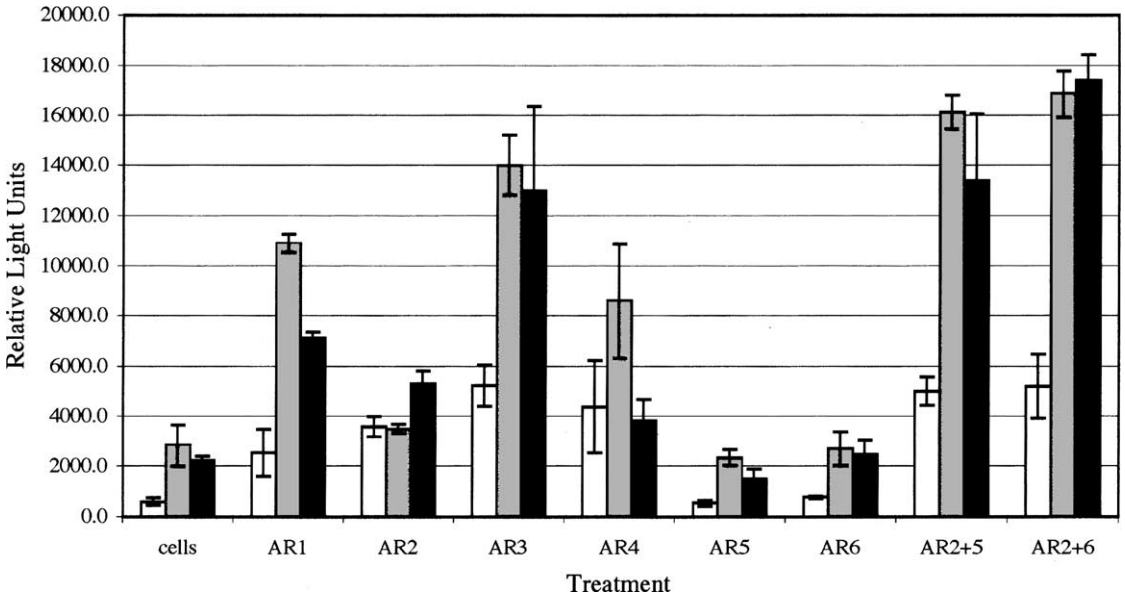

Fig. 6. C_2C_{12} myoblast proliferation 48 h after the addition of 1 nM T (grey bars), 100 nM E_2 (black bars), or no added hormones (white bars), as measured by chemiluminescent BrdU incorporation after transfections with the various AR mutant constructs. Data is presented as the mean of triplicate samples ± S.E.M.

