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Stimulation of lysyl oxidase (EC 1.4.3.13) activity by testosterone and characterization of androgen receptors in cultured calf aorta smooth-muscle cells

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Previous studies have indicated a greater incidence of atherosclerotic cardiovascular disease in men than in women of child-bearing age, suggesting that vascular interactions with sex steroids may effect pathogenesis in these cases. In the present study, it was found that the presence of 10–100 nM-testosterone in the growth medium of calf aortic smooth-muscle cells in culture stimulates lysyl oxidase activity approx. 2.5-fold in the medium and 5.5-fold in the fraction bound to the cell layer. Androgen receptors were identified in these cultured smooth-muscle cells, and their properties were very similar to those in the cytosolic fraction of whole bovine aortic tissue. These receptors appeared to be specific for androgen, of high affinity (K_d = 0.4 nM) and of low capacity (9000 sites/cell). The present results indicate that the aortic smooth-muscle cell is a cellular target for androgens, and thus raise the possibility that the development of fibrotic arterial lesions involving the deposition of excess collagen may in part be regulated by androgen-mediated stimulation of collagen cross-linkage formation as catalysed by lysyl oxidase.

INTRODUCTION

Although sex-related differences in the incidence of coronary heart disease are well documented, the physiological mechanisms underlying such differences remain poorly understood (McGill & Stern, 1979). Specific androgen- or oestrogen-induced changes in cardiovascular tissues which have been described include alterations in blood-vessel wall morphology and changes in the metabolism of collagen and elastin (Wolinsky, 1972; Fischer & Swain, 1978) and in cholesterol esterase activity (Tomita *et al.*, 1982) in rat aorta.

The effects of steroid hormones on the growth and function of target cells in cardiovascular tissue are likely to be mediated by steroid-specific intracellular receptors in a manner similar to the known mechanism of action of steroids in other target tissues. Indeed, receptors for oestrogens, androgens, progestin and glucocorticoid have been demonstrated in arterial tissues of various animal species (McGill & Sheridan, 1981; Horwitz & Horwitz, 1982; Lin *et al.*, 1982, 1985, 1986; Lin & Shain, 1985).

Nevertheless, a specific receptor-mediated biochemical effect of sex-steroid hormones directly on vascular tissue has yet to be demonstrated. The present study was undertaken to investigate the effects of androgens on the activity of lysyl oxidase (protein-lysine 6-oxidase, EC 1.4.3.13) in arterial cells. Lysyl oxidase is the only enzyme known to be required for the formation of lysine-derived cross-links in both collagen and elastin (Eyre *et al.*, 1984; Kagan, 1986). We have used bovine aortic smooth-muscle cells in culture for these purposes, since previous studies have indicated that oestrogens alter collagen metabolism in these cultured cells (Beldekas *et al.*, 1981), and in consideration of the fact that the investigation of the effect of sex hormones in cell culture avoids complexities inherent to animal models. Thus we have characterized androgen receptors in cultured bovine aortic smooth-muscle cells as well as in the cytosol of bovine aorta, in order to correlate receptor binding with androgen-mediated biological activity. A preliminary report of these studies has been presented (Bronson *et al.*, 1986).

MATERIALS AND METHODS

Chemicals

[17α-*methyl*-^{3}H]Mibolerone (7α,17α-dimethyl-19-nortestosterone) (82–88 Ci/mmol) and L-[4,5-^{3}H]lysine were obtained from Amersham, Arlington Heights, IL, U.S.A. Methyltrienolone (R1881) was obtained from New England Nuclear Corp., Boston, MA, U.S.A. Other unlabelled steroids were purchased from Steraloids, Wilton, NH, U.S.A. Fetal bovine serum was purchased from Biofluids, Rockville, MD, U.S.A. Media and reagents used for cell-culture purposes were obtained from Grand Island Biologicals, Grand Island, NY, U.S.A.

Bovine aortic tissue

Aortic arches of 2–3-week-old male calves were obtained from a local slaughterhouse. The aortas were excised immediately after slaughter, placed in tissue-culture medium maintained at 0 °C on ice, cleaned of fat and mesenteric tissue and transported to the laboratory. The tissues were used fresh for binding studies *in situ* and for preparation of cultured smooth-muscle cells.

Abbreviations used: DHT, dihydrotestosterone; DMNT, 7α,17α-dimethyl-19-nortestosterone; Tes/GME buffer, 0.02 M-Tes/10% (v/v) glycerol/10 mM-sodium molybdate/1.5 mM-sodium EDTA, pH 7.2, at 2 °C.

