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RESEARCH PAPER

Alanine Aminotransferase Regulation by Androgens in Non-hepatic Tissues

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ABSTRACT

Purpose Alanine amino-transferases (ALTs) play a crucial role in drug development as a surrogate marker of liver injury where elevations in serum ALT activity are used to diagnose drug-induced liver damage. Two ALT isoforms have been characterized with disparate but overlapping tissue expression. ALT1 is primarily expressed in live; ALT2 is found in muscle and prostate tissues. We investigate ALT gene expression in diverse rodent tissues following administration of the steroidal androgen receptor (AR) agonist dihydrotestosterone and a novel tissue selective nonsteroidal agonist S-23.

Methods Putative AR regulation of ALT expression was determined in silico by an orthologous promoter androgen response element (ARE) search. Regulation was evaluated by transient transfection of ALT promoter region constructs and qRT-PCR experiments in cultured cells and in tissues following androgen administration.

Results Several putative AREs were found in the proximal promoter regions of ALT1 and ALT2. AREs in ALT2 but not ALT1 were capable of AR-mediated transcription. ALT2 expression was affected by castration and androgen administration in muscle and prostate but not liver tissues.

Conclusions Androgen action in non-hepatic tissues, as opposed to xenobiotic toxicity alone, may contribute to increases in serum ALT activity following androgen administration.

KEY WORDS ALT · androgen receptor · selective androgen receptor modulator

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INTRODUCTION

Glutamate pyruvate transaminases (GPTs, also known as alanine amino-transferases or ALTs) catalyze the reversible transamination between alanine and 2-oxoglutarate to form pyruvate and glutamate and play an essential role in the intermediary metabolism of glucose and amino acids (36). In tissues, such as muscle, where amino acids are a primary fuel source, amino groups are collected from glutamate via transamination. ALTs transfer the α-amino group from glutamate to pyruvate to form 2-oxoglutarate and alanine, the major amino acid present in fasting blood. Circulating alanine is then absorbed by the liver for glucose generation from pyruvate, reversing the ALT reaction and completing the alanine-glucose cycle (7). Two ALT isoforms sharing 80% homology and nearly undistinguishable transaminase activities have been characterized to date (36,37). ALT1, the first characterized, is primarily expressed in intestine, liver, adipose, and colon while ALT2 is expressed primarily in muscle, brain, liver and prostate tissues (35,37).

ALT is best known as a surrogate marker of liver injury where several criteria involving serum ALT elevation are used to diagnose both hepatocellular disease and drug-induced liver damage (1,37). While serum ALT is elevated in a variety of liver diseases, it is also elevated in conditions un-related to hepatocellular distress such as severe muscle injury, muscle disease and even apparently healthy individuals (2,5,18,25,26). Likewise, in preclinical drug toxicity screening, serum ALT elevation is sometimes observed in rodents without supportive histological evidence of liver injury (24,37). Toxicity-induced ALT elevations are thought to result from porous necrotic liver tissue leaking enzymes into the systemic circulation. Clinically, serum ALT levels are measured by the catalytic activity of the enzyme in serum with no way to monitor each isoform's contribution. However, rodents administered known liver toxins showed

elevated serum ALT activity and increases in both ALT-1 and ALT-2 serum protein content (37). Interestingly, increases in serum ALT1 protein content correlated better with increases in serum activity than did ALT2, suggesting that ALT1 is the principal contributor to increased serum ALT activity following liver damage, not ALT2.

The androgen receptor (AR) is a member of the nuclear hormone receptor family and functions as a ligand-activated transcription factor in diverse tissues throughout the body including muscle, prostate, testes and brain (19). Unliganded AR resides in the cytoplasm bound by heat-shock proteins, held in an inactive state. Upon ligand binding, the receptor translocates to the nucleus, homo-dimerizes, and binds specific DNA sequences known as androgen response elements (AREs) in the promoter or enhancer region of target genes resulting in changes in gene expression (33). The primary endogenous androgens, testosterone (T) and its 5α reduced form 5α-dihydrotestosterone (DHT), act through the AR to promote the development of secondary sexual characteristics in males and the maintenance of skeleton and musculature in both males and females (23). Androgens are used therapeutically in a limited number of disease states, namely hypogonadism, due in part to their undesirable side-effects. The recent development of novel nonsteroidal, orally available selective androgen receptor modulators with improved side effect profiles has greatly expanded potential patient populations and initiated a re-evaluation of the side effects of traditional steroidal androgen administration (3,8,31). Serum ALT elevations following various exogenous steroidal androgen administration paradigms, particularly oral, have been reported in both clinical trials and animal models (9,11,30,34). As previously discussed, several factors are known to confound diagnoses of hepatotoxicity based solely on increases in serum ALT activity. In the following study, we show that ALT2 expression is AR regulated by both DHT and the tissue selective agonist (*S*)-*N*-(4-cyano-3-trifluoromethyl-phenyl)-3-(3-fluoro, 4-chlorophenoxy)-2-hydroxy-2-methyl-propanamide (S-23) in diverse rodent tissues (15). Thus, AR action in non-hepatic tissues may contribute to increases in serum ALT following androgen administration.

MATERIALS AND METHODS

Chemicals

S-23 was synthesized as outlined by Marhefka *et al.* (20). Chemical purity was confirmed using a combination of elemental analysis, mass spectrometry, and proton nuclear magnetic resonance. 5α-DHT was purchased from Sigma-Aldrich (St. Louis, Missouri). Bicalutamide was purchased from Tocris BioScience (Ellisville, Missouri).

