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Androgen Receptors, 5 Alpha-Reductase Activity and Androgen-dependent Proliferation of Vascular Smooth Muscle Cells

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To assess the direct effect of androgen on the development of atherosclerosis, we investigated the effect of androgen and its receptor expression in rat vascular smooth muscle cells (VSMC) isolated from rat aorta. We detected the androgen receptor mRNA in VSMC by reverse transcription of the total RNA coupled with amplification of the resulting cDNA by polymerase chain reaction. Binding studies revealed the presence of a single class of binding sites for testosterone (K_d 7.37 nM, B_{max} 10.59 fmol/mg protein) and dihydrotestosterone (DHT; K_d 4.89 nM, B_{max} 11.37 fmol/mg protein) in VSMC. Measurement of 5α-reductase activity suggested that testosterone is converted to DHT in VSMC (K_m 0.36 μM, V_{max} 623 fmol/mg protein/h). Moreover, in the present study, DHT significantly stimulated DNA synthesis of VSMC (120–160% of control). The mitogenic activity of testosterone is much less potent than that of DHT. Considering these results, we concluded that androgen may directly accelerate atherosclerosis by stimulating the proliferation of VSMC.

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INTRODUCTION

In the human population, males have a much higher prevalence of coronary heart disease than females [1]. In females, the incidence of atherosclerosis is high in the postmenopausal period. Considering these results, androgens have been thought to be a causal factor in the developments of atherosclerosis. There are some reports that an increase of plasma testosterone concentration leads to a decrease in high-density lipoprotein (HDL) cholesterol, which is a well-known risk factor of atherosclerosis [2, 3]. However, the study group of the European Atherosclerosis Society reported that even when other risk factors like plasma cholesterol, hypertension and smoking are accounted for, male sex is an independent cardiovascular risk factor [4]. Moreover, Fischer *et al.* [5] found that the aortic production of collagen and elastin was increased in cholesterol-fed rabbits in which testosterone promoted atherosclerosis, and an effect of testosterone directly on the arterial wall was suggested. These results indicated that androgens may directly affect the mechanisms of atherosclerosis.

Vascular smooth muscle cells (VSMC) play an important role in atherogenesis. They migrate from the media into the intima and proliferate with the formation of atherosclerotic plaques. However, there have only been a few reports regarding the effect of androgen on the cells. The aim of this study is to assess the existence of androgen receptor (AR) in VSMC from rat aorta and the effect of androgen on the cells.

EXPERIMENTAL

Cell culture

VSMC were isolated by modifications of the methods of Ross [6]. Male Wistar rats (Kyuudou, Kumamoto, Japan) were killed by cervical dislocation and the thoracic aorta were excised and placed in a

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35 mm Petri dish (Falcon 3001, Oxnard, CA, U.S.A.) containing phosphate-buffered saline (PBS) (Ca^{2+}, Mg^{2+} free). Fat, adventitia and the intima were gently removed with fine forceps. The media was cut into smaller pieces (~ 1 mm^2) with scissors and incubated in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY) supplemented with 20% fetal calf serum (FCS; Nipro Co., Osaka, Japan), penicillin G (75 units/ml) and streptomycin (50 ng/ml) (Gibco) in an atmosphere of 95% O_2–5% CO_2 at 37°C. VSMC began to grow out from the pieces after 7–10 days. When the cells became confluent, they were subcultured and maintained in the same culture medium supplemented with 10% FCS. The cells at the 4–10th passage were used for experiments.

AR mRNA detection by reverse transcription (RT) coupled to polymerase chain reaction (PCR)

Total RNA was isolated from VSMC by a single step method [7]. Reverse transcription (RT) of the total RNA was performed on 5 μg of RNA. The components of the final 20 μl reaction were as follows: 10 mM $MgCl_2$, 50 mM KCl, 50 mM Tris–HCl, pH 8.3, 10 mM dithiothreitol, 1 mM levels of each dNTPs (Pharmacia Biotech., Uppsala, Sweden), 1 units/μl of RNasin (Promega Co., Madison, U.S.A.), 100 ng of random primer (Takara Shuzo Co.) and 0.5 units/μl of avian myeloblastoma virus reverse transcriptase (Takara Shuzo Co., Kyoto, Japan). Reaction tubes were incubated for 1 h at 37°C in a Program Tempcontrol System (Astec, Fukuoka, Japan). At the end of the incubation period, the reaction was stopped by heating for 5 min at 90°C. This heat treatment denatures RNA–cDNA hybrids and inactivates the reverse transcriptase.