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Preparation of bovine aortic cytosol and nuclear fractions

Cytosolic and nuclear fractions were prepared as described previously for calf uterine tissue (Muller *et al.*, 1982; Traish *et al.*, 1986). Briefly, aortic tissue slices or tissue powder (1 g/3 ml) were homogenized at 0–2 °C in Tes/GME buffer. Monothioglycerol (10 mM), leupeptin (1 mM) and phenylmethanesulphonyl fluoride (0.5 mM) were included in the homogenization buffer just before use. The homogenate was centrifuged at 800 *g* for 10 min to isolate the crude nuclear myofibrillar fraction. The supernatant was centrifuged at 100000 *g* for 30 min at 2 °C to obtain the high-speed supernatant (cytosol fractions). The nuclear pellet was washed three times by resuspension in buffer and centrifugation. The pellet was then resuspended by homogenization in the same buffer and used for binding assays.

Binding of [^{3}H]DMNT to bovine aortic cytosol

To determine the optimal time required for [^{3}H]DMNT binding to reach equilibrium, 0.1–0.2 ml portions of cytosol were incubated in triplicate at 0 °C for various time periods with 2 nM-[^{3}H]DMNT in the absence (total binding) or presence (non-specific binding) of a 1000-fold molar excess of unlabelled DHT. Protein-bound radioactivity was determined by an hydroxyapatite adsorption assay as described (Traish *et al.*, 1984, 1985). Specific binding was calculated from the difference between total binding and non-specific binding. These preliminary experiments indicated that a time of 16–20 h was required for [^{3}H]DMNT to saturate unoccupied androgen-binding sites in bovine aorta cytosol. To determine the equilibrium binding constants for [^{3}H]DMNT interaction with bovine aorta androgen receptors, portions of cytosol were incubated at 0 °C for 20 h with increasing concentrations of [^{3}H]DMNT (0.1–5.0 nM) in the absence (total binding) or presence (non-specific binding) of a 1000-fold molar excess of unlabelled DHT. At the end of the incubation, protein-bound radioactivity was assayed by the hydroxyapatite adsorption technique (Traish *et al.*, 1984), and the specific binding data were treated as described by Scatchard (1949).

Sucrose-density-gradient analysis of bovine aortic androgen receptors

Linear 5–20 % sucrose density gradients were prepared in Tes/GME buffer with a Beckman gradient former, as previously described (Traish *et al.*, 1985). Portions of [^{3}H]DMNT-labelled cytosol were treated with dextran-coated-charcoal pellets (Traish *et al.*, 1985) for 20 min at 0 °C to remove free and loosely bound steroid, and then centrifuged at 1000 *g* for 10 min at 2 °C. Samples (0.3 ml) of the dextran-coated-charcoal-treated cytosols were layered on each gradient. ^{14}C-labelled γ-globulin (7 S) and bovine serum albumin (4.6 S) were included in each gradient as internal sedimentation markers. The gradients were centrifuged at 300000 *g* for 20 h at 0 °C in a Beckman SW60 rotor. The gradients were fractionated into individual 0.1 ml fractions, and radioactivity of each was quantified in 4 ml of Liquiscent scintillation fluid (National Diagnostic, Somerville, NJ, U.S.A.).

Analysis of bovine aortic androgen receptors by gel filtration on Sephacryl S-300

Sephacryl S-300 (Pharmacia, Piscataway, NJ, U.S.A.) was packed in a 2.6 cm × 80 cm column, which was then equilibrated with Tes/GME buffer containing 0.4 M-KCl, pH 7.3, at 2 °C. The column was calibrated with Blue Dextran 2000 (V_0), ^{14}C-labelled γ-globulin (5.2 nm), bovine serum albumin (3.55 nm), ovalbumin (2.8 nm) and glycine (V_t), as described by Traish *et al.* (1985).

[^{3}H]DMNT-labelled cytosols were treated with dextran-coated charcoal pellets, and samples (3 ml) were individually applied to the Sephacryl S-300 column and eluted with Tes/GME buffer containing 0.4 M-KCl at a flow rate of 18 ml/h. Fractions (3.2 ml) were collected, and radioactivity in 1 ml of each fraction was counted.