Orthologous Promoter Androgen Response Element (ARE) Search

Twenty eight well-characterized AREs were compiled from Nantermet et. al. (21) to create a position weight matrix (PWM) using the MatInd method (27). Sequence data corresponding to 8 kilobases (kb) surrounding the transcriptional start site (TSS, -6 kb to +2 kb) was then retrieved for the human, mouse and rat ALT1 and ALT2 promoters according to the genomic addresses of exon 1 (TSS) of the 3′ most transcriptional isoform of each gene. Using a modified version of the Perl script MatScan (4), human sequences were searched, 15 base pairs at a time, using the MatInspector (27) methodology. If a PWM match of 90% or greater was detected, then a 1,600 bp window centered on the first position in the human sequence was searched in the mouse and rat sequences. Conserved AREs were reported with matches greater than 85% in either rodent sequences.

Plasmid Constructs and Transcriptional Activation Assay

Full length human androgen receptor expression construct (AR) was previously described (16). The mammalian expression vector pCR3.1 (Invitrogen) was utilized as a negative control. Genomic DNA fragments approximately 300 bp in size, centered on putative AREs determined by the above bioinformatic approach, were amplified from LNCaP genomic DNA and cloned into the multiple cloning region of the firefly reporter vector pGL4.26(Promega) utilizing the Sac I and Xho I restriction sites. The primers pairs for these elements are as follows (forward primer, reverse primer); **ALT1-ARE1** (TATATCTCG-AGGAGTGCATC GAGGCCGTGATC, TATAGAGCTCGCACG CACTCGCCCATGTAG); **ALT1-ARE2** (TATATCTC GAGGCACAAGGCTGTGTCAGAAGCTC, TATAGA-GCTCGCTGAGCCAGGAGGGAGAAC); **ALT1-ARE3** (TATATCTCGAGCAGAG-GACCCAGGAGAGCTCATG, TATAGAGCTCCAGAAGCACCAGGCATCTGTC); **ALT2-ARE1** (GACATCTCGAGGTGCTTTGCAAATTG TACAGTG, TATAGAGCTC GACCTTGACTTGCATG TAATGTCC); **ALT2-ARE2** (TATATCTCGAGC-CAAACCCAGCGAGCTACACATG, TATA GAGCTCGTGTGCTCCCACGTCATCACTG); **ALT2-ARE3** (TATATCTCGAGTGGGAGCAGCT CATGGGACAC, GACAGAGCT CCACATAGCTGGA GAGTCATATG); **ALT2-ARE4** (TATATCTCGAGT-CAGGCTCCCTCGAGAGCAAGAG, GACA GAGCTCGTAGATTCTGTACGGTCCATGC). 300 bp genomic regions from both ALT-1 and ALT-2 promoters not containing a putative ARE were similarly cloned into pGL4.26. The primer pairs for these elements were

as follows; **ALT1-NC** (TATATCTCGAGGTGTCCACAGG-TAGGCCTGCT, TATAGAGCTC-CCTGTCCTAGATCCTGTCATTAAG); **ALT2-NC** (TATATCTCGAGCCTGGA-CCATAAGCCAAGGC, TATAGAGCTCGATGCCCAGGCAGG-AGAATCA). The previously described ARE containing enhancer element from the prostate specific antigen (PSA) enhancer region (12) was similarly cloned into pGL4.26 using the following primers; **PSA-Enhancer** (TATATCTCGAGCATGTTCACATTAGTACACCTTGC, TATAGAGCTCCGTTGAGACTGTCCTGCAGACAAG). The sequence and orientation of each construct was confirmed by sequencing. The pGL4.73-SV40-hRLuc (Promega) vector was used as a transfection control.

For transient transfection, HEK-293 (ATCC) cells in log-phase growth were plated at 1 million per dish cells in 6 well dishes (Corning) in phenol-red free DMEM (MediaTech) supplemented with 5% charcoal-stripped fetal bovine serum (csFBS, Atlanta Biologicals). These cells were then transfected with 1 μg of either empty pGL4.26 or one of the pGL4.26 constructs described above(firefly luciferase), 5 ng pGL4.73-SV40-hRLuc (renilla luciferase), and either 5 ng pCR3.1 or 5 ng of AR expression vector using FugeneHD (Roche) in accordance with the manufacturer's protocol. After 10 h, the cells were trypsinized and re-plated at 40,000 cells per well into 96-well plates (Corning). Once attached, the cells were treated as indicated for 24 h. Firefly and renilla luciferase activities were determined using the Dual-Luciferase Assay Reporter system (Promega) and a BioTek Synergy 4 plate reader. Firefly luciferase values were normalized by renilla luciferase values for triplicate data and represented as ratio of treated to vehicle (Fold, mean ± SD) for each transfection condition.

LNCaP Prostate Cancer Cells Gene Expression

Low passage LNCaP cells were cultured according to ATCC's guidelines. Once seeded, cells were maintained in phenol red-free RPMI supplemented with 1% charcoal-dextran stripped fetal bovine serum (cFBS) for 4 days. On day five, the media was replaced and the cells were treated as indicated. Cells were harvested using Trizol and total RNA extractions performed. cDNA was generated using the High Capacity cDNA Kit with RNase inhibitor (Applied Biosystems) and 1 μg of total RNA from each sample. All genes were assayed using Taqman Assays (Applied Biosystems) and 2x Universal Master Mix qRT-PCR reagents on a 7300 Real Time PCR System (Applied Biosystems). Data was analyzed using the $2^{-\Delta\Delta Ct}$ method (17), normalized to 18S or GAPDH. Positive fold change values were represented as $2^{-(\Delta\Delta Ct)}$ (Low Error Bar: $2^{-(\Delta\Delta Ct\ +\ SD\Delta\Delta Ct)}$, High Error Bar: $2^{-(\Delta\Delta Ct\ -\ SD\Delta\Delta Ct)}$) as suggested by Yuan *et al.* (38). Negative fold change values are represented as $-2^{\Delta\Delta Ct}$ (Low Error Bar: $-2^{(\Delta\Delta Ct\ -\ SD\Delta\Delta Ct)}$, High Error Bar: $-2^{(\Delta\Delta Ct\ +\ SD\Delta\Delta Ct)}$) for easier interpretation of down regulated transcripts. One-way ANOVA followed by pair-wise two-tailed Student's *t*-tests (Fisher's Least Significant Difference [LSD]) were performed on ΔΔCt values with a threshold set at $p<.05$ when determining significant differences.

