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The Nongenotropic Synthetic Ligand 4-Estren-3α17β-diol Is a High-Affinity Genotropic Androgen Receptor Agonist

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ABSTRACT

The nongenotropic ligand estren (*Science* **298:**843–846, 2002) was evaluated for its transcriptional activity mediated by the human androgen receptor (AR). Our results show that estren can bind, translocate, transactivate, and regulate two known target genes of AR in androgen-responsive cell lines. Estren binds recombinant AR with 10-fold higher affinity than either estrogen receptor (ER)-α or ERβ. Estren-bound AR can translocate AR to the nucleus and stimulate the androgen response element-luciferase reporter activity with an efficacy similar to that of androgen. Estren also increased the expression of prostate-specific antigen (PSA) in a dose-dependent manner in human LnCaP cells. Using chromatin immunoprecipitation analysis, we show that the estren-bound AR coimmunoprecipitates with a region of the PSA gene promoter. Therefore, cotreatment with an AR antagonist, bicalutamide, blocked the estren-induced increase in PSA expression. In contrast, phosphoinositol 3-kinase inhibitor wortmannin, or extracellular signal-regulated kinase inhibitor 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophynyltio)butadiene (U0126), and ER antagonist ICI-182780 failed to block the effects of estren. In vivo analysis of estren's action on male-orchidectomized ICR mice revealed estren's AR agonist actions on the levator ani and seminal vesicle target tissues. Taken together, our results reveal the hitherto unidentified genotropic action of estren mediated by AR in androgen-responsive cells and tissues.

The synthetic ligand estren activates the estrogen receptor (ERα and ERβ)/androgen receptor (AR) via nongenotropic pathways, relying primarily on protein-protein interactions with members of the ERK (Kousteni et al., 2001, 2002, 2003) and PI3K (Simoncini et al., 2000; Kousteni et al., 2003) enzyme complex. These studies provided an exciting opportunity to obviate the risk associated with the uterotrophic and mammary gland-stimulating activities typically associated with the genotropic pathways induced by ERα (Harris et al., 2002; Frasor et al., 2003) while continuing to protect against ovariectomy-induced bone loss. Thus, the prototypical molecule estren displays a profile that is unique from selective estrogen receptor modulators and estrogen because it harbors no uterotrophic activity in vivo and reverses ovariectomy-induced bone loss. It is interesting that the effect of estren on bone mineral density is distinct from both estrogen and selective estrogen receptor modulators when evaluating the cortical bone changes induced by these treatments (Kousteni et al., 2002). This prompted us to examine the potential interactions between estren and AR because androgens have been reported to possess cortical bone formation, enhancing activity in vivo (Turner et al., 1990). Hence, we evaluated the role of AR in mediating the biological action of estren. In this study, we show that estren can bind, translocate, transactivate, and induce endogenous target genes of AR in two distinct androgen-responsive cell types using the conventional genotropic signaling. These findings add a layer of complexity to this ligand previously claimed to exclusively work via nongenotropic pathways (Kousteni et al., 2003).

Materials and Methods

All chemicals used were purchased from Sigma-Aldrich (St. Louis, MO) except for estren (Steraloids, Newport, RI) or as otherwise indicated.

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ABBREVIATIONS: ER, estrogen receptor; ARE, androgen response element; Ab, antibody; AR, androgen receptor; ChIP, chromatin immunoprecipitation; PSA, prostate-specific antigen; ERK, extracellular signal-regulated kinase; PI3K, phosphoinositol 3-kinase; DAPI, 4,6-diamidino-2-phenylindole; ELISA, enzyme-linked immunosorbent assay; DMEM, Dulbecco's modified Eagle's medium; ORX, orchidectomized; IGF, insulin-like growth factor; LA, levator ani; LH, luteinizing hormone; R1881, methyl trienolone, a synthetic stable androgen; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophynyltio)butadiene; ICI-182780, Faslodex.