Primary cell cultures

Cultures of bovine aortic smooth-muscle cells were established as described by Beldekas *et al.* (1981). Explants of calf aortic medial segments were incubated for 7 days in an atmosphere of O_2/CO_2 (19:1) in 10 ml of Dulbecco & Vogt (1954) medium supplemented with 3.7 g of $NaHCO_3$/l, 10% fetal bovine serum, 1% non-essential amino acids, 1.0 mM-sodium pyruvate, 20 mM-Hepes, pH 7.4, and 100 units each of penicillin and streptomycin sulphate/ml, in 75 cm^2 plastic flasks. Conditioned medium was replaced with 10 ml of the same fresh medium once per week. Cultures were subcultivated into first passage after 3 weeks of explant growth. For this purpose, the conditioned medium was removed and the cells were freed from the matrix by incubation of each cell layer with 1.5 ml of 0.05% trypsin/0.02% EDTA for 10 min before transfer. Cells cultured in first passage reached confluency by day 6 and were then subcultivated into second passage by seeding at a density of 5×10^5 cells in each 25 cm^2 flask. Cells in second passage were cultured in 5 ml (per flask) of Dulbecco & Vogt (1954) medium supplemented as described above and further supplemented with 50 μg of sodium L-ascorbate/ml. The medium was changed twice during each week of culture. The cells reached confluency between 3 and 4 days in second passage.

Treatment of cell cultures with androgen

The effect of testosterone was assessed in smooth-muscle cell cultures which had been incubated to day 4 in second passage as described above and which then received fresh 5 ml portions of medium per 25 cm^2 flask, supplemented with 1 μl of stock solutions of testosterone in ethanol so that the final concentrations of the androgen ranged from 10 to 100 nM, as specified. Control cultures received equal quantities of ethanol. Fetal bovine serum, present at 10% (v/v) in testosterone-treated or control cultures, was freed of endogenous steroids before use by treatment with charcoal, followed by aseptic filtration through filters (0.2 μm pore size) in accordance with a published procedure (Raaka & Samuels, 1983). Cultures received fresh androgen-supplemented or control medium twice per week of subsequent incubation. The rate of change in cell numbers occurring between days 4 and 12 of second passage was not altered from the control by the presence of 10 nM-testosterone during this time period. The medium from sets of four flasks for each control or testosterone-treated condition was then changed on day 12 to fresh 2.5 ml portions of the appropriate control or steroid-supplemented medium, and incubation was continued for 24 h. The 24 h-conditioned medium was decanted and immediately supplemented with 0.1 mM-

phenylmethanesulphonyl fluoride as an inhibitor of serine proteinases and stored frozen at −80 °C until assays were performed. The cell layers were rinsed *in situ* three times with physiological saline (0.9% NaCl), suspended in 2 ml of 4 M-urea/0.016 M-potassium phosphate (pH 7.7) per cm² flask, and then manually and intermittently homogenized in this urea/phosphate buffer at 4 °C in a ground-glass conical homogenization tube for 30 min. The homogenates were centrifuged at 12000 *g* for 10 min, and the supernatants were dialysed against 0.1 M-sodium borate/0.15 M-NaCl, pH 8.0, at 4 °C.

Enzyme assays

Lysyl oxidase activities were determined against a [^{3}H]elastin substrate prepared from 16-day chick-embryo aortae which had been pulsed in organ culture with L-[4,5-^{3}H]lysine as described by Kagan & Sullivan (1982). Enzyme assays included 125000 c.p.m. of the saline-insoluble aortic [^{3}H]elastin, 0.5 ml of conditioned medium or dialysed cell-layer extract as the enzyme source, and 0.1 M-sodium borate/0.15 M-NaCl, pH 8.0, in a final volume of 0.75 ml. Assays were incubated at 37 °C for 4 h and then distilled *in vacuo* to isolate ^3H_2O, the radioactivity of which was then quantified by liquid-scintillation spectrometry. All assays were corrected for background rates of ^{3}H release in the absence of enzyme, and all activities reported were fully inhibitable by the presence of 50 μM-β-aminopropionitrile in the assays. Enzyme-dependent ^{3}H release was shown to be linear with time for the duration of the 4 h assay period. Enzyme activities were assayed in duplicate for each of two flasks of cells for each growth condition assessed. DNA was determined by the method of Burton (1956).

Pulsing with [^{35}S]methionine

Cells were grown to day 13 in second passage in medium supplemented with ascorbate and 10% charcoal-stripped fetal-calf serum in the presence or absence of 0.1 μM-testosterone as described above. The medium was removed, and the cell layers were rinsed three times with 10 mM-sodium phosphate/0.15 mM-NaCl (pH 7.4) and then incubated in the presence or absence of testosterone for 1.5 h in methionine-free medium containing ascorbate and dialysed charcoal-stripped 10% fetal-calf serum. This medium was then removed and replaced with the same testosterone-supplemented or -deficient medium further supplemented with 5 μCi of [^{35}S]methionine/μl, and incubation was continued for 2 h. The medium of each flask was removed and the cell layers were rinsed in saline and homogenized in buffer composed of 0.625 M-Tris/HCl, 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, pH 6.8. These extracts were directly resolved by electrophoresis on 10% cross-linked SDS/polyacrylamide slab gel, performed by the method of Laemmli (1970). Radioactive protein bands were localized by autoradiography of the developed gels, as described by Bonner & Laskey (1974).

