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SELECTIVE INHIBITION OF ANDROGEN ACTION BY FERULIC ACID IN THE RAT PROSTATE

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Abstract

Slight but significant reduction occurred in the binding of testosterone to cytosol protein of ventral prostate and seminal vesicles by ferulic acid *in vitro*, but not in the binding of 5α-dihydrotestosterone. Incorporation of amino acids into either cytoplasmic particles or cytoplasmic soluble proteins was not inhibited by ferulic acid administered at a daily dose of 25 mg/kg for 3 days, but the uptake into acid-soluble cytosol fraction was significantly suppressed exclusively in the ventral prostate. Ferulic acid reduces the intracellular pool size of free amino acids in the ventral prostate and subsequently affects the diverse cellular functions, while the transport systems of neutral amino acids and the receptor mechanism of androgen action remain intact.

INTRODUCTION

Most of the synthetic antiandrogens so far reported are steroidal and possess other hormonal properties. Nonsteroidal compounds such as flutamide[1] and DIMP[2] were also reported to inhibit the weight gains of androgen responsive organs in androgen-replaced castrated rats. We reported recently that ferulic acid (*trans*-4-hydroxy-3-methoxycinnamic acid, FA) inhibits the weight gain of the ventral prostate in rats by both endogenous and exogenous androgens without any significant effects on the seminal vesicle and levator ani muscle[3]. Such a selective effect on the rat prostate was also reported using *p*-coumaric acid[4].

Major sites of the action of currently available antiandrogens were proposed to be in the following stages[5] : entry of testosterone into target cells ; intracellular conversion of testosterone into 5α-dihydrotestosterone (DHT) ; and specific binding of DHT to cytosol and nuclear receptor proteins. Our *in vivo* study[3], however, indicated that these three stages were not involved as a site

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of the action of FA. The present investigation was undertaken to clarify the mechanism of selective inhibitory effect of FA on the rat prostate as compared with its effect on the seminal vesicle.

MATERIALS AND METHODS

Male Sprague-Dawley rats (CLEA Japan, Inc.) were used in this study. Orchidectomy in rats was performed through scrotal incision under light ether anesthesia.

In vitro effect of FA (Sigma) on the receptor binding of testosterone and DHT was examined using the cytosol preparation[6] of ventral prostate and seminal vesicles of 24 hr castrated rats. Cytosol proteins were incubated with different concentrations of ^{3}H-androgens in 650 μl of TEM buffer solution (10 mM Tris-HCl, 0.1 mM EDTA, 0.5 mM 2-mercaptoethanol, and 1 mM $MgCl_2$, pH 7.4) containing 0.25 M sucrose at 0-1°C for 60 min in the presence or absence of 0.77-360 μM FA, and the protein-bound radioactivities were measured after removal of free androgens with 200 μl of dextran-coated charcoal (0.5% (w/v) dextran T-70 and 5% (w/v) Norit A in TEM buffer). Nonspecific binding was measured in the presence of 300-fold molar excess of respective radioinert androgens.

Protein synthesis was measured using mince incubation[7]. Ventral prostate and seminal vesicles were removed and cut up with scalpels into small pieces (ca. 10 mg) in Hanks' medium supplemented with 10 mM HEPES-NaOH buffer, pH 7.4. The minced tissue fragments were incubated with ^{14}C-labeled amino acid mixture (0.48 μCi/ml) in Hanks' medium at 37°C, and washed three times with 0.15 M NaCl. The tissues were homogenized with a Polytron PT 10 (Kinematica, Luzern) at setting dial 7 for 15 sec in 1.5 ml of chilled TEM containing 0.25 M sucrose and subjected to differential centrifugations at 800×g for 15 min and subsequently at 100,000×g for 60 min (Hitachi 5PR and 55P-2). Cytoplasmic particles (100,000×g pellet) were washed three times with TEM and dissolved in 0.5 M NaOH. Cytoplasmic soluble proteins were sedimented by centrifugation at 1,000×g for 10 min following to the addition of equal volume of 20% (w/v) perchloric acid (PCA) at 0-1 °C. The precipitate was washed twice with 10% (w/v) PCA and dissolved in 0.5 M NaOH. Crude nuclear fraction (800×g pellet) was washed, resuspended, and used for the determination of DNA.

Whole tissue uptake of amino acids was measured as follows : Minced tissue fragments of the ventral prostate from noncastrated rats were incubated with ^{14}C-labeled 2-aminoisobutyric acid (AIB), 2-aminobicyclo (2,2,1) heptane-2-carboxylic acid (BCH), and amino acid mixture in Hanks' medium at 37°C,

and washed four times with 0.15 M NaCl. The wet weight was measured in a vial, and the tissue was digested with NCS solubilizer (Radiochemical Centre, Amersham).