Then, PCR was performed with rat AR specific primers. AR primer 1 (antisense) was defined by bases 1842–1859 and primer 2 (sense) encompassed bases 723–742. The sequence of primer 1 was 5′-CAGAGTCATCCCTGCTTC-3′ and that of primer 2 was 5′-AGTGCCAAGGAGTTGTGTAA-3′. The cDNA sequence for AR was described by Chang *et al.* [8]. The predominant cDNA amplification products were predicted to be 1137 basepair (bp) in length (the distance between primers plus primer length). To carry out the PCR, 80 μl of PCR reagent mixture was added to each reaction tube. Thus, the total PCR volume was 100 μl, including the original 20 μl from the RT reaction mixture. The PCR mixture contained the following components (final concentrations): 50 mM KCl, 10 mM Tris–HCl, pH 8.3, 1 mM $MgCl_2$, 200 μM levels of each dNTPs, 0.1 μM levels of each primer and 2.5 units/100 μl Taq DNA polymerase (Wako Pure C.I., Osaka, Japan). The PCR was performed in a Program Tempcontrol System (Astec) for 30 cycles. The standard conditions were as follows: denaturation for 1 min at 95°C, annealing for 2 min at 63°C and extension for 1–5 min at 72°C. The amplification products were electrophoresed through 1–1.3% (w/v) agarose gels, and were stained with 0.5 μg/ml of ethidium bromide. DNA was visualized with a u.v. transilluminator and photographed.

Receptor binding studies

VSMC were seeded at a concentration of 15×10^4 cells/3 ml of DMEM with 10% FCS in a 35 mm Petri dish (Falcon). After 6 h, when the cells had become attached to the dish, the medium was replaced with the same volume of phenol red-free DMEM, supplemented with 10% charcoal-treated FCS to deplete endogenous steroid hormones, and the cells were allowed to grow subconfluently for 4–5 days. Then the medium was removed and the cells were washed three times with 1 ml of phenol red-free DMEM supplemented with 2% bovine serum albumin (BSA; Wako Pure C.I.). The cells were incubated in DMEM with 2% BSA for 2 h at 37°C with 2–10 nM [1,2,6,7,17,18-3H]testosterone ([^{3}H]testosterone; 137 Ci/mmol New England Nuclear, Boston, MA) or [1,2,4,5,6,7-3H]dihydrotestosterone ([^{3}H]DHT; 127.5 Ci/mmol, New England Nuclear) in the presence or absence of 500 nM of unlabeled hormones to determine the total and nonspecific binding, respectively. After the incubation, the medium was removed by aspiration and the cells were washed four times with 2 ml of serum free, phenol red-free DMEM. 2 ml of 0.1 M NaOH was added and 1.5 ml of the content was transferred to a scintillation vial containing liquid scintillator EX-H (Dojin Chemicals Co. Ltd., Kumamoto, Japan). The radioactivity was counted with a liquid scintillation counter (Aloka LSC-3500E, Aloka Co. Ltd., Tokyo, Japan). 0.5 ml of the remaining content was used for the determination of protein by a Bio-Rad protein assay kit (Bio-Rad Lab., Richmond, CA) using BSA as standard. Each experiment was performed in duplicate, and results were analyzed by the method described by James E. Griffin *et al.* [9].

5α-Reductase activity

5α-reduction in VSMC was carried out using our previously described method [10], with some modifications. The cells were grown on a 35 mm Petri dish (Falcon) until near confluency and then serum free DMEM was substituted for DMEM with 10% FCS and incubated one more day. Then the cells were washed with serum free DMEM and then incubated in 1 ml of DMEM containing 0.1–10 μM [^{3}H]testosterone. After incubation for 2 h at 37°C, the medium was removed to a test tube for purification of DHT and the cells were dissolved in 1 ml of 0.1 N NaOH to determine the protein content by a Bio-Rad protein assay kit. Blanks were run for each assay, using a medium containing [^{3}H]testosterone incubated in culture dishes without cells. Each medium was extracted three times with 5 ml ethyl ether after the addition of

a known quantity of [^{14}C]DHT (57 mCi/mmol, New England Nuclear) diluted 5000 dpm to a monitor recovery. The extracts were evaporated to dryness, the residues were redissolved in 30 μl ethanol containing 10 μg radioinert steroids and these were purified by the following procedures as described by us [10]. The purification of DHT was performed by a two-step silica gel thin layer chromatography, first using chloroform–methanol (98:1.75), then using chloroform–ethyl acetate–ethanol (85:15:3). After the second chromatograph, the areas corresponding to DHT were scraped off, transferred to vials and eluted with ethyl acetate. Each of the samples was dried, scintillation fluid was added and the radioactivity was counted in a scintillation counter. The amount of each steroid produced, corrected for recovery losses, was expressed as fmol/mg protein/h. The scintillation counter had a [^{3}H]-counting efficiency of 45% and a [^{14}C]-counting efficiency of 65%. The [^{14}C] to [^{3}H] spillover was 1.0% and the [^{3}H] to [^{14}C] spillover was less than 0.01%.