Primary Rat Hepatocyte Gene Expression

Cryo-preserved primary male Sprague–Dawley rat hepatocyte suspensions (CellzDirect Durham, NC) were plated in collagen-coated 6 well plates (Beckton Dickinson) in William's E-medium supplemented with 5% FBS, 0.1 U/ml penicillin/streptomycin, 4 μg/ml bovine insulin, 1 μM dexamethasone, 17.5 mg/ml *L*-glutamine and 15 mM HEPES in accordance with the vendor's protocol (Supplement Pack, CellzDirect). Four hours after plating, the media was replaced with phenol red-free William's E Media supplemented with 0.1 U/ml penicillin/streptomycin, 100 nM dexamethasone, 1x insulin-transferrin-selenium solution, 17.5 mg/ml *L*-glutamine and 15 mM HEPES (Supplement Pack, CellzDirect). Twelve hours later, the media was refreshed and the cells were treated as indicated. Following treatment, cells were harvested and total RNA extracted using Trizol. Samples where qRT-PCR assayed for ALT1 and ALT2 expression as described previously.

Animal Studies

All procedures were reviewed and approved by the Institutional Laboratory Animal Care and Use Committee of The University of Tennessee. Sprague–Dawley rats were purchased from Harlan Biosciences (Indianapolis, IN). Male rats (12 weeks old) were randomized by pre-surgery body weight into treatment groups of five and then orchidectomized (ORX) or sham-operated to remain as intact controls. Animals were treated the day following surgery, and then daily thereafter, via subcutaneous injections of each compound (80:20 (v:v) PEG300:DMSO). At the end of the indicated treatments, animals were anesthetized, and sacrificed within 8 h after the last dose. Tissues of interest were harvested and weighed at sacrifice including prostate, seminal vesicles, soleus muscle, levator ani muscle, and liver. After weighing, tissues were flash frozen in a dry ice ethanol bath and stored at −80°C prior to RNA extraction and subsequent qRT-PCR analysis. One-way ANOVA followed by pair-wise two-tailed Student's *t*-tests (Fisher's LSD) were performed on body weight normalized tissue masses represented as a percentage of mean normalized intact control tissue mass with a threshold set at $p<.05$ to determining significant differences.

Western Blotting

Western blots were performed as previously described (22). All tissues were from intact untreated animals with rat prostate and levator ani representing pooled samples. An aliquot (75 μg) of each sample was probed first with AR-PG21 (Millipore) to detect AR expression. The membrane was then probed with a pan-actin antibody (Clone C4, Millipore) to gauge loading. Densitometry of the resulting image was performed using ImageQuant TL software (Amersham Biosciences).

RESULTS

The Regulatory Regions of Both ALT1 and ALT2 Contain Androgen Response Elements (AREs)

A bioinformatics search for AREs was performed in an effort to evaluate ALT1 and ALT2 as putative direct (cis) targets of AR regulation. Simple sequence matching to the canonical ARE is too rigid to incorporate what is known to be rather diverse ARE sequence content (21). Also, only slightly more complicated mis-match allowance techniques do not account for known invariant bases required for AR binding (28). The MatInd methodology was employed as an improved approach previously shown to predict *bone fide* nuclear hormone receptor DNA binding sites (4). When the regulatory regions of ALT1 and ALT2 were searched for AREs, several strong putative androgen receptor binding sites were detected (Table I). The functional relevance of these sites is supported by their conservation across species lines.

The ability of the AR to drive transcription via each ARE was interrogated by transient transfection (Fig. 1). The empty firefly reporter construct (pGL4.26) demonstrated a small receptor- and ligand-dependent induction of approximately 3-fold when cotransfected with AR and treated with 100 nM DHT, whereas no induction was apparent in either the ALT1-NC or ALT2-NC constructs. Treatment with either DHT or S-23 resulted in receptor and ligand dependent induction of ~24- and 19-fold, respectively, of the PSA-

Table I Bioinformatic Search for Conserved Androgen Response Elements (AREs)

	Species	Gene symbol	RefSeq	Location (Δ TSS)	Strand	Chr.	PWM score	Sequence
A								
ARE-1	Human	ALT1	NM_005309	+1963	Plus	8	.958	AGGACAACGTGTACG
ARE-1	Mouse	ALT1	NM_182805	+1631	Plus	15	.914	GGCACACAGAGTGGT
ARE-1	Rat	ALT1	NM_031039	+1698	Plus	7	.909	AGGACAACGTGTATG
ARE-2	Human	ALT1	NM_005309	−1373	Plus	8	.906	GGCACAATGTGTCAG
ARE-2a	Mouse	ALT1	NM_182805	−710	Minus	15	.912	AGAACAGGAAGTGAG
ARE-2b				−531	Plus		.958	AGGGCACCAAGTCTT
ARE-2a	Rat	ALT1	NM_031039	−877	Minus	7	.872	GGGACAGAGGGATCA
ARE-2b				−551	Plus		.958	AGAACTAGCTGTCCC
ARE-3	Human	ALT1	NM_005309	–5536	Minus	8	.953	AGAACAGGCCGTGCT
ARE-3	Mouse	ALT1	NM_182805	−6006	Plus	15	.862	AGGGCACCAAGTCTT
ARE-3	Rat	ALT1	NM_031039	−5375	Minus	7	.909	AGAACTAGCTGTCCC
B								
ARE-1	Human	ALT2	NM_133443	−1827	Minus	16	.908	TGTGCTTGTTGTTCT
ARE-1	Mouse	ALT2	NM_173866	−1437	Plus	8	.945	GGCACAGTTGGTGCT
ARE-2	Human	ALT2	NM_133443	−2002	Minus	16	.934	AATACTGCCTGTGCT
ARE-2	Mouse	ALT2	NM_173866	−1437	Plus	8	.945	GGCACAGTTGGTGCT
ARE-3	Human	ALT2	NM_133443	−2976	Minus	16	.932	GAAACTGTATGTTCT
ARE-3	Mouse	ALT2	NM_173866	−2978	Minus	8	.917	AGCACTGATTGCTCT
ARE-4	Human	ALT2	NM_133443	−3977	Minus	16	.912	CTCACTTTCTGTTCT
ARE-4	Mouse	ALT2	NM_173866	−4535	Plus	8	.984	GGGACAAGCTGTTCT
ARE-4	Rat	ALT2	XM_001052974	−4334	Plus	19	.926	GGTACACGCGGTCCA