Quantitation of radioactivities was carried out using a solution containing 5.5 g DPO, 0.1 g POPOP, 667 ml toluene and 333 ml Triton X-100 with a Searle Mark III (model 6880) scintillation counter. For aqueous alkaline sample, the scintillation solution was added after neutralization with acetic acid.

Protein was measured by the method of Lowry *et al.*[8] with crystalline bovine serum albumin (Armour Pharmaceutical Co.) as a standard. DNA was determined using the diphenylamine procedure of Burton[9] with calf thymus DNA (Sigma Chemical Co.) as a standard. Student's *t*-test was used to calculate statistical significance.

[1, 2, 6, 7-^{3}H]Testosterone (80 Ci/mmole), 5α-dihydro[1, 2, 4, 5, 6, 7-^{3}H] testosterone (114 Ci/mmole), and uniformly ^{14}C-labeled amino acid mixture (57 mCi/mAtom carbon) were perchased from the Radiochemical Centre. [1-^{14}C]AIB (51.6 mCi/mmole) and [carboxyl-^{14}C]BCH (4.95 mCi/mmole) were obtained from the New England Nuclear.

RESULTS

Since many antiandrogens, both steroidal and nonsteroidal, were reported to antagonize the receptor binding of androgens[5,10], FA was tested whether it inhibits the binding of androgens to cytosol receptor. As shown in Fig. 1, binding of [^{3}H]testosterone to the prostatic cytosol protein was slightly inhibited by 7.7 μM FA in competitive manner, while that of [^{3}H]DHT was not inhibited by FA up to 36 μM. The similar results were obtained using the cytosol protein of seminal vesicles. The binding parameters, which are obtained by the Scatchard analysis[11], in the absence of FA are as follows (apparent dissociation constant in nM; binding sites in fmoles/mg protein) : binding of testosterone, 1.75 ; 74 and 2.75 ; 38 : binding of DHT, 1.30 ; 72 and 1.55 ; 37 in the ventral prostate and seminal vesicles, respectively.

When 48 hr castrated rats were given subcutaneously 6 mg/kg of testosterone and the ventral prostate was removed 24 hr thereafter, incorporation of ^{14}C-labeled amino acids into cytosol proteins was markedly enhanced (2.2-fold increase as compared with castrated control). The androgen-dependent enhancement of the incorporation per nuclear DNA was also observed in cytoplasmic particles and PCA-soluble cytosol fraction : 2.2-fold and 1.6-fold increase, respectively. However, treatment of intact rats with FA *in vivo* did not affect the uptake of amino acids into cytoplasmic particles and PCA-precipitable

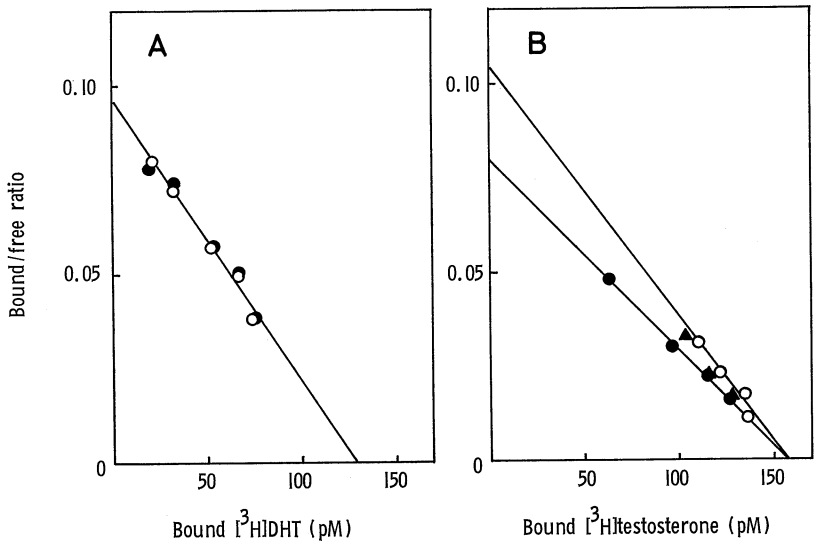

Fig. 1. Binding of androgens to cytoplasmic receptor of ventral prostate in the presence or absence of FA. [^{3}H]DHT (A) and [^{3}H]testosterone (B) were incubated with the prostatic cytosol (A, 1.74 ; B, 2.13 mg protein/ml) of 24 hr castrated rats. Specific binding is plotted according to Scatchard. Final concentrations of FA in the incubation mixture were 0 (○), 0.77 (▲), and 7.7 (●) μM. Mean of duplicate.