The effect of androgen on DNA synthesis

VSMC were harvested with 0.1% trypsin solution (Sigma, St Louis, MO) from subconfluent cultures and cells were plated at 3–5 × 10^4 cells/well in 96-well tissue culture plates (Falcon). After 6 h, when the cells had become attached to the plates, the medium was replaced by 200 μl of serum free, phenol red-free DMEM supplemented with 2% BSA and the cells were incubated for 48 h at 37°C. Then the medium was removed and 100 μl of the medium supplemented with 2% BSA containing testosterone or DHT was added. The concentration of the hormones ranged from 1 nM–100 nM. The cells were incubated in 5% CO_2 and 95% air for 24 h at 37°C. After stimulation the medium was replaced by phenol red-free DMEM supplemented with 0.5 μCi/100 μl [^{3}H]thymidine (2.0 Ci/mmol, Amersham International plc, Bucks, U.K.) and the cells were incubated for 2 h. Then the cells were washed twice with PBS, trypsinized and harvested to glass papers by a cell-harvester. The radioactivity was counted using a liquid scintillation counter (Aloka LSC-3500E).

RESULTS

Detection of the AR gene by RT-PCR

We could not detect AR mRNA in rat VSMC by Northern blot hybridization. Therefore, we tried to visualize the AR mRNA by RT of the total RNA coupled with amplification of the resulting cDNA by PCR. Fig. 1 shows that VSMC RNA gene rises to a low but distinctive 1137 bp band. When the PCR procedure was carried out in the absence of reverse transcription, this band was not seen and there were no recognizable bands. This indicated that the 1137 bp band originated from mRNA, not from genomic DNA.

Receptor binding studies

The expression of functional AR protein in VSMC was demonstrated by measuring the specific binding of [^{3}H]testosterone and [^{3}H]DHT to confluent cells incubated in an androgen-depleted medium. The saturation analysis of their binding in VSMC is illustrated in Fig. 2. The specific bindings of [^{3}H]testosterone [Fig. 2(A)] and [^{3}H]DHT [Fig. 2(B)] to VSMC were saturable. Scatchard analysis revealed a single class of binding sites for testosterone with an apparent equilibrium dissociation constant (K_d) of 7.37 nM and an apparent equilibrium maximum number of binding sites (B_{max}) of 10.59 fmol/mg protein, and for DHT with a K_d of 4.89 nM and a B_{max} of 11.37 fmol/mg protein. The B_{max} of binding values were similar for

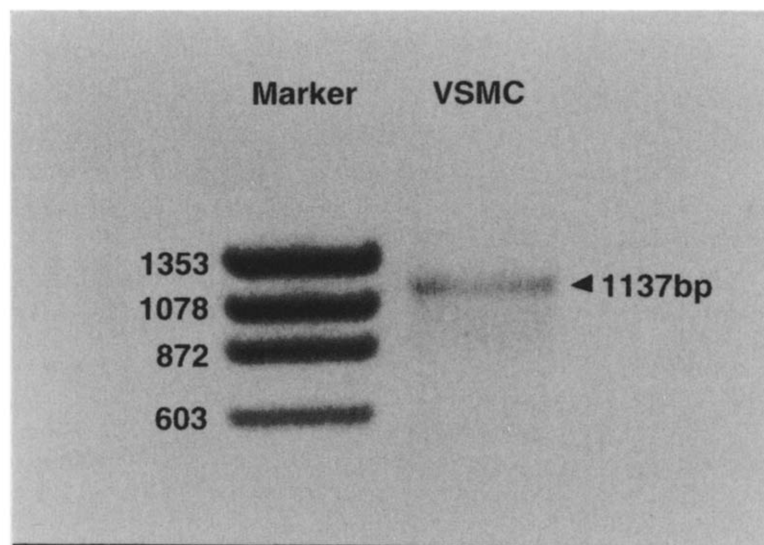

Fig. 1. Detection of the AR mRNA signal in total RNA from VSMC by RT-PCR. Total RNA (5 μg) from VSMC was reverse transcribed and PCR amplified to generate a 1137 bp cDNA fragment, as described in the text.