28 biochemically characterized AREs were used to create an ARE position weight matrix (PWM). The promoter regions of ALT1 and ALT2 were searched for matches >=86% in human mouse and rat sequences. Matches found within 800 bp, relative to the transcription start site (TSS), between species were considered conserved and likely functionally relevant. (A) Conserved AREs in the promoter region of ALT1. 3 AREs in the regulatory region of ALT1 were conserved across human, mouse and rat. (B) Conserved AREs in the promoter region of ALT2. 4 conserved AREs were located upstream of the TSS in the human ALT2 genomic sequence. 3 were conserved between human and mouse, and one across all three species.

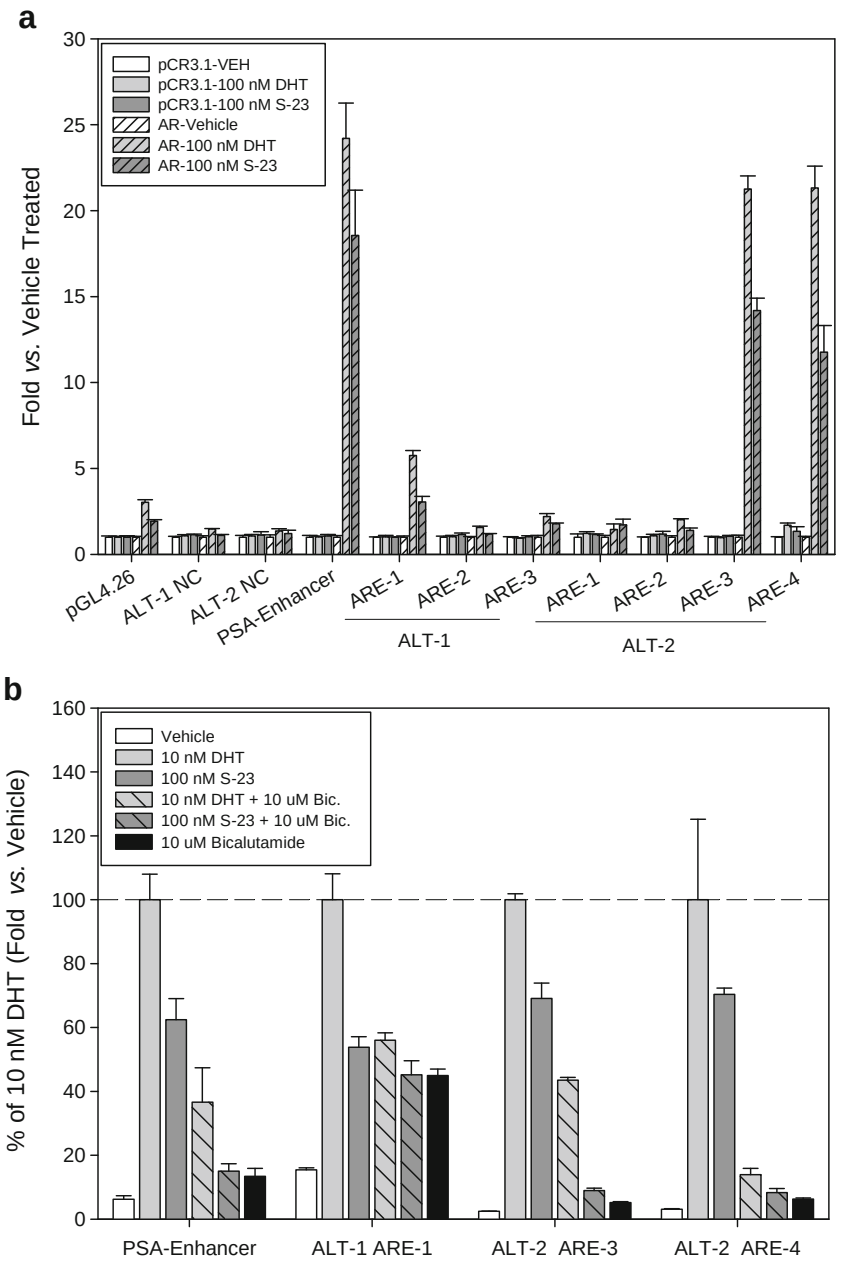

Fig. 1 (**a**) AR transcriptional activation assay. HEK-293 cells were transiently transfected with reporter constructs and an empty pCR3.1 expression vector (*open bars*) or AR (*hatched bars*) expression plasmid then treated with vehicle or ligand for 24 h. Fold induction (Mean ± SD) *versus* vehicle is reported. (**b**) HEK-293 cells were transfected with an AR expression plasmid and reporter constructs then treated with vehicle or ligand(s) for 24 h. The fold induction over vehicle was calculated and reported as a percentage of the induction resulting from 10 nM DHT treatment (Mean ± SD).