RESULTS

Effect of testosterone on lysyl oxidase activity of cultured calf smooth-muscle cells

Cultures of calf aortic smooth-muscle cells were maintained from day 4 until day 12 of second passage in medium supplemented with ascorbate (50 μg/ml) and 10% charcoal-stripped fetal-calf serum in the presence or absence of testosterone, with twice-weekly changes of the medium during this period, as described in the Materials and methods section. The medium was changed on day 12, incubation was continued for an additional 24 h, and the 24 h-conditioned medium and urea-soluble fractions of the cell layers were assayed for lysyl oxidase activity. As shown in Fig. 1, the activity of the enzyme is increased in both the medium and urea extract of the cell layer at both 0.01 μM- and 0.1 μM-testosterone, with increases in the medium of 2.5-fold and in the cell layer extract of 5.5-fold when 0.1 μM androgen was present during cell culture. The increased enzyme activity was essentially fully inhibited by the presence of 0.1 mM-β-aminopropionitrile in the assay, as shown in Fig. 1, consistent with the known sensitivity of lysyl oxidase to this agent. The total DNA as well as cell numbers per flask were separately determined in sets of flasks incubated in the presence or absence of testosterone from days 4 to 12 in second passage and then re-fed with fresh androgen-supplemented medium for 24 h as described above. Neither of these parameters was changed within the limits of experimental error (±5%) by incubation in the presence of testosterone, indicating that the androgen had no significant effect on the rate of growth of the cultured cells during the period of exposure as defined.

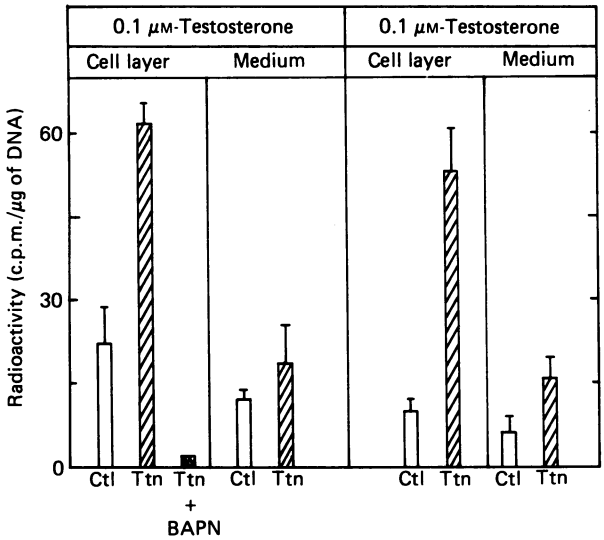

Fig. 1. Effect of the presence of testosterone in cultures of bovine aortic smooth-muscle cells on lysyl oxidase activity

Cultures were incubated in duplicate with or without the specified concentrations of testosterone, and each fraction of each flask was assayed in duplicate. Bars represent the average ± S.D. of four assays per condition. Key: Ctl, control; Ttn, grown in testosterone; TTn+BAPN, 0.1 mM-β-aminopropionitrile present during assay.

The effect of testosterone on the profile of proteins synthesized by the smooth-muscle cells was assessed by pulsing cultures, grown under the testosterone-stimulated or control conditions, with [^{35}S]methionine as described above. SDS/polyacrylamide-gel electrophoresis of extracts of the pulsed cell layers was analysed by autoradiography. The densities and number of resolvable bands in a set of three testosterone-stimulated cultures