Receptor Binding and Cotransfection Assay. K_i values were determined using competitive binding assays. [^{3}H]17β-estradiol- and baculovirus-expressed ERα or ERβ receptors were used for binding to ER receptor. For AR binding assays, [^{3}H]methyltrienolone (R1881 or androgen) and extracts from 293 cells overexpressing human AR receptor were used.

C2C12 cells (50,000 cells/well) were transiently transfected with a plasmid expressing human AR (1.6 μg/ml) as well as a plasmid with 3× androgen response element (ARE)-luciferase (5 μg/ml). Results are expressed as relative efficacy compared with 100 nM R1881.

Immunofluorescence Assay. LA20 cells were treated for various times with 10 nM estren or 10 nM androgen, fixed (4% paraformaldehyde), and permeabilized (1% Triton X-100), followed by incubation with an anti-AR rabbit polyclonal Ab (PG-21) 1:100 dilution or IgG 1:100 dilution, followed by a secondary fluorescein isothiocyanate-labeled anti-rabbit Ab (1:200 dilution). Cells were visualized using confocal microscopy (confocal system, Bio-Rad MRC 1024-UV, Bio-Rad, Hercules, CA; microscope, Diaphot 200, Nikon, Tokyo, Japan; 20× magnification), and nuclei were counterstained using DAPI. The increase in nuclear AR signal for each time point after the addition of ligand (standardized to DAPI) as measured by quantitative confocal microscopy (acquisition software, LaserSharp 2000; Bio-Rad) is shown in the table in Fig. 2 as the percentage of vehicle control.

LnCaP Prostate-Specific Antigen ELISA Assay. LnCaP cells were seeded into 96-well plates overnight at 4×10^4/well. Cells were treated for 48 h with various concentrations of androgen or estren. Later, 10 μl of the media from each well was assayed for prostate-specific antigen (PSA) using the TOTAL PSA Kit (Diagnostic Systems Labs, Webster, TX).

Chromatin Immunoprecipitation Assay. The chromatin immunoprecipitation (ChIP) assay was performed with slight modifications to the protocol described elsewhere (Shang et al., 2002). LNCaP cells were seeded in DMEM/F-12 media supplemented with 5% charcoal-dextran–stripped fetal bovine serum. Cells were cultivated for 2 days and stimulated twice with the appropriate ligands (once the night before, and once 2 h before cross-linking). Cross-linking was performed using 1% formaldehyde followed by incubation at 37°C for 10 min. Cells were washed twice with 1× Dulbecco's phosphate-buffered saline (Invitrogen, Carlsbad, CA) and then collected in 5 ml of Dulbecco's phosphate-buffered saline. Pellets were resuspended in 0.1 ml of lysis buffer (SDS lysis buffer) (Upstate Biotechnology, Waltham, MA) and 1× protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN), incubated, diluted properly, and sonicated three times for 20 s per set. Supernatants were collected and diluted. Anti-AR (Upstate Biotechnology) was added to a final dilution of 1:100 and rotated overnight at 4°C. Immunoclearing was carried out with 0.05 ml of sheared salmon sperm DNA and protein Sepharose A (Upstate Biotechnology) for 1 h. Sepharose beads were then washed sequentially for 3 min each with the various salts provided by the ChIP kit (Upstate Biotechnology). Beads were finally washed with an elution buffer (0.1 M $NaHCO_3$, 10% SDS) for 30 min at room temperature. Supernatants were transferred and de–cross-linked by a 1-h incubation at 65°C using the de–cross-linking buffer containing 5 M NaCl, 0.5 M EDTA, 1 M Tris-Cl, and proteinase K (Invitrogen). A standard DNA precipitation method was used, and pellets were resuspended in 0.050 ml of water. Polymerase chain reaction parameters and primers were used according to the procedures followed by Shang et al. (2002).