Enhancer reporter construct. Among the putative AREs in the promoter region of ALT1, only ARE-1 displayed the ability to drive AR mediated transcription with a modest ~6- and 3-fold induction following DHT and S-23 treatment, respectively. A similar analysis of putative AREs in the promoter region of ALT2 revealed robust induction of ARE-3 and ARE-4 reporter constructs. Treatment with 100 nM DHT resulted in ~21-fold increase in firefly signal for both constructs, whereas 100 nM S-23 demonstrated a reduced effect ~14- and 11-fold for ARE-3 and ARE-4, respectively. The 15-bp core ARE sequence is identical between the ARE-3 and ARE-4 constructs (Table IB) further supporting the importance of this binding element in mediating transcriptional activation.

The specificity of the AR:ARE interactions were confirmed by co-treatment with the AR antagonist bicalutamide (Fig. 1B). The PSA-Enhancer, ALT1 ARE-1, ALT2 ARE-3 and ALT2-ARE-4 constructs were co-transfected with the AR expression plasmid and treated with 10 nM DHT or 100 nM S-23 alone or in the presence of 10 μM bicalutamide. Consistent with previous reports DHT was more potent than S-23 demonstrating ~%30–40 greater induction at 10 nM than was seen with 100 nM S-23(15). Bicalutamide co-treatment reduced the DHT mediated signal between ~%44(ALT1 ARE-1) and%86 (ALT2 ARE-4). In all cases, the luciferase signal generated with 100 nM S-23 was antagonized by co-treatment with

bicalutamide resulting in levels similar to 10 μM bicalutamide treament alone.

AR Expression

AR expression was readily apparent in the prostate cancer cell line LNCaP, rat prostate tissue and rat hepatocytes (Fig. 2). AR expression was present but barely detectable in liver and levator ani tissue. When adjusted for loading, AR abundance was given the following rank order: LNCaP > rat prostate = rat hepatocyte > rat liver > rat levator ani. The finding that liver tissue expression is lower than prostate and contained primarily to the hepatocyte in the rat is consistent with reports from human tissues (29).

ALT1 and ALT2 Regulated by Androgens in LNCaP Prostate Cancer Cells

LNCaP cells express AR in abundance (Fig. 2) and are responsive to androgen treatment making them a suitable *in vitro* model to study potential androgen-mediated transcriptional regulation (6). Basal androgen signaling was reduced by "serum-starving" the cells for several days in media supplemented with hormone-depleted 1% serum. At 24 h, DHT and S-23 demonstrated small but significant induction of both ALT isoforms (Fig. 3). Prostate specific antigen (PSA) expression was also induced, a hallmark of AR action in prostate, across all time points with both ligands. The induction of ALT1 waned with time resulting in slight repression by both ligands after 3 days. ALT2 however showed induction by both ligands through 48 h and 72 h of treatment with S-23.

Expression of ALT Not Regulated by Androgens in Liver

As male rat liver tissue expresses both AR (Fig. 1) and an abundance of ALTs, androgen's ability to regulate the ALTs was evaluated in both rodent primary hepatocytes and rodent liver tissue (32,37). When male rat primary hepatocytes were treated with up to 1 μM concentrations of both DHT and S-23 for 48 h no significant changes were evident in either ALTs' expression (Fig. 4a–b). However, a small but significant repression of ALT2 resulted from treatment with 10 μM bicalutamide that was also present when the hepatocytes were treated with 1 μM DHT and 10 μM bicalutamide concurrently (Fig. 4a). To evaluate the effects of endogenous and exogenous androgens on ALTs' expression in liver tissue, a hormone-depleted male rat model was employed. In this model male rats were surgically castrated to effectively remove circulating endogenous androgens. After 3 days, castration had no apparent effect on the hepatic expression of either ALT1 or ALT2 (Fig. 4b). Likewise, exogenous androgen administration produced no significant change in hepatic ALT expression.

ALT2 Regulated by Androgens in Prostate and Muscle Tissue

While ALTs are primarily thought of as derived from liver, their expression has been reported in numerous non-hepatic

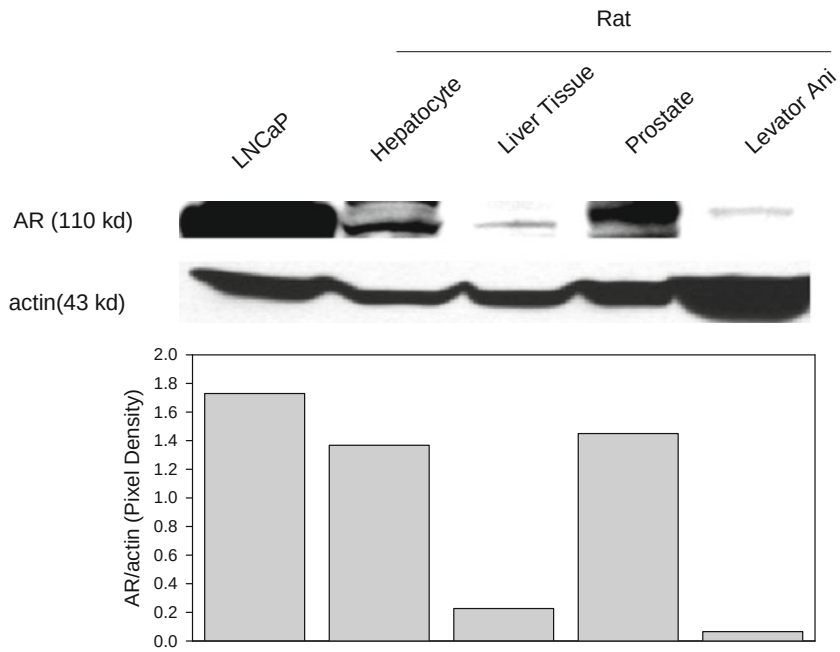

Fig. 2 Cell and tissue androgen receptor expression as determined by western blot. Densitometry was performed on digitized images and the ratio of signals presented.