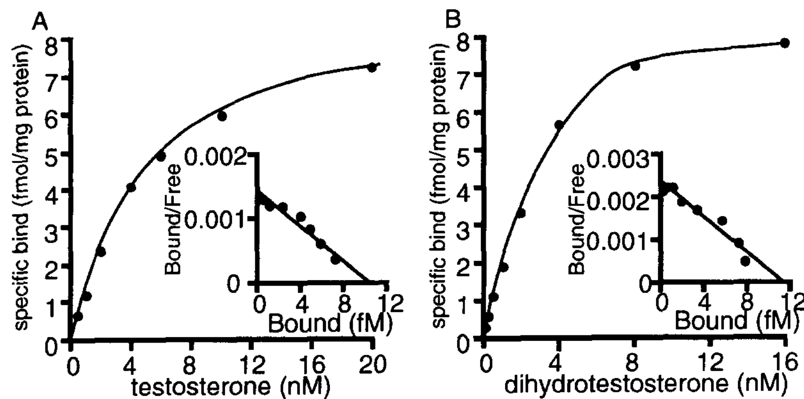

Fig. 2. Saturation curves and Scatchard analyses of [^{3}H]testosterone (A) and [^{3}H]dihydrotestosterone (B) binding to VSMC. VSMC were incubated in medium containing each [^{3}H]steroid with or without a 500 nM of unlabeled steroid, as described in Experimental. Binding is plotted as a function of each steroid's concentration. Each point represents a mean value from duplicate experiments.

both ligands and the half-maximal saturation concentration observed with DHT was 1.5-fold higher than that seen with testosterone.

5α-Reductase activity

The results of 5α-reductase activity assay in VSMC are presented in Fig. 3. The DHT formation in VSMC was increased in proportion to the amount of the testosterone added and almost reached a plateau. The apparent K_m was 0.36 μM and the maximum velocity was 623 fmol/mg protein/h.

The effect of androgen on DNA synthesis

DNA synthesis, as determined by [^{3}H]thymidine uptake, is shown in Fig. 4. DHT, the concentration of which was from 1 to 100 nM, significantly stimulated the DNA synthesis of VSMC (approx. 120–160% of control). Testosterone also stimulated the DNA synthesis slightly (approx. 107–112%), but it was significant.

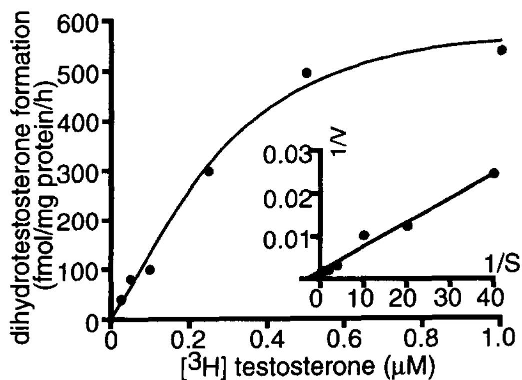

Fig. 3. 5α-Reductase activity in VSMC. Cells were incubated in duplicate for 2 h at 37°C in Dulbecco's Modified Eagle's Medium with increasing amounts of [^{3}H]testosterone (see Experimental). The apparent K_m and maximum velocity were found by regression lines computed by the methods of least squares.

DISCUSSION

The effect and mechanism by which androgenic steroids affect muscles remain unknown. In the case of skeletal muscle, it is well known that androgens directly initiate a variety of biochemical changes, including an increase in RNA polymerase activity, enhanced glycogen synthesis and enhanced uptake and phosphorylation of 2-deoxyglucose. However, in smooth muscles, especially in the vascular smooth muscle, the action of androgen is still unclear. It is generally accepted that steroid hormones, including androgens, interact with their receptors. Actually, the existence of AR in muscles has been reported in skeletal [11] and smooth muscle cells of the accessory sex organ [12–14], but, to our knowledge, there is no report on the existence of that in VSMC.