IGF-1 bDNA mRNA Analysis. Rat LA20 cells were seeded at 8000 cells/96-well in DMEM/F-12 medium (Invitrogen) with 10% charcoal-stripped FBS. Compounds in 0.1% dimethyl sulfoxide were dosed after 24 h and dissolved in 10% charcoal-stripped DMEM/F-12 media. After 72 h, the Quantigene (bDNA) assay was performed according to manufacturer's protocol (Genospectra, Inc., Fremont, CA). In brief, 50 μl of Quantigene lysis mixture is added to cells with culture media at the end of the incubation period, followed by transfer of 100 μl of this mix to capture plates containing the universal probes (Table 1). The lysis mixture contains the capture extender and label extender probes. The mixture was incubated overnight (16 h) at 56°C, followed by the addition of branch label and luminescence substrate. Later, the luminescence from each well was read using an MLX luminometer (Dynex Technologies, Inc., Chantilly, VA). Results are plotted as relative light units/8000 cells for each treatment group.

In Vivo LA Assay. All protocols pertaining to animal use and storage for experimentation were approved by the Lilly Animal Care protocol. Twelve-week-old male ICR mice were bilaterally orchidectomized (ORX) or sham-operated. At 6 weeks postsurgery, they received treatment with 10 mg/kg/day s.c. estren for 14 days (n = 8). Testosterone enanthate (TE) 2 mg/kg/day was used as positive control. One group of ORX and sham control received vehicle alone. At

TABLE 1
Probe list

Probe	Function	Sequence
rIGF001	CE	ccgaatgctggagccatagctttttctcttggaaagaaagt
rIGF002	CE	cgagctgactttgtaggcttcatttttctcttggaaagaaagt
rIGF003	CE	gctgggcccggatggaatttttctcttggaaagaaagt
rIGF004	CE	tttctgttcctcgggaggctttttctcttggaaagaaagt
rIGF005	CE	caggtgttccgatgttttgcatttttctcttggaaagaaagt
rIGF006	CE	ggtattgaactcattattgatatttggtttttctcttggaaagaaagt
rIGF007	LE	tctggtccggctgtggctttttaggcataggacccgtgtct
rIGF008	LE	cagccccgcaaagggtctttttaggcataggacccgtgtct
rIGF009	LE	gcccgtctgtggtgccctttttaggcataggacccgtgtct
rIGF010	LE	gcggagcacagtacatctccatttttaggcataggacccgtgtct
rIGF011	LE	ggttcctcaagcagcaaaggatttttaggcataggacccgtgtct
rIGF012	BL	ctgaagagcgtccaccagct
rIGF013	BL	ccccttggtccacacacgaa
rIGF014	BL	ctgtgggcttgttgaagtaaaag
rIGF015	BL	ggaagcaacactcatccacaat
rIGF016	BL	gcctcctcagatcacagctcc
rIGF017	BL	ttgggcatgtcagtgtggc
rIGF018	BL	tcttcaagtgtacttccttctgagtc
rIGF019	BL	ttcctgcacttcctctacttgtgt
rIGF020	BL	tcctcctacattctgtaggtcttgt
rIGF021	BL	cttgcggtgacgtggcat
rIGF022	BL	gggaaatgcccatctctgaaat
rIGF023	BL	ggaatgtttacttgtgtatttcattga
rIGF024	BL	ttaacaaacactcctaaagacaatgtc

CE, capture extender; LE, label extender; BL, blocker probe.

the end of the treatment period, the levator ani muscle and seminal vesicles were removed and weighed. Statistical analysis was performed using Dunnett's test.

