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Inhibition of steroid 5α-reductase by specific aliphatic unsaturated fatty acids

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Human or rat microsomal 5α-reductase activity, as measured by enzymic conversion of testosterone into 5α-dihydrotestosterone or by binding of a competitive inhibitor, [^{3}H]17β-*NN*-diethylcarbamoyl-4-methyl-4-aza-5α-androstan-3-one ([^{3}H]4-MA) to the reductase, is inhibited by low concentrations (< 10 μM) of certain polyunsaturated fatty acids. The relative inhibitory potencies of unsaturated fatty acids are, in decreasing order: γ-linolenic acid > *cis*-4,7,10,13,16,19-docosahexaenoic acid = *cis*-6,9,12,15-octatetraenoic acid = arachidonic acid = α-linolenic acid > linoleic acid > palmitoleic acid > oleic acid > myristoleic acid. Other unsaturated fatty acids such as undecylenic acid, erucic acid and nervonic acid, are inactive. The methyl esters and alcohol analogues of these compounds, glycerols, phospholipids, saturated fatty acids, retinoids and carotenes were inactive even at 0.2 mM. The results of the binding assay and the enzymic assay correlated well except for elaidic acid and linolelaidic acid, the *trans* isomers of oleic acid and linoleic acid respectively, which were much less active than their *cis* isomers in the binding assay but were as potent in the enzymic assay. γ-Linolenic acid had no effect on the activities of two other rat liver microsomal enzymes: NADH:menadione reductase and glucuronosyl transferase. γ-Linolenic acid, the most potent inhibitor tested, decreased the $V_{max.}$ and increased K_m values of substrates, NADPH and testosterone, and promoted dissociation of [^{3}H]4-MA from the microsomal reductase. γ-Linolenic acid, but not the corresponding saturated fatty acid (stearic acid), inhibited the 5α-reductase activity, but not the 17β-dehydrogenase activity, of human prostate cancer cells in culture. These results suggest that unsaturated fatty acids may play an important role in regulating androgen action in target cells.

INTRODUCTION

In many androgen-responsive organs, such as prostate and skin, testosterone is converted into 5α-dihydrotestosterone (5α-DHT) by 5α-reductase. 5α-DHT then binds to androgen receptors and functions in the nucleus to regulate specific gene expression (Liao *et al.*, 1989). Since 5α-DHT promotes the development of acne, male pattern alopecia, benign prostatic hyperplasia and female hirsutism, inhibitors of 5α-reductase may be useful for treatment of these conditions. Certain 4-azasteroids are potent competitive inhibitors of 5α-reductase (Liang & Hess, 1981; Liang *et al.*, 1984*a*, 1985*a*) and have been shown to have therapeutic value (Vermeulen *et al.*, 1989; Rittmaster *et al.*, 1989; Gormley *et al.*, 1990; Imperato-McGinley *et al.*, 1990). We report here that some naturally occurring unsaturated fatty acids can inhibit 5α-reductase in cultured cells and in cell-free systems. Our results suggest that these fatty acids could function as endogenous inhibitors of 5α-reductase.

MATERIALS AND METHODS

Materials

Lipids, NADH, NADPH, UDP-glucuronate, carotenes, retinoids and prostaglandins, as well as detergents, polyoxyethylene ether W-1 and CHAPS were obtained from Sigma. [1,2,4,5,6,7,16,17-^{3}H(n)]5α-DHT (153 Ci/mmol) and [1,2,6,7-^{3}H(n)]testosterone (105 Ci/mmol) were products of New England Nuclear. [1,2-^{3}H]17β-*NN*-diethylcarbamoyl-4-methyl-4-aza-5α-androstan-3-one ([^{3}H]4-MA) (60 Ci/mmol) was prepared as previously described (Liang *et al.*, 1983). Prostate cancer cells, LNCaP and PC-3 were obtained from the American Type Culture Collection, and culture medium RPMI 1640 was from GIBCO.

Preparation of microsomal fractions

Microsomes were prepared at 4 °C from a buffered 0.32 M-sucrose homogenate of human liver and from the livers of adult Sprague–Dawley female rats by differential centrifugation as described previously (Liang *et al.*, 1990), and were used in the assay of 5α-reductase activity. In some experiments, microsomes were solubilized with 0.1% polyoxyethylene ether W-1 as described previously (Liang *et al.*, 1990), except for the substitution of polyoxyethylene ether W-1 for Lubrol-WX.

To obtain microsomal fractions that contain 5α-reductase inhibitors, rat liver microsomes (30 mg of protein) were suspended in 5 ml of 20 mM-potassium phosphate buffer, pH 7.0, mixed with 20 ml of acetic acid, and then 4 vol. of methanol were added. After 30 min, precipitated protein was removed by centrifugation at 8000 *g* for 10 min and the supernatant was evaporated at 50 °C with a stream of nitrogen. The residue was resuspended in 1 ml of acetic acid and then centrifuged at 14000 *g* for 4 min. The supernatant was applied to a Sephadex G-50 column (1.5 cm × 94 cm) equilibrated with 70% acetic acid. The column was then eluted with 70% acetic acid, and 4 ml fractions were collected. To assay 5α-reductase inhibitor activity, 0.1 ml of each fraction was evaporated under a stream of nitrogen, and the residue was dissolved in 10 μl of ethanol and then combined with the [^{3}H]4-MA binding assay mixture. Alternatively, rat liver microsomes (2.5 mg of protein) were vigorously shaken with 0.5 ml of methylene chloride at 25 °C for

Abbreviations used: 5α-DHT, 5α-dihydrotestosterone (17β-hydroxy-5α-androstan-3-one); 4-MA, 17β-*NN*-diethylcarbamoyl-4-methyl-4-aza-5α-androstan-3-one. The numerical symbols for fatty acids were named according to the IUPAC–IUB recommendations [Lipids (1976) **12**, 455–468]; for example, $C_{18:3,cis-6,9,12}$ is the symbol for γ-linolenic acid and represents the structure of a C_{18} unbranched fatty acid with three double bonds at positions 6, 9 and 12, all in *cis* configuration. The carbon atoms were numbered with the *C*-terminal carbon as 1.