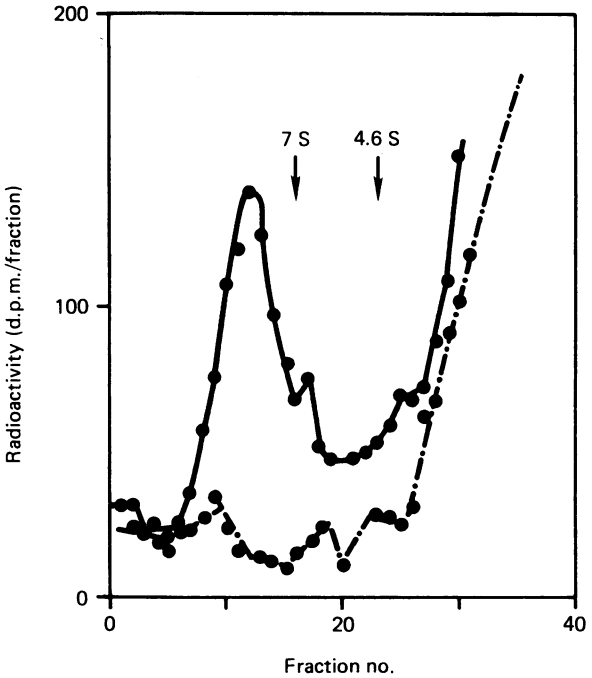

Fig. 2. Sucrose-density-gradient analysis of androgen receptors of calf aorta tissue cytosol

Calf aorta cytosol was labelled with [^{3}H]DMNT at 0 °C for 24 h in the absence (total binding; ——) or presence (non-specific binding; —•—) of a 100-fold molar excess of unlabelled DHT.

appeared to be essentially the same as those in a corresponding set of three control cultures. The total incorporation of [^{35}S]methionine into proteins was determined by isolating and counting the radioactivity of the protein fraction precipitated from the soluble extract of the cell layers with 10% (w/v) trichloroacetic acid, this analysis being performed in each of three control and three androgen-treated cultures. The testosterone-stimulated cultures contained $2.01(\pm 0.13) \times 10^5$ c.p.m./flask, and the control cultures contained $2.26(\pm 0.11) \times 10^5$ c.p.m./flask in acid-precipitable protein. These results indicate that the presence of testosterone exerted little effect on total protein synthesis in these cultured cells. The stimulation of lysyl oxidase activity is clearly not due to a general increase in protein synthesis induced by the androgen.

Characterization of androgen receptors in bovine aortic cytosol

Although aortic tissue from various animal species has been shown to contain specific steroid receptors, it has not yet been demonstrated that bovine aortic tissue or smooth-muscle cells derived from this tissue contain specific androgen receptors with characteristics similar to those isolated from other tissues. Therefore we investigated the binding characteristics of androgen receptors in bovine aortic tissue and in bovine aortic smooth-muscle cells.

Analysis of [^{3}H]DMNT-labelled bovine aortic cytosol on low-salt sucrose density gradients revealed a binding component sedimenting in the 8–9 S region of the gradient (Fig. 2). This binding is displaced with an excess of unlabelled DHT, suggesting that bovine aortic cytosol contains androgen-binding sites with sedimentation properties similar to those of androgen receptors of rat ventral prostate (Traish *et al.*, 1985) and calf seminal vesicles (A. M. Traish, unpublished work).

To characterize further the parameters for the binding of [^{3}H]DMNT to androgen receptors of bovine aortic tissue, cytosol was incubated with [^{3}H]DMNT at 0 °C for various time periods. [^{3}H]DMNT binding reached a plateau between 16 and 20 h at 0 °C and remained constant for at least 70 h at 0 °C (results not shown). On the basis of these observations, we then examined the interactions of [^{3}H]DMNT with bovine aortic androgen receptor under equilibrium conditions. Cytosol was incubated at 0 °C for 20 h with increasing concentrations of [^{3}H]DMNT. Specific binding data were determined

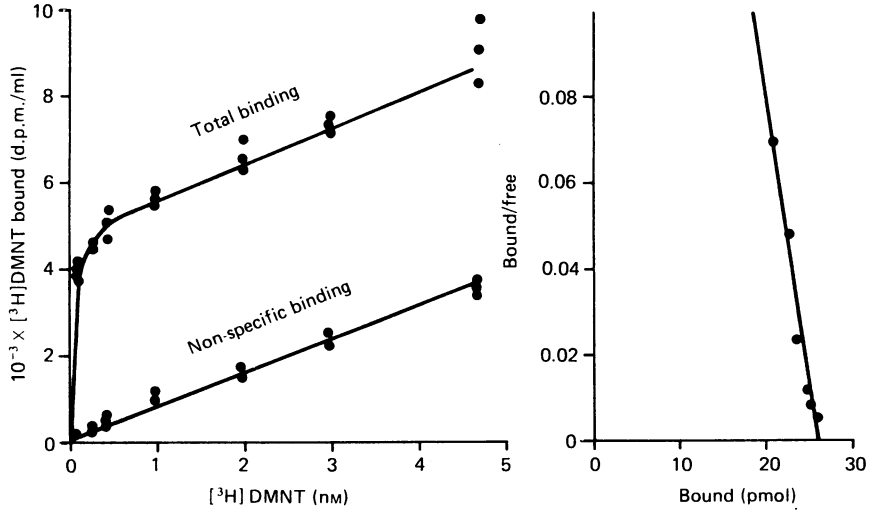

Fig. 3. Androgen receptors in calf aorta tissue cytosol

Portions (0.1 ml) of cytosol (7.4 mg of protein/ml) were incubated in triplicate at 0 °C for 24 h with equal volumes of buffer containing various concentrations of [^{3}H]DMNT. Specific binding was determined and linearized by the method of Scatchard (1949); K_D = 0.08–2 nM. See the Materials and methods section for other details.