cytosol proteins either in the ventral prostate or in the seminal vesicles (Table 1). On the other hand, the uptake into PCA-soluble cytosol fraction was significantly decreased by FA pretreatment in the ventral prostate but not in the

TABLE 1.
Effect of FA on the uptake of ^{14}C-labeled amino acids into subcellular fractions

Tissues	Treatment	Uptake of amino acids ($\times 10^3$ dpm/mg DNA)		
		Cytoplasmic particles	Cytosol	
			PCA precipitable	PCA soluble
Ventral prostate	Control	10.2±0.3	1.58±0.11	405±17
	Ferulic acid	8.2±0.9	1.63±0.08	331±24*
Seminal vesicle	Control	5.7±0.5	1.40±0.11	254± 9
	Ferulic acid	4.7±1.1	1.11±0.12	226±24

Rats (160-210 g) received subcutaneously 25 mg/kg of FA for 3 days. Tissues were removed 24 hr after the last injection, minced in Hanks' medium, and incubated with 0.48 μCi/ml ^{14}C-labeled amino acid mixture at 37°C for 40 min. Mean±SEM of 5 animals. *Significantly different from control, $P<0.05$.

seminal vesicles. Fig. 2 shows the time course of the uptake. The initial rate seems not to be influenced by FA pretreatment. Remarkable effect of FA was observed in more than 20 min of incubation, and the saturation level

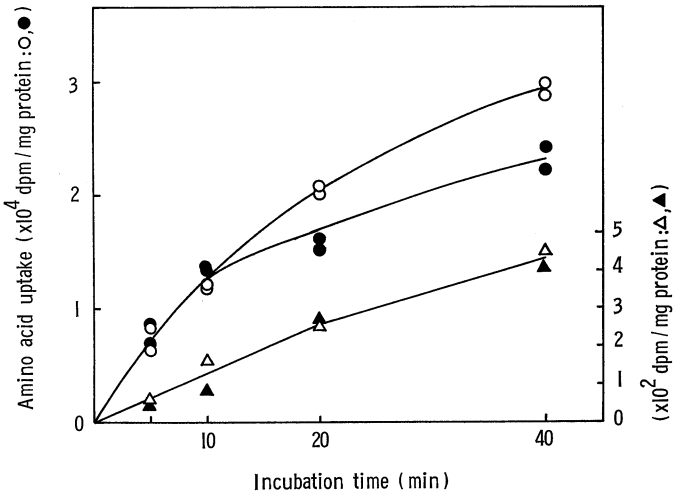

Fig. 2. Effect of FA on the uptake of amino acids into PCA soluble and precipitable fractions of the prostatic cytosol. Minced ventral prostate from rats (210-260 g) given FA (solid symbols) or vehicle (open symbols) was incubated with ^{14}C-labeled amino acid mixture as in the footnote for Table 1, except that incubation time was changed as indicated. Amino acid uptake into PCA soluble pool (circles) and cytosol protein (triangles) is plotted against incubation time after correction with the value in 0 min incubation.

appeared to be depressed. Since the amino acid uptake into the PCA-soluble cytosol fraction of ventral prostate was not inhibited with a dose as high as 0.1 mM FA added *in vitro* (data, not shown), it is unlikely that FA could directly inhibit the uptake process of amino acids. This was assured by the following experiments using nonmetabolizable amino acids, such as AIB and BCH.

In vivo effect of FA on the whole tissue uptake of amino acids was examined using ^{14}C-labeled AIB and BCH instead of amino acid mixture, and the results are shown in Fig. 3 There was no inhibitory effect on the tissue uptake of AIB or BCH over the incubation periods of up to 30 min. In FA-treated rats, the uptake of amino acid mixture by the ventral prostate per wet weight was also significantly inhibited in 60 min incubation, whereas