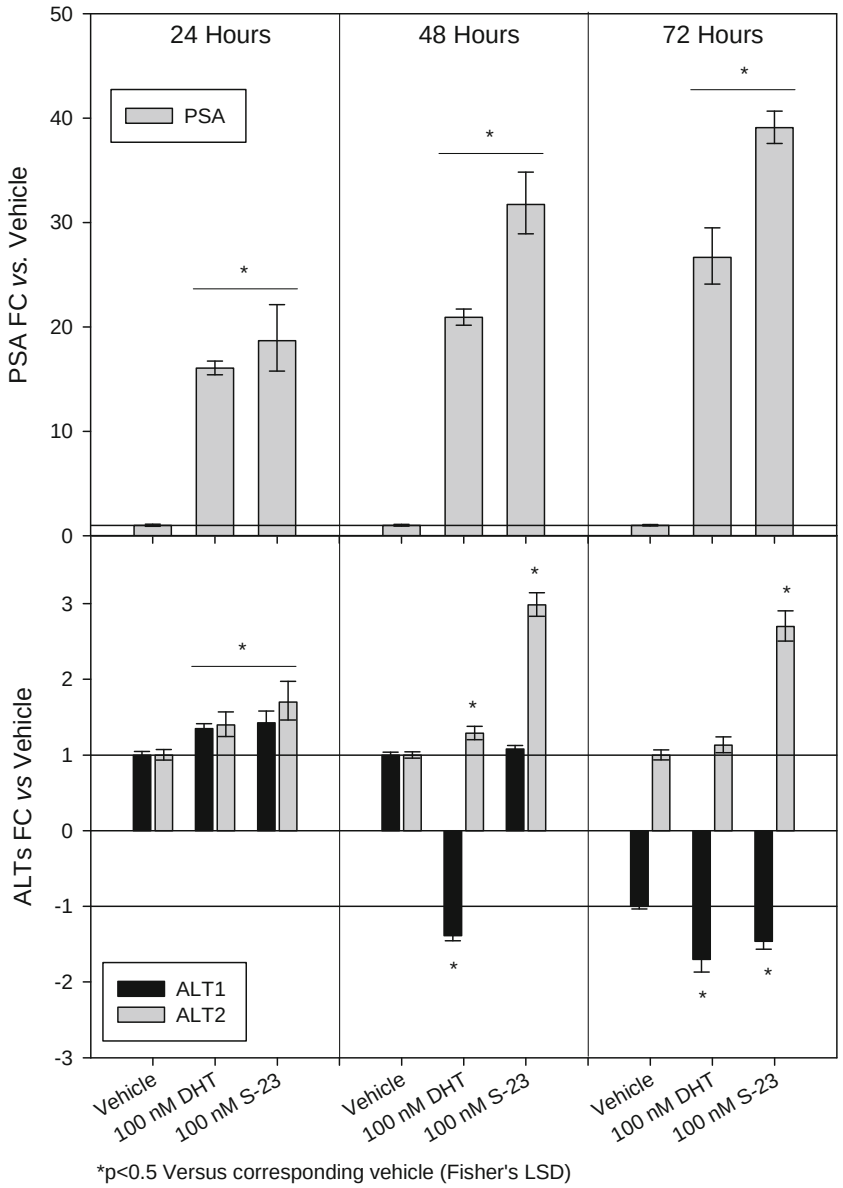

Fig. 3 LNCaP cells were "serum-starved" in media devoid of steroid hormones for 5 days to reduce basal AR signaling. Cells were then treated with 100 nM DHT or 100 nM S-23 for 24, 48, or 72 h. Following treatment qRT-PCR was performed, monitoring prostate specific antigen (PSA), ALT1 and ALT2 expression. The results are represented as mean fold change (FC) ± SD (n=3), relative to the vehicle group, as described in the methods section. * $p < .05$ *versus* corresponding vehicle (Fisher's LSD) at each time point.

tissues (35,37). Two such tissues, prostate and muscle, have well characterized androgen dependencies and were therefore chosen to study potential *in vivo* androgen-mediated regulation of the ALTs. The rat prostate undergoes drastic remodeling and atrophy in as little as 3 days following orchidectomy (ORX, data not shown). In prostate, castration caused a near 100-fold down regulation of ALT2 message when compared to intact controls (Fig. 5a). Treatment by both DHT and S-23 for 3 days in ORX animals maintained prostatic ALT2 expression at intact levels. However, neither castration alone or in combination with androgen administration had any effect on ALT1 expression. It is possible that increases in the ratio of prostate stroma to epithelium known to occur following orchidectomy contributed to changes in ALT2 expression as these cell types were not considered separately (13). However, treatment with both ligands maintained ALT2 message at intact levels but only DHT treated animals maintained prostate mass suggesting that organ remodeling alone cannot explain the reported changes in prostatic ALT2 expression (Fig. 5c). Amongst skeletal muscles, AR expression is most prevalent in the levator ani. As such this tissue is sensitive to androgen treatment (14,15). In levator ani, 3 days after castration a smaller 4-fold reduction of ALT2 expression was detected (Fig. 5b). Interestingly, DHT and S-23 treatment not only maintained, but led to significant increases in ALT2 mRNA expression in the levator ani as compared to intact control animals. As in prostate, no changes were seen in ALT1 expression.