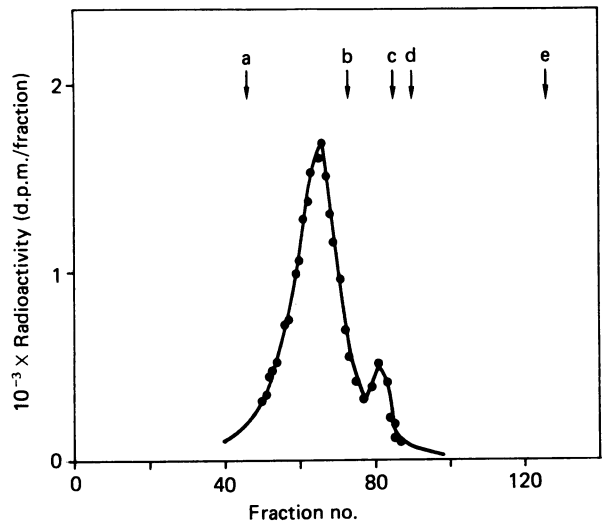

Fig. 4. Chromatography of [^{3}H]DMNT-labelled cytosolic androgen receptors of calf aorta on Sephacryl S-300

Cytosol was incubated at 0 °C for 24 h with 5 nM-[^{3}H]DMNT in the absence or presence of excess unlabelled DHT and applied to columns of Sephacryl S-300. Only specifically bound [^{3}H]DMNT is represented. M_r markers are: a, Blue Dextran 2000 ($\geqslant 2 \times 10^6$; V_0); b, γ-globulin (158000); c, bovine serum albumin (67000); d, ovalbumin (45000); e, glycine (V_t).

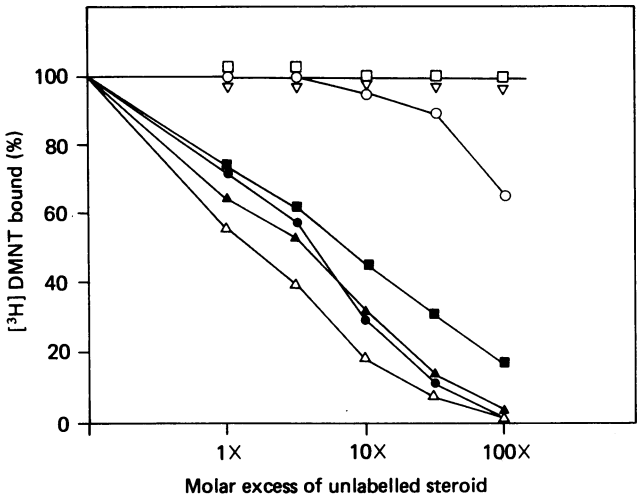

Fig. 5. Steroid specificity of androgen-binding sites in calf aorta tissue cytosol

Calf aorta cytosol was prepared and incubated at 0 °C for 24 h with 5 nM-[^{3}H]DMNT in the absence (total binding) or presence (non-specific binding) of 500 nM unlabelled DHT. An identical set of samples were incubated in parallel with 5 nM-[^{3}H]DMNT in the presence of increasing concentrations of unlabelled DMNT (△), DHT (●), R1881 (▲), testosterone (■), oestradiol (○), progesterone (▽) or cortisol (□). Protein-bound radioactivity was determined by the hydroxyapatite assay, and specific binding was plotted as a percentage of control binding versus the molar excess of unlabelled steroid.

and analysed by the method of Scatchard (1949) (Fig. 3). In three separate experiments, binding of [^{3}H]DMNT to bovine aortic cytosol was found to be of high affinity (K_D = 0.08–0.2 nM) and limited capacity (8 ± 3 fmol/mg of protein). These results suggest that bovine aortic tissue contains high-affinity binding sites for androgens. It should be pointed out that [^{3}H]DMNT does not bind specifically to plasma proteins (Traish *et al.*, 1986). Therefore the binding to bovine aortic tissue seen here represents interaction of [^{3}H]DMNT with androgen receptors.