Results and Discussion

Using recombinant human AR and radiolabeled androgen ([^{3}H]methyl trienolone) in a competitive binding assay, we measured direct binding of estren to AR. Our results show that estren binds to AR (K_i = 27 nM) and transactivates (EC_{50} = 1.5 nM) a luciferase reporter linked to an ARE in C2C12 cells (Fig. 1A). Estren's binding affinity to recombinant human AR is approximately 10-fold higher than that to recombinant human ERα (K_i = 242 nM) or ERβ (K_i = 272 nM) (Fig. 1A, inset). Furthermore, the relative efficacy (101%) of estren compared with androgen in the ARE-dependent reporter assay shows that it is a full AR agonist. Similar results were obtained with a human prostate cancer cell line PC-3 cotransfection assay (data not shown).

The hallmark of conventional type II genotropic ligands is the translocation of the receptor from the cytoplasm to the nucleus (Jenster et al., 1993). Hence, to visualize the translocation of AR protein from its cytoplasmic apo-receptor location to the estren-bound location, we performed immunofluorescent analysis using an AR-specific antibody. Our results (Fig. 1B) clearly demonstrate the nuclear translocation and accumulation of AR after 15 min of treatment with 10 nM estren. This translocation of AR-bound estren is further evident after 6 h and is similar to that of androgen. In contrast, treatment with E2 failed to elicit this effect on AR translocation (data not shown). Furthermore, after 24 h of treatment with estren or androgen, we observed an increase (3-fold) in nuclear AR levels using quantitative confocal microscopy (Fig. 1B). Taken together, these results show that estren behaves like a classic genotropic ligand of AR.

To extend these observations to endogenous target genes of genotropic androgen signaling, we used the LnCaP androgen-responsive human prostate cancer cell line. We measured PSA in the medium of cells treated with various concentrations of estren for 48 h. PSA is a genotropic target of AR activity, and its promoter elements responding to liganded AR have been studied extensively (Luke and Coffey, 1994; Cleutjens et al., 1996). Results from these experiments (Fig. 2A) clearly show the potent and efficacious AR agonist activity of estren relative to androgen (relative efficacy = 90%, EC_{50} = 0.4 nM). Furthermore, this increase in PSA levels was completely blocked by the AR antagonist bicalutamide (Fig. 2C). Reversal of estren action by AR antagonist is also evident in the ARE reporter assay (data not shown). However, cotreatment with the ERK inhibitor U0126 (Fig. 2C) or PI3K inhibitor wortmannin (Fig. 3C) failed to reduce this activity. The ERK and PI3K pathways were reported as prominent mediators of estren's nongenotropic activity (Kousteni et al., 2003). In addition, cotreatment with ICI-182780, the "pure" ERα/ERβ antagonist, also failed to affect the estren-induced increase in PSA (Fig. 2C). Hence, the lack of reduction after cotreatment with either the ERK or PI3K inhibitor of estren-induced PSA levels is indicative of the absence of nongenotropic signaling of estren in stimulating PSA levels in LnCaP cells. To ascertain that the estren-induced expression of PSA is indeed mediated by a genotropic mechanism, we used the ChIP assay described previously (Shang et al., 2002). The clear recruitment of estren-bound AR to the promoter of the PSA gene is comparable with R1881-bound AR and is indicative of a classic transcriptional regulation of PSA gene expression by AR (Fig. 2D) and confirms the genotropic action of estren on a target gene of AR.

We extended these observations to another target gene of AR by evaluating changes in IGF-1 mRNA after treatment of LA20 muscle cells with estren and androgen. IGF-1 is a reliable target gene of AR in the LA20 cell line (K. Chen, T. Moore, unpublished results). The LA20 cell line is derived from the rat levator ani (LA) and is a highly responsive target tissue of androgen (Nnodim, 2001). Our results show that estren can induce the expression of IGF-1 mRNA in a dose-dependent manner in LA20 cells with a relative efficacy comparable with that of androgen (Fig. 2B). To study the role of nongenotropic signaling in estren-induced IGF-1 stimula-