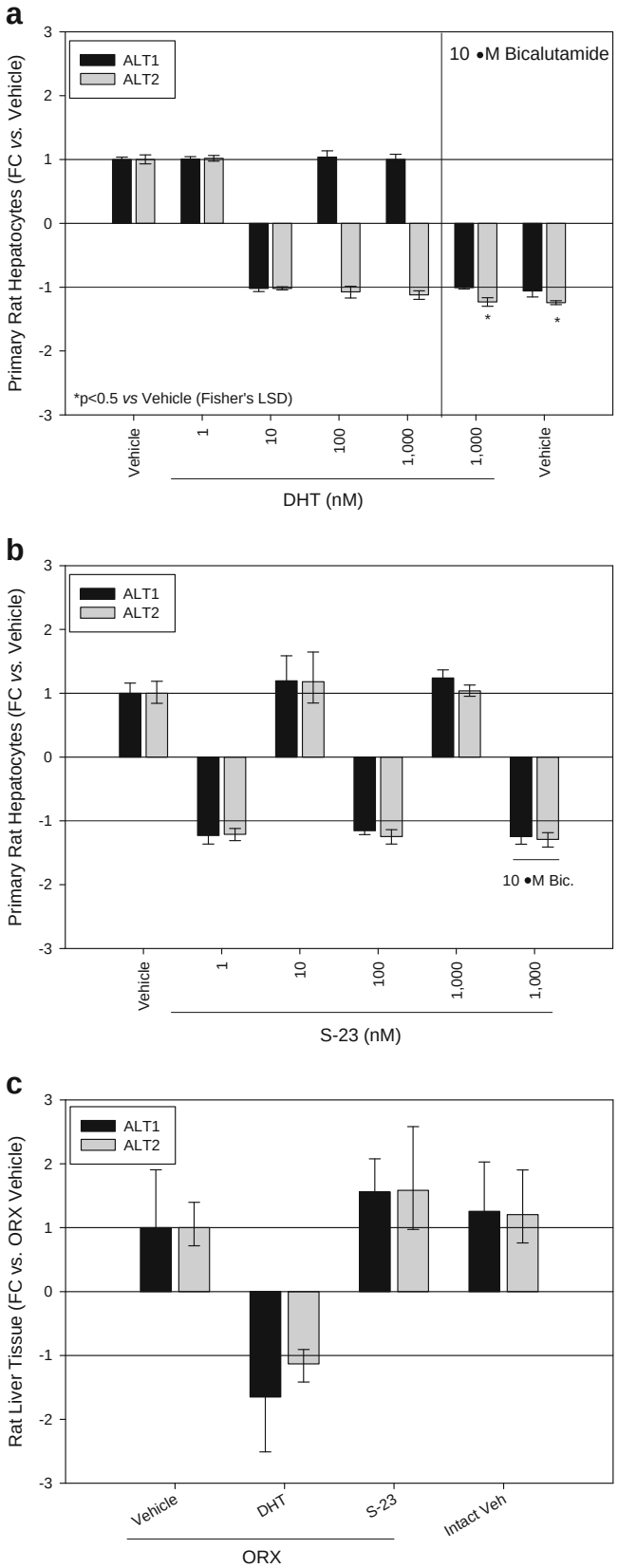

Fig. 4 (**a**) Primary male rat hepatocytes were treated with 1 – 1,000 nM DHT, 1,000 nM DHT + 10 μM bicalutamide or 10 μM bicalutamide alone for 48 h and then qRT-PCR was performed measuring ALT1 and ALT2 expression. The results are represented as mean fold change ± SD (n=3) as described in the methods section. (**b**) Primary male rat hepatocytes were treated with 1 – 1,000 nM S-23 or 1,000 nM S-23 + 10 μM bicalutamide for 48 h and analysis performed as in (**a**). (**c**) Twelve-week-old surgically castrated rats were treated subcutaneously with 5 mg/day DHT, S-23, or an equal volume of vehicle for 3 days. The intact group was sham operated and treated with vehicle alone. Within 8 h of the final dose animals were sacrificed and tissue flash frozen for subsequent qRT-PCR analysis. The results are represented as mean fold change ± SD (n=5), relative to the ORX vehicle group, as described in the methods section.

DISCUSSION

Numerous studies have shown increases in serum ALT activity following androgen administration (9,11,30,34). Elevated serum ALT activity is considered a surrogate marker of liver toxicity and thought to occur when necrotic liver tissue leaks enzyme into circulation. To date very little work has been done to characterize the actual mechanisms leading to increased serum ALT activity, while at the same time its utility as a stand alone marker in diagnosing liver damage is increasingly disputed (10,24). Recent work by Yang *et al.* in rat demonstrated that both ALT1 and ALT2 proteins are present in liver and serum, and their protein content in serum increases when liver damage occurs (37). Interestingly, the principal serum ALT both before and after liver damage was ALT1. Yang *et al.* further reported that ALT1 serum protein levels were also better correlated with serum ALT activity, suggesting that ALT1 is primarily responsible for increased serum ALT activity in response to liver damage. Conversely, elevations in serum ALT activity due primarily to ALT2 induction may not reflect the occurrence of hepatoxicity.