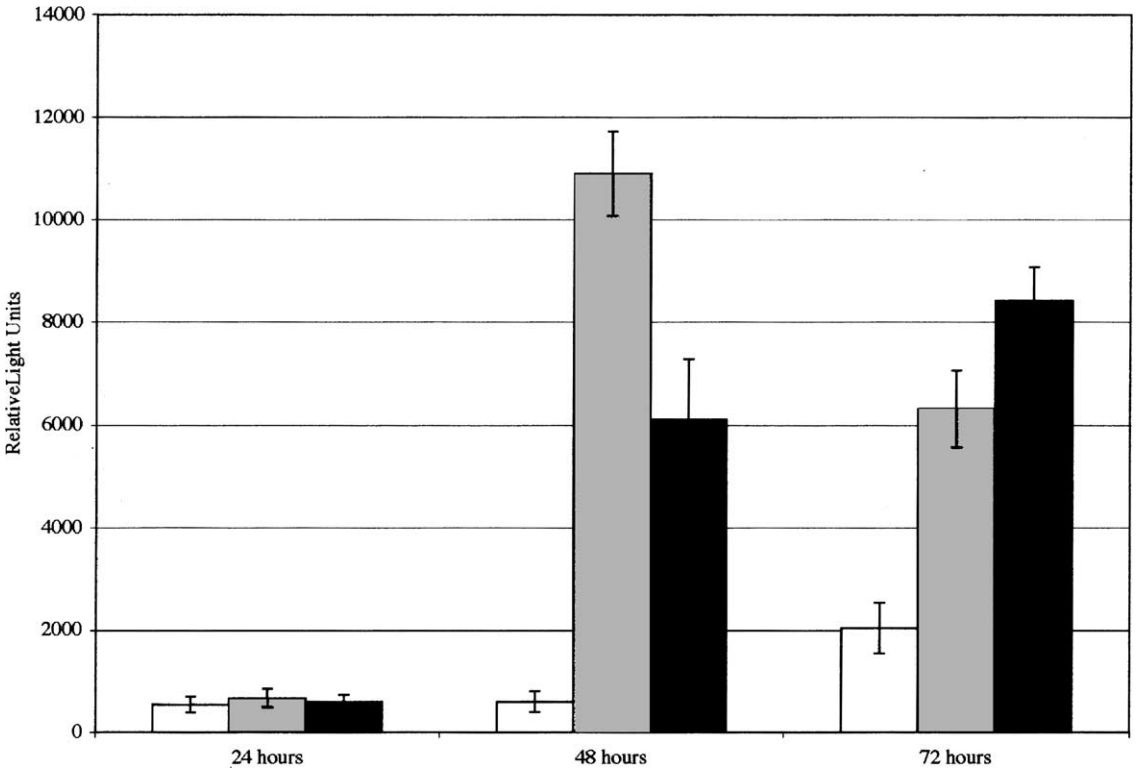

Fig. 7. AR1-transfected C_2C_{12} myoblast proliferation after the addition of 1 nM T (grey bars), 100 nM E_2 (black bars), or no hormone added control (white bars), as measured by chemiluminescent BrdU incorporation at three different time points. Data is presented as the mean of triplicate samples ± S.E.M.

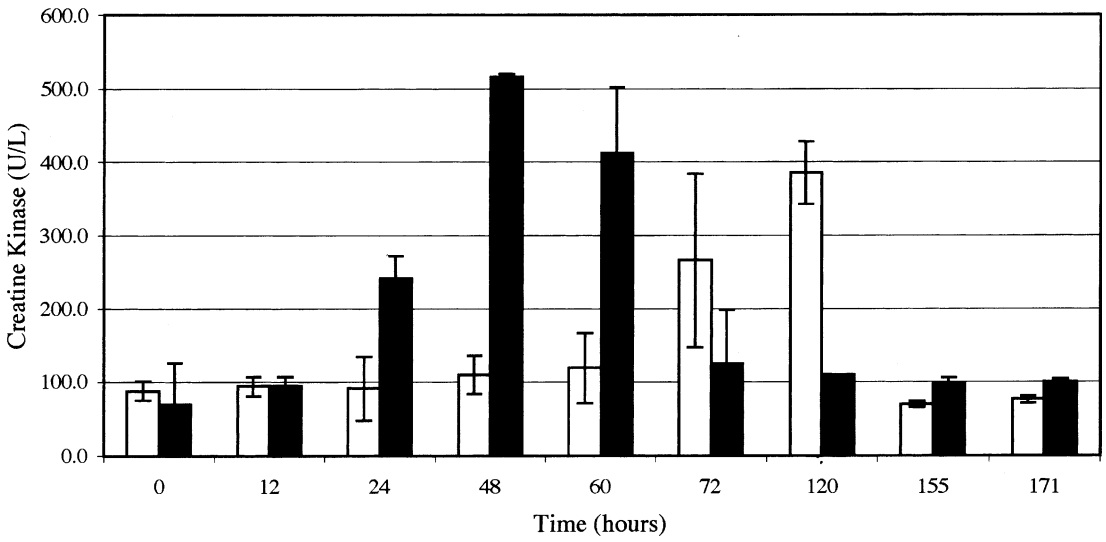

Fig. 8. AR1-transfected C_2C_{12} myoblast fusion as measured by creatine kinase from 0 to 171 h after addition of testosterone (1 nM; black bars) or no hormone added (white bars). Data is presented as the mean of triplicate samples ± S.E.M.

Despite the fact that Sui et al. [8] demonstrated that the AR LBD-p65 construct was able to interact with the full length AR. In experiments presented here, the AR LBD-p65 constructs were not able to drive expression of the $(ARE)_2$TATA-Luc reporter. There are two possibilities to account for the inability to replicate this observation. First, in the experiment by Sui et al. [8] the AR LBD-p65 was cotransfected with wild type AR. It is possible that the levels of endogenous AR in HeLa cells are insufficient to interact with the AR LBD and induce expression of the target gene. Observations of masked AR expression in C_2C_{12} myoblasts have been reported [19]. Secondly, because the AR LBD lacks the DNA binding domain, the dimerization with AR and lack of binding to the response element may not be stable enough for the proper recruitment of necessary transcription factors. Given that HeLa cells have been used for classical studies involving steroid signaling and that the lack of reporter gene expression was noted in both the C_2C_{12} and L_6 myoblasts, it is suggested that the endogenous AR levels are not insufficient in unaltered cell types.