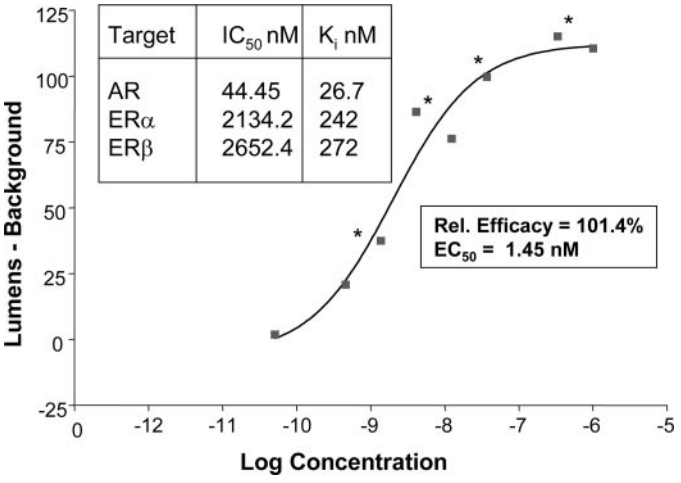
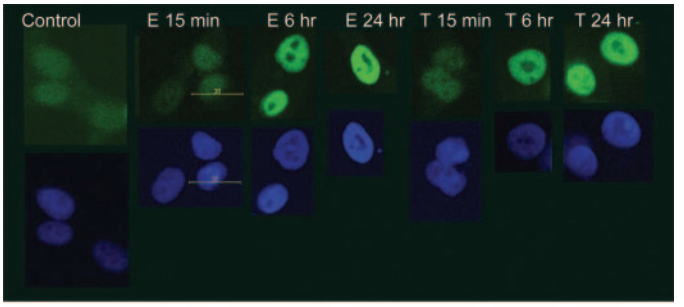


Fig. 1. Receptor activation (EC_{50}) was determined in C2C12 cells transiently transfected with a plasmid expressing AR as well as a plasmid with ARE-luciferase. In addition, percentage efficacy was determined versus activity of 100 nM methyltrienolone. Inset, K_i values were determined in competitive binding assays. [^{3}H]17β-estradiol- and baculovirus-expressed ERα or ERβ receptors were used for binding to ER receptor. For AR binding assays, [^{3}H]methyltrienolone and extracts from 293 cells overexpressing AR receptor were used.



Time	Estren	T
15 min	104%	106%
6 hr	202%	220%
24 hr	320%	295%

Fig. 2. LA20 cells were treated for various times with 10 nM estren (E) or 10 nM R1881 (T) and subjected to immunofluorescence analysis using a rabbit polyclonal Ab (PG-21) 1:100 dilution or IgG 1:100 dilution, followed by a secondary fluorescein isothiocyanate-labeled anti-rabbit Ab (1:200 dilution). Cells were visualized using confocal microscopy, and nuclei were counterstained using DAPI. The increase in AR signal (standardized to DAPI) as measured by quantitative confocal is shown in the table as the percentage of vehicle control.

tion, we used the identical set of ERK and PI3K inhibitors along with estren in LA20 cells. As observed in the LnCaP PSA assay, the inhibitors had no effect on estren-induced IGF-1 stimulation, thus ruling out the proposed nongenotropic signaling on this target gene (data not shown).

Finally, to evaluate the role of AR in mediating some of estren's in vivo activity, we induced LA muscle atrophy (Krieg, 1976; Nnodim, 2001) by orchidectomy in weight-matched male ICR mice (Fig. 4). The LA muscle and the seminal vesicles are primary targets of AR-mediated genotropic signaling in male mice (Krieg, 1976). Orchidectomy-induced atrophy of the LA muscle was reversed by TE or estren after 2 weeks (Fig. 3A). Similar results were seen with changes in seminal vesicles in these animals after 2 weeks of treatment with estren (Fig. 3B). Taken together, these results point to potent and efficacious androgenic activity in vivo in male ICR mice that is consistent with the genotropic AR activity in vitro.