In this report we present clear evidence that ALT2 expression is regulated in non-hepatic tissues by androgens. Several conserved AREs in the genomic regulatory region of human ALT2 suggested the potential for direct regulation by AR which was then confirmed by transcriptional activation assays. Androgen-mediated regulation in an endogenous cellular context was confirmed in LNCaP though its nature, direct or indirect, was not evaluated. Further evidence of *in vivo* regulation was detected in prostate and levator ani muscle tissues, where castration suppressed ALT2 expression that was restored by androgen treatment. DHT and S-23, two structurally distinct AR agonists, demonstrated similar stimulation of ALT2 expression *in vivo* further supporting that this effect is AR mediated. The magnitude of

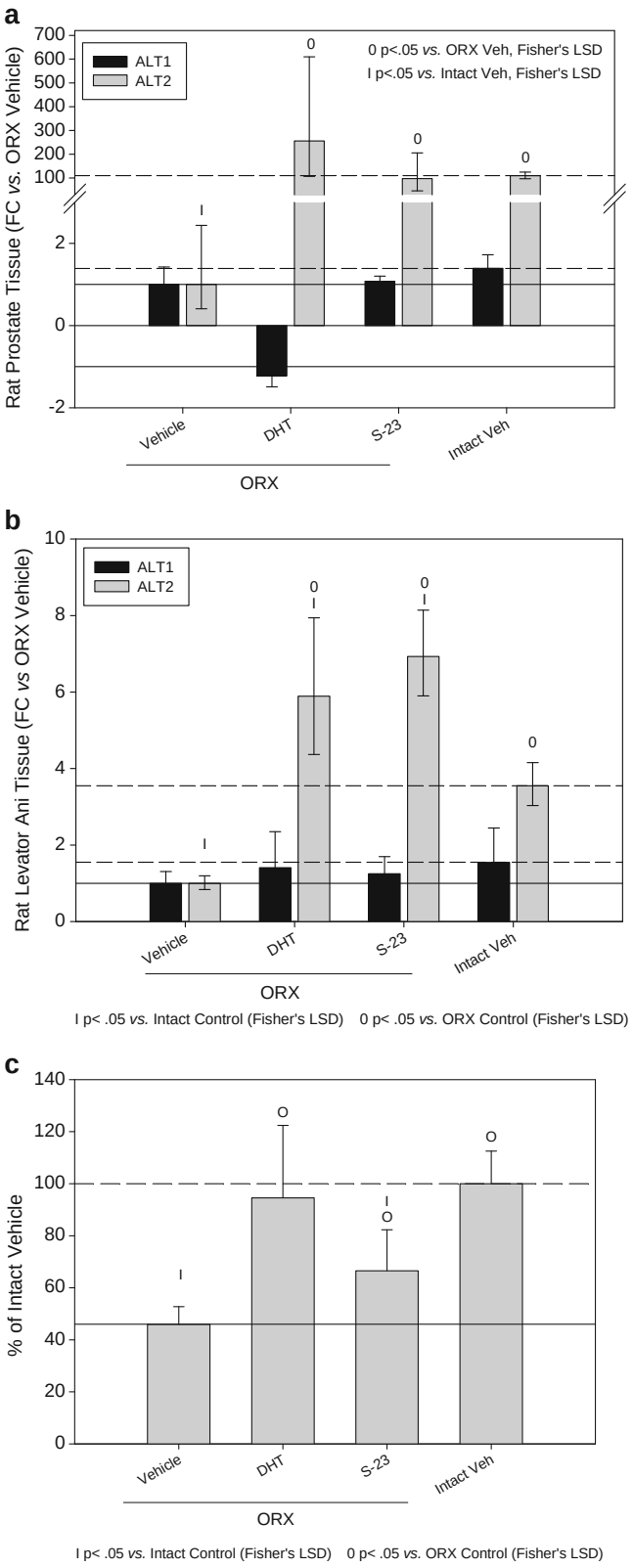

Fig. 5 Androgen regulation of ALT1 and ALT2 in prostate and muscle. Twelve-week-old surgically castrated rats were treated subcutaneously with 5 mg/day DHT, S-23, or an equal volume of vehicle for 3 days. The intact group was sham operated and treated with vehicle alone. Within 8 h of the final dose animals were sacrificed and tissue flash frozen for subsequent qRT-PCR analysis. The results are represented as mean fold change (FC) ± SD ($n=5$), relative to the ORX vehicle group, as described in the methods section. 0 $p < .05$ *vs.* ORX vehicle, I $p < .05$ *vs.* Intact vehicle (Fisher's LSD) (**a**) Prostate tissue ALT1 and ALT2 expression. (**b**) Levator ani muscle tissue ALT1 and ALT2 expression. (**c**) Prostate Weights.

ALT2 regulation trended with AR expression except in hepatic tissues. ALT2 regulation was observed in levator ani despite the relatively scant AR expression in this tissue. This suggests AR expression was not limiting in hepatic ALT2 regulation. The biological significance of extra-hepatic ALT2 regulation remains an open question. Recent evidence that ALT2 is a mitochondrial protein, primarily expressed in muscle, suggests its physiological function maybe distinct from ALT1 but more in-depth study is required to further understand differences in the isoenzymes' biological roles (37).

Discovery of several conserved AREs in the promoter region of ALT1 suggested a similar capacity for direct AR regulation though only near background levels of transcriptional activation were detected. Likewise ALT1 showed only very moderate regulation in prostate cancer cells and no regulation in prostate or levator ani muscle. Neither isoenzyme's expression was significantly affected by androgens in either primary hepatocytes or liver tissue. In the castrated rat study, both DHT and S-23 were administered subcutaneously reducing the amount of drug reaching the liver when compared to an equivalent oral dose. Therefore hepatic ALT regulation following oral androgen administration has not been excluded.

Consistent increases in serum ALT activity were not observed in this experimental paradigm (data not shown). While the administered doses likely fall in the supra-physiological range, 3 days may have been insufficient time to affect serum activity as other studies have shown (34). Unfortunately ALT protein content was not examined in this study due to the lack of readily available immunological methods, though studies where both ALT mRNA and protein were assayed showed a strong correlation (37). Further studies monitoring ALT isoenzyme tissue and serum contributions following androgen administration preclude the establishment of a firm link between tissue expression and serum protein content. Nevertheless, our data support the potential contribution of AR action in non-hepatic tissues, as opposed to xenobiotic toxicity alone, to increases in serum ALT activity following androgen administration.

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