Chromatographic analysis of [^{3}H]DMNT–receptor complexes

Cytosols labelled with 5 nM-[^{3}H]DMNT in the absence (total binding) or presence (non-specific binding) of 5 μM unlabelled DHT were analysed on Sephacryl S-300 molecular-sieving columns equilibrated on Tes/GME buffer containing 10 mM-monothioglycerol and 0.4 M-KCl. As shown in Fig. 4, the specifically bound [^{3}H]DMNT was eluted as a single peak, with an apparent Stokes radius of 6.5 nm. These results are similar to those obtained in our laboratory with cytosolic androgen receptors from rat ventral prostate analysed under identical conditions (Traish *et al.*, 1985). The present results clearly indicate that calf aortic tissue contains androgen receptors with properties similar to those in other androgen target tissues.

Steroid specificity of androgen receptors of bovine aorta

Bovine aortic cytosol was incubated at 0 °C for 20 h with [^{3}H]DMNT in the absence or presence of various concentrations of unlabelled steroids. Non-specific binding was determined by incubation with 5 μM unlabelled DHT, and specific binding was determined by subtraction of non-specific binding. As shown in Fig. 5, unlabelled DMNT, DHT, R1881 and testosterone were each effective competitors for [^{3}H]DMNT binding. Progesterone, oestradiol and cortisol did not effectively compete for [^{3}H]DMNT binding, however. These data thus indicate that [^{3}H]DMNT binds to androgen receptors in bovine aortic cytosol.

Binding of [^{3}H]DMNT to androgen receptors in aortic tissue slices at 37 °C

To determine if binding of [^{3}H]DMNT to cytosolic androgen receptors of bovine aortic tissue causes translocation of androgen-receptor complexes into the nucleus (or increases the affinity of nuclear androgen receptors for nuclear components), we incubated aortic tissue slices with 5 nM-[^{3}H]DMNT at 37 °C for 1 h. Tissues were then fractionated into cytosolic and nuclear fractions, and specifically bound radioactivity was determined in each fraction. In two experiments, 70% of total androgen receptors was found associated with the crude nuclear fraction, and 30% was found in the cytosolic fractions. These observations suggest that binding of androgen to its receptor in bovine aortic tissue induces translocation of androgen-receptor complexes into nuclei.

Binding of [^{3}H]DMNT to intact bovine aortic smooth-muscle cells

Bovine aorta smooth-muscle cells were incubated at 37 °C in serum-free media with various concentrations of [^{3}H]DMNT in the absence (total binding) or presence (non-specific binding) of a 1000-fold molar excess of unlabelled DHT. After 16 h at 37 °C, the cell layers were treated with trypsin, and the cells were transferred to silicone-coated tubes. The cells were washed three times

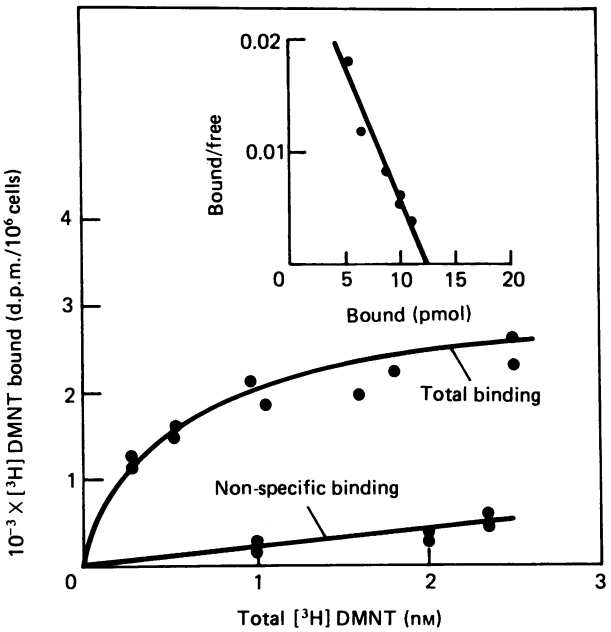

Fig. 6. Androgen receptors on calf aorta smooth-muscle cells

Smooth-muscle cells of calf aorta were incubated at 37 °C for 16 h in serum-free medium with various concentrations of [^{3}H]DMNT in the absence (total binding) or presence (non-specific binding) of a 100-fold molar excess of unlabelled DHT. Cell layers were treated with trypsin, transferred into silicone-coated tubes and washed three times with ice-cold phosphate-buffered saline. The cell pellets were extracted with ethanol (2 ml) at 22 °C for 16 h, and the extract was removed and counted for radioactivity. The residual pellets were washed with ice-cold 5% trichloroacetic acid and analysed for DNA content. The inset shows a Scatchard plot of the results: $K_A = 2.4 \times 10^9$ M^{-1}, $K_D = 0.4 \times 10^{-9}$ M; $n = 9000$ sites/cell.