In addition, treatment of ovariectomized female SD rats with estren results in a suppression of the luteinizing hormone (LH) levels that are greatly induced as a result of ovariectomy (data not shown). We also found that in the previously reported morphine withdrawal-induced hot-flush model (Simpkins et al., 1983), estren fails to produce any of the hot-flush ameliorating effects of estrogen, despite lowering LH levels at these doses (data not shown). Earlier reports have clearly demonstrated that genotropic AR ligands such as dihydrotestosterone fail to ameliorate the hot-flush endpoint in this model despite lowering LH levels (Panidis et al., 1994; Merchenthaler et al., 1998). Hence, we believe that this unique profile of estren in both of these in vivo experiments using female SD rats is primarily attributed to its androgen-like properties found in the previous male ICR mice experiment.

In conclusion, estren was found to be a potent AR ligand using recombinant human AR and labeled androgen in competitive AR binding assays. In addition, estren was found to translocate AR to the nucleus and possess full AR agonist activity as observed in two distinct androgen-responsive cell-based assays. It was reported recently that the conversion of estren to 19-nortestosterone was responsible for some of the androgenic effects of estren (Centrella et al., 2004). However, in our binding assays, we use purified human androgen receptor to quantify the binding affinity of the compound, and we hypothesize that in addition to metabolism to 19-nortestosterone in certain cells, estren can directly bind AR and elicit a genotropic effect. We predict that the genotropic action of estren on AR is responsible for mediating the in vivo