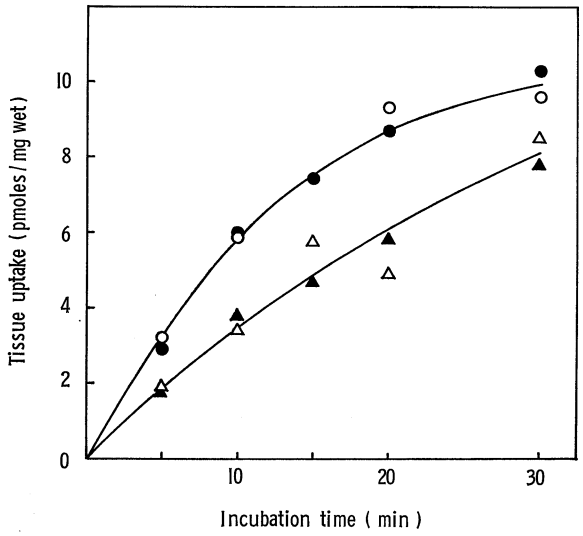

Fig. 3. Effect of FA on the tissue uptake of BCH and AIB by ventral prostate. Minced ventral prostate of control (open symbols) or FA-treated rats (solid symbols) weighing 240-270 g was incubated with ^{14}C-labeled BCH (22 μM) or AIB (22 μM) at 37°C for 0-30 min. Others were the same as in Table 2. Whole tissue uptakes of BCH (circles) and AIB (triangles) are plotted against incubation time.

no effect was observed on the uptake of AIB or BCH (Table 2). Furthermore, the uptake of AIB or BCH in the ventral prostate was not suppressed with 0.1 mM FA added *in vitro*.

TABLE 2. Effect of FA on the tissue uptake of ^{14}C-labeled BCH, AIB, and amino acid mixture by ventral prostate

Treatment	Incubation time (min)	Tissue uptake		
		BCH (pmoles/mg wet)	AIB (pmoles/mg wet)	Amino acids (pAtoms carbon/mg wet)
Control	20	5.78±0.16	4.66±0.30	5.65±0.22
	60	7.64±0.20	8.60±0.36	8.77±0.52
Ferulic acid	20	5.74±0.42	5.06±0.43	5.05±0.14
	60	7.03±0.26	8.43±0.68	7.26±0.14*

Rats (170-240 g) were treated as in Table 1. Minced prostate was incubated in Hanks' medium with either 11 μM BCH, 11 μM AIB, or 0.48 μCi/ml (8.42 μAtoms carbon/l) amino acid mixture at 37°C. Data are presented as the means±SEMs of 6 animals after correction with the values obtained in 0 min incubation. *Significantly different from control, $P<0.05$.

DISCUSSION

FA has a unique antiandrogenic property in the ventral prostate of rats, but neither in the seminal vesicles nor in the levator ani muscle. This is quite different from other antiandrogens which antagonize androgen both in the ventral prostate and in the seminal vesicles about the same extent in rats[12]. It is interesting, therefore, to elucidate the mechanism of action of FA in the rat prostate.

The possible sites of action of antiandrogens in male accessory sexual glands are present in the stages of penetration of testosterone into target cells, conversion of testosterone to DHT, and binding of DHT to cytoplasmic and nuclear receptors[5]. As reported here and elsewhere[3], we could not find the antagonistic effect of FA, either *in vivo* or *in vitro*, at these three points. Therefore, it is unlikely that above three stages are involved in the mechanism of inhibitory action of FA.

If FA affected any site within the sequential events of androgen action through receptor mechanism, the androgen-induced enhancement of the bulk protein synthesis should be inhibited by the daily treatment with FA. However, any evidence could not be obtained that FA did inhibit the incorporation of labeled amino acids into cytoplasmic soluble protein or cytoplasmic particles (Table 1). It is suggested further that nuclear translocation of androgen-receptor complex and subsequent stimulation of transcriptional and translational processes were not involved in the mechanism of the action of FA. If any one of these stages was involved, all other male accessory organs should be suppressed.

In the experiments to see the effect on protein synthesis, the significant reduction of the uptake of labeled amino acids into a PCA-soluble cytosol fraction was found in the ventral prostate from FA treated rats, but not in the seminal vesicle (Table 1). This is in agreement with the suppression of weight gain solely of ventral prostate[3]. The inhibition of the uptake of free amino acids by rat prostate was dependent on an incubation time (Fig. 2), and equivocal within 20 min of incubation (Table 2), suggesting that transport process of amino acid through the prostatic cell membrane is unaffected, but that intracellular pool size of amino acids may be reduced under the influence of FA *in vivo*. This suggestion was further supported by the following evidence. Inhibition by FA, *in vivo* and *in vitro*, could not be demonstrated during 5-60 min incubation using labeled AIB and BCH (Table 2 and Fig. 3), which have been used as the model amino acids having high affinities for sodium-dependent and sodium-independent transport systems, rescpectively, in Ehrlich ascites tumor[13] and isolated rat hepatocytes[14]. It is concluded, therefore, that the decrease in the weight of prostate by FA may be attributable to the

fall of intracellular pool size of free amino acids in the prostatic cells which subsequently results in the decreased synthesis of bulk proteins *via* lowered supply of available amino acids. As to the selective activity of FA, the inhibition of the uptake of free amino acids may be secondary to the suppression of the unknown cellular functions which are intrinsic to the prostatic glands.

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