with ice-cold 10 mM-sodium phosphate/0.15 M-NaCl (pH 7.4), the cell pellets were extracted with ethanol at 22 °C, and the radioactivity in the extract was assessed. As shown in Fig. 6, bovine aortic cells bind [^{3}H]DMNT with high affinity ($K_D = 0.4$ nM) and limited capacity (9000 ± 1000 sites/cell). These observations indicate that bovine aortic smooth-muscle cells contain androgen receptors and corroborate the data obtained with aortic tissue cytosols.

The binding of [^{3}H]DMNT to bovine aortic smooth-muscle cells was displaced with unlabelled DMNT, DHT, testosterone and cyproterone acetate, but not by progesterone, oestradiol or cortisol (results not shown), suggesting that [^{3}H]DMNT binding occurs specifically to androgen receptors in aortic smooth-muscle cells.

DISCUSSION

The present paper demonstrates that androgens increase the activity of lysyl oxidase in bovine aortic smooth-muscle cells in culture. Although the level at which this regulatory effect is expressed has yet to be established, possibilities include androgen-mediated alterations in the rate of transcription or translation and/or in the degree of stabilization of lysyl oxidase-specific mRNA. Previous studies have described evidence for the stimulation of the release of intracellular storage forms of lysyl oxidase by changing of the growth medium of cultured rabbit smooth-muscle cells (Gonnerman *et al.*, 1981), and preliminary evidence has also been obtained for intracellular forms which are considerably larger than the 32 kDa species of lysyl oxidase purified from bovine aorta (Bronson *et al.*, 1985). Thus lysyl oxidase activities may also be regulated by alterations in rates of proteolytic processing of enzyme precursors and/or in rates of secretion into the medium. Nevertheless, it does appear that the androgen-induced increase in lysyl oxidase is not due to a non-specific stimulus of protein synthesis, since the profile of proteins newly synthesized by the cultured bovine aortic cells was apparently unchanged by the presence of androgen in the culture. This result is in agreement with that of Nichlos *et al.* (1983), who similarly found no obvious change in the profiles of labelled proteins synthesized by androgen-stimulated rat aortic smooth-muscle cells.

Steroid hormones regulate growth and functions of their target cells by their interaction with specific intracellular receptor proteins. Androgen receptors were identified and characterized in whole bovine aortic tissue and cultured bovine aortic smooth-muscle cells, thus indicating the potential for such regulatory pathways in each of these biological systems. Mibolerone, a synthetic androgen, was used for these purposes, since it binds to androgen receptors with high affinity, but does not bind specifically to serum proteins (Traish *et al.*, 1986), nor is it metabolized in tissue culture at 37 °C (Liao *et al.*, 1973). However, it does bind to progesterone receptors with low affinity (Traish *et al.*, 1986). We therefore used unlabelled DHT in these studies as a competitor to assess for DHT-displaceable androgen-binding sites. The data presented here demonstrate that bovine aortic tissue contains androgen-specific binding sites which have high affinity for [^{3}H]DMNT, sediment as an 8 S species in low-salt sucrose gradient and which are eluted from Sephacryl S-300 as a macromolecule with a Stokes radius of 65 nm. These properties are similar to those reported for the androgen receptor in rat prostate (Traish *et al.*, 1985), a classical androgen target organ. Androgen receptors with quite similar characteristics were also identified in the cultured bovine aortic smooth-muscle cells. Thus bovine aortic tissue and bovine aortic smooth-muscle cells are androgen targets.

Lysyl oxidase is the only enzyme known to be required for cross-linkage formation in elastin and collagen. This enzyme can thus play an important regulatory role in the formation of insoluble fibres of these connective-tissue proteins. Previous studies have documented that lysyl oxidase activity is under hormonal control in mouse cervix (Ozasa *et al.*, 1981) and rat skin (Sanada *et al.*, 1978). The present observation that the expression of this enzyme can be regulated by androgen in aortic cells suggests at least one contributory biochemical basis to the sexual dimorphism seen in the development of fibrotic cardiovascular disease. Moreover, since the cultured bovine aortic smooth-muscle cells retain the key physico-chemical properties of the androgen receptors of bovine aortic tissue and express androgen-sensitive lysyl oxidase activity, these cultured cells represent a reasonable model system for further investigation of hormone-mediated modulation of connective-tissue protein metabolism.

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