Increased expression of the reporter gene in HeLa cells treated with C/T can be accounted for even in the presence of the antiandrogen because the combination was added simultaneously. The high affinity of the androgen for the AR bound before the antiandrogen was able to block all of the binding sites on the ARs. These data demonstrates the necessity of pre-treating the cells with the antiandrogen before adding the androgen to allow the antiandrogen to bind first and block the binding site for the androgen. L_6 rat myoblasts have been shown to fuse and differentiate into multinucleated skeletal muscle myotubes [20] and bear similar biochemical and histological properties to normal skeletal muscle cells [21]. The full length constructs and truncated constructs cotransfected with a full length AR were able to significantly induce expression of the reporter gene indicating that the constructs were not performing in a species dependent manor. The ability of AR2 and AR6 to activate the target gene with significant expression suggests that not only were the molecules able to achieve proper folding with androgens and estradiol, but they were able to properly coordinate the recruitment and stabilization of the cofactors required to achieve maximal activation and induce expression.

All transfections treated with DHT with the exception of the AR2 + 6 had surprisingly low levels of expression of the response gene. It is suggested that the high levels of 3α-hydroxysteroid oxireductase found in skeletal muscle cells rapidly converts DHT to 3α-diol [22] and that 3α-diol is unable to properly activate the various AR constructs that were tested. It is possible that the 3α-hydroxysteroid oxireductase has a greater affinity for the DHT than the AR and majority of the DHT is converted into the inactive 3α-diol rather than activating the AR pathway. The inability of R5020 and DHT to activate the transfected AR in C_2C_{12} cells is similar to that found in HeLa cell.

The antiandrogens maintained their inhibitory properties in all transfections. In AR4 transfection, the casodex and flutamide treatments appear to have elevated levels of expression of the target gene, but the levels are not significant over wild type controls. Similarly, the C/T treatment appeared elevated, but they too were not significant. In all studies, the hormone treatment was added for 48 h. In the C/T treatment of C_2C_{12} myoblasts, the casodex was added for 24 h, then the T was added to the media for the final 24 h of treatment. This method allowed the antiandrogen to have time to bind to the AR before the addition of the T, which has a higher affinity for the receptor.

AR1 cells treated with T have rapid increase in cell numbers seen at 48 h, which is shown to be due to an increase in proliferation. This data suggests that an increase in the

AR activity is at least partially responsible for the anabolic activity of the cells. Those cells displaying proliferative activity correspond to those that are fusing at higher rates as well. Anabolic activity as measured by the degree of proliferation was influenced by the various AR mutants tested. Constructs that were able to induce reporter gene expression were also able to initiate anabolic activity.

Although the work presented by Lee [19] suggests that the AR mechanisms alter the levels of differentiation by up-regulating myogenin and do not change the levels of proliferation, the data presented here suggests that both overexpressing the AR and various mutations within the ligand-binding domain, in fact, do influence the proliferation of C_2C_{12} cells. Studies have indicated that androgens may increase skeletal satellite cell or myogenic precursor cell proliferation [23,24] while suppressing myoblast proliferation and inducing differentiation. Additionally, AR signaling pathways have had both positive and negative effects on cell growth [15] suggesting cell environment has a role. These observations taken together suggest that the role of the AR needs to be studied further.

In this study, increased expression of the AR results in increased muscle cell proliferation and fusion. A point mutation at amino acid 865 of AR changed the specificity of the receptor to allow estradiol to bind and induce activation. When fused to the p65 transactivation domain, the full length AR was capable of high levels of increased reporter expression. Although the AR LBD-p65 alone was not able to induce expression, when cotransfected with the full length AR, it induced high expression of the reporter gene. Therefore, both the mutation at amino acid 865 and the fusion of p65 transactivation domain to the AR provides a means of altering the mechanisms of androgen sensitive gene expression. It also provides a model to investigate the mechanisms in which the growth patterns of muscle can be manipulated.

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