The results obtained in the present study provide evidence for the presence of AR in VSMC. First, AR mRNA was detected by PCR coupled to RT. Second, the results of the Scatchard analysis indicate a single class of receptors for testosterone and DHT,

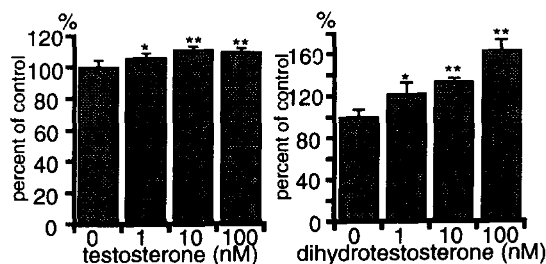

Fig. 4. Effects of testosterone and dihydrotestosterone on [^{3}H]thymidine uptake by VSMC during 24 h incubation. Cultures of VSMC in 96-well plates were incubated for 24 h in phenol red-free DMEM supplemented with indicated concentrations of testosterone and DHT. Thymidine uptake was measured in last 2 h of incubation. Data are means + SD of 4 samples. $^*P < 0.01$, $^{**}P < 0.001$ compared with control cells. See text for definitions.

respectively, and the bindings of [^{3}H]testosterone and [^{3}H]DHT to VSMC are both saturable and specific for each steroid hormone.

Gonzalez *et al.* [14] detected AR mRNA in smooth muscle cells from rat penis by Northern blot analysis and the mean value for the AR B_{max} was about 36 fmol/mg protein and the K_d was about 0.15 nM in their receptor binding assay in which [^{3}H]R-1881 was used. In the present study we failed to detect AR mRNA on VSMC by Northern blots and PCR coupled to RT was required to demonstrate it. We also identified the existence of AR in VSMC by receptor binding assay, but in the results of DHT binding to the receptor, the respective values of B_{max} (11.37 fmol/mg protein) and K_d (4.89 nM) were smaller than those of smooth muscle cells from rat penis. These results suggest that AR exists in VSMC, but only in small amounts.

In many androgen target tissues, DHT is the active form of androgen and testosterone is reduced to DHT by the enzyme 5α-reductase before binding to AR [15]. However, Powers and Fionni [16] found that testosterone, not DHT, stimulates mitotic activity in a myoblast culture system and this result suggests a possibility that testosterone affects muscles by direct interaction with the AR. This is supported by the finding by Wilson and Gloyna [17] that 5α-reductase activity in muscle tissue was lower than that in accessory sex organs.

In our binding study, testosterone and DHT had similar B_{max} binding values in VSMC and the affinity of binding of DHT was greater than that of testosterone. These results were in agreement with that of Grino *et al.* [18] in fibroblasts from the genital skin. However, it could not be denied that testosterone was converted into DHT before binding to the receptor. Then we measured 5α-reductase activity of VSMC to examine the conversion of testosterone to DHT, and the enzyme activity was lower than those of fibroblasts [10] and the rat prostatic duct [19]. This result seemed to support the hypotheses of Powers and Fionni [16] that testosterone, not DHT, may directly affect muscles. However, in VSMC, according the result of our receptor binding assay, there is a possibility that the enzyme was present in sufficient amounts to convert testosterone to DHT which produces androgenic effects.

It is well known that VSMC play an important role in atherogenesis by their migration from the media into the intima and by proliferation with the formation of atherosclerotic plaques. To assess the direct effect of androgens on the growth of VSMC, we examined its effect on the DNA synthesis of the cells by measuring the tritiated thymidine incorporation. We found that DHT was moderately able to stimulate DNA synthesis in growing VSMC and that testosterone also stimulated DNA synthesis, but the effect was smaller than that of DHT.

Previous studies that examined the effects of androgen on proliferation of muscle cells gave various results. In an *in vivo* study, Kochakian and Tillotson [20] reported that androgens lead to a significant reduction in muscle weight and cell size and in the skeletal muscle, while cell number and collagen content do not change. Neubauer and Mawhinney [21] reported a similar result in the smooth muscle cell of the guinea pig seminal vesicle. In contrast, in the case of *in vitro* studies, Powers and Florini [16] found that androgen stimulates DNA synthesis in a myoblast culture system and Gonzalez *et al.* [22] also reported a similar result in smooth muscle cells from rat penis. The differences in these results have been attributed to the difference in the situations of studies.

On the other hand, Nakao *et al.* [23] reported that testosterone did not have any significant effect on the proliferation of VSMC. They only examined the effect of testosterone, not DHT, but the effect of testosterone at high concentrations was also smaller than that of DHT in our study. The reason for the difference in the effects between testosterone and DHT was unclear. We found that androgens clearly enhance DNA synthesis of VSMC significantly, but in some experiments the enhancement was marginal. Consequently, an important cause to be considered might be the difference in the condition or the stage of VSMC in culture. Actually, the biological characteristics of the cells have been reported to vary according to the stage of differentiation [24].

In conclusion, we detected AR mRNA in VSMC and found specific binding sites for androgen. DHT significantly stimulated the proliferation of VSMC and its effect was stronger than that of testosterone. These results suggest that androgen may directly accelerate atherosclerosis by stimulating the proliferation of VSMC.

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