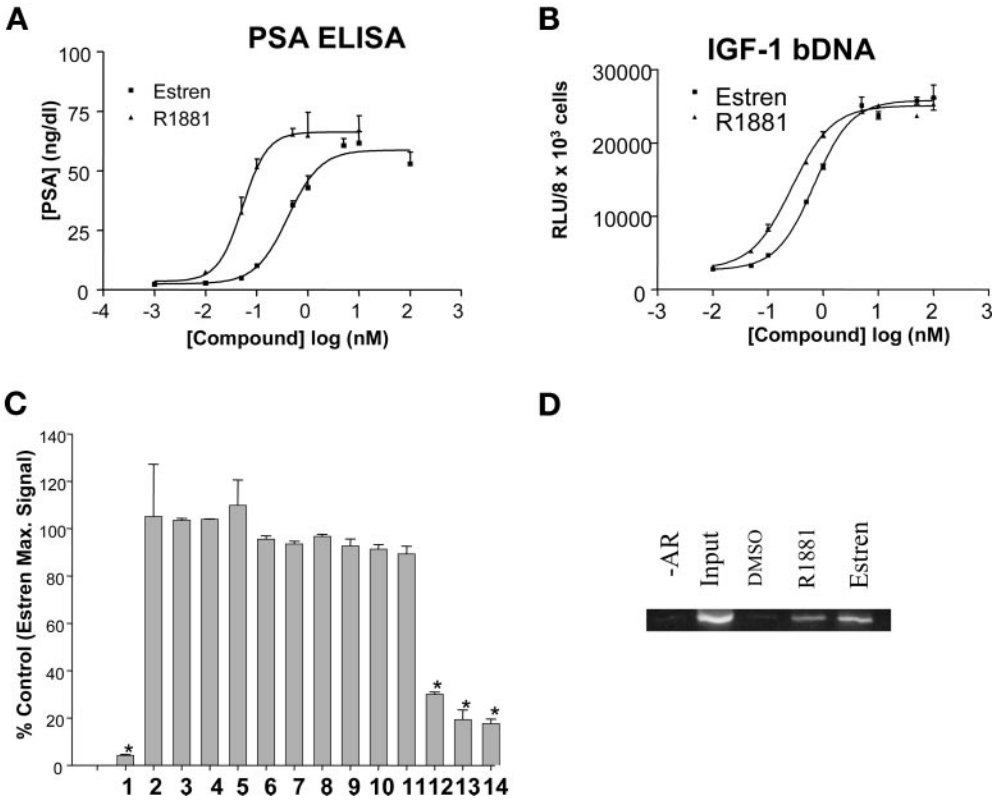


Fig. 3. A and B, LnCaP (A) or LA20 (B) cells were seeded into 96-well plates overnight at 4 × 104/well. Cells were treated for 48 h with estren or R1881 at various doses. Ten microliters of the media from each well was assayed for PSA by ELISA (Total PSA Kit, Diagnostic Systems Labs). Then, 100 ml of the LA20 lysate was used in the bDNA assay (⋆, $p < 0.05$ significantly lower than estren 10 nM control as determined by analysis of variance Fisher's post hoc test). C, LnCaP cells were seeded into 96-well plates overnight at 4 × 104/well. Cells were cotreated for 48 h with 1) dimethyl sulfoxide 0.2%, 2) R1881 1 nM, 3) estren 10 nM + 0.5 nM wortmannin, 4) estren 10 nM + wortmannin 5 nM, 5) estren 10 nM + wortmannin 50 nM, 6) estren 7.5 nM + 0.1 μM ICI-182780, 7) estren 7.5 nM + 1.0 μM ICI-182780, 8) estren 7.5 nM + 10 μM ICI-182780, 9) estren 7.5 nM + 0.1 μM U0126, 10) estren 7.5 nM + 1.0 μM U0126, 11) estren 7.5 nM + 10 μM U0126, 12) estren 7.5 nM + 1 μM bicalutamide, 13) estren 7.5 nM + 5 μM bicalutamide, and 14) estren 7.5 nM + 10 μM bicalutamide. A 5-μl sample of the media from each well was assayed for PSA by ELISA (Total PSA Kit). ⋆, $p < 0.05$ significantly lower than estren control (analysis of variance Fisher's post hoc test). D, ChIP assays of AR occupancy of the promoter region of the PSA gene. LnCaP cells were treated with 10 nM R1881, estren, and an equivalent of vehicle alone. Soluble chromatin was prepared and formaldehyde cross-linked. Antibodies raised against AR were used to immunoprecipitate protein-bound DNA fragments. Fragments shown were amplified using polymerase chain reaction methods.

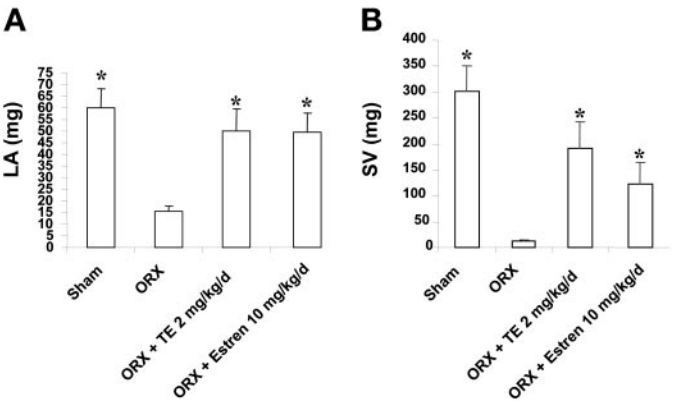


Fig. 4. Eight-week-old male ICR mice were bilaterally ORX or sham-operated. At 6 weeks after surgery, they received treatment with and 10 mg/kg/day s.c. estren for 14 days ($n = 8$). TE (2 mg/kg/day) was used as positive control. One group of ORX and sham control received vehicle alone. At the end of the treatment period, blood samples were collected and the levator ani muscle (A) and seminal vesicles (B) were removed and weighed. ⋆, $p < 0.05$ compared with ORX control (Dunnett's test).

and in vitro effects of estren in both female SD rats and male ICR mice. Future use of this class of ligands for therapeutic intervention in treatment of osteoporosis via its nongenotropic action on ERα/ERβ must factor in the potential involvement of the genotropic AR function described in this study.

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