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1 min. The methylene chloride extract was collected and the solvent was evaporated to dryness. The residue was dissolved with 0.2 ml of ethanol, and 5 μl of the solution was used for the assay.

[^{3}H]4-MA binding assay for 5α-reductase

The procedure was described in detail previously (Liang *et al.*, 1983, 1990). Briefly, the binding assay solution, in a final volume of 0.15 ml, contained microsomes (2–20 μg of protein), 0.08 μCi of [^{3}H]4-MA, 0.1 mM-NADPH, 1 mM-dithiothreitol and 50 mM-potassium phosphate, pH 7.0, with or without the indicated amount of a lipid or an inhibitor preparation. Lipids were dissolved in ethanol and added in 1–5 μl volumes. Control tubes received the same amount of ethanol. After incubation at 0 °C for 1 h, the [^{3}H]4-MA bound to microsomes was determined by collecting microsomes on a Whatman GF/F glass fibre filter and washing with 10 ml of 20 mM-potassium phosphate, pH 7.0, containing 0.01% CHAPS to remove unbound [^{3}H]4-MA.

Assay of the enzymic activity of microsomal 5α-reductase

The standard reaction mixture, in a final volume of 0.15 ml, contained microsomes (1 μg of protein), 0.1 μCi of [^{3}H]testosterone, 0.5–3.0 μM non-radioactive testosterone, 0.1 mM-NADPH, 1 mM-dithiothreitol and 50 mM-potassium phosphate, pH 7.0, with or without the indicated amount of a lipid. The reaction was started by the addition of microsomes and the incubation was carried out at 37 °C for 15 min. Steroids were extracted and separated by t.l.c. as described previously (Liang & Heiss, 1981; Liang *et al.*, 1984*a*, 1985*a*). Radioactive steroids were located by fluorography and the amount of radioactivity present was determined by scintillation counting. The 5α-reductase activity was measured by analysing the extent of the conversion of [^{3}H]testosterone to [^{3}H]5α-DHT.

Assay of the 5α-reductase activity of prostatic cancer cells in culture

Human prostate cancer cell lines (androgen-insensitive PC-3 and androgen-sensitive LNCaP) were grown to 2×10^6 cells in 2 ml RPMI 1640 of cell culture medium containing 10% fetal calf serum. For the measurement of 5α-reductase activity, cells were washed twice with culture medium and incubated with 0.3 μCi of [^{3}H]testosterone in the presence or absence of γ-linolenic or stearic acid in 2 ml of culture medium at 37 °C for 2 h. The fatty acids were added in 20 μl of ethanol, and controls received the same volume of vehicle. The steroids in the culture medium and cells were extracted twice with 2 ml of ethyl acetate, separated by t.l.c. and analysed as described above.

Electron microscopic examinations of microsomes

Rat liver microsomes (66 μg/ml) were incubated with 10 and 50 μM-γ-linolenic acid or -stearic acid in the presence of 0.08 μCi of [^{3}H]4-MA, 0.1 mM-NADPH, 1 mM-dithiothreitol and 20 mM-potassium phosphate, pH 7.0, in a final volume of 12 ml. The lipids were added in 62.5 μl of ethanol solution. Controls received same volume of ethanol. After incubation at 0 °C for 1 h, microsomes were pelleted by centrifugation at 100000 *g* for 1 h. The pellets were immediately prepared for electron microscopic examinations as described by Fleischer & Kervina (1974).

Other methods

NADH:menadione reductase activity was assayed in a 3.0 ml reaction mixture containing rat liver microsomes (1 μg of protein), 0.2 mM-NADH, 1 mM-$MgCl_2$, 10 mM-NaF, 1 mM-dithiothreitol, 0.6 mM-menadione and 50 mM-potassium phosphate, pH 7.0, in the presence or absence of γ-linolenic acid. The reaction was carried out at 25 °C and was monitored by the decrease in the absorbance at 340 nm (Liang *et al.*, 1990). UDP-glucuronic acid:5α-DHT glucuronosyltransferase activity was measured by monitoring the formation of 5α-DHT glucuronide. The reaction mixture, in a final volume of 0.15 ml, contained 0.1 μCi of [^{3}H]5α-DHT, 1 mM-UDP-glucuronic acid, rat liver microsomes (10 μg of protein), 1 mM-$MgCl_2$, 20 mM-Tris/HCl, pH 7.5, with or without γ-linolenic acid. After incubation at 37 °C for 15 min, the reaction was terminated by the addition of 1 ml of ice-cold water and immediately extracted twice with 1 ml of ethyl acetate. The radioactivity of the aqueous phase that contained [^{3}H]5αDHT glucuronide was measured. Protein concentrations were determined by the method of Bradford (1976).

RESULTS

Assays for 5α-reductase activity in microsomal fractions

In mammalian cells, 5α-reductase is very tightly associated with microsomal membranes. Solubilization and purification of active 5α-reductase have not been very successful. The assay of 5α-reductase activity, therefore, has been performed by measuring the rate of conversion of testosterone to 5α-DHT by whole cells or by microsomal preparations in the presence of NADPH (enzymic assay). Alternatively, 5α-reductase activity can be reliably assayed by measuring NADPH-dependent non-covalent binding of a potent radioactive inhibitor, such as [^{3}H]4-MA, which strongly competes with testosterone for binding to the reductase. The results of the two assays correlate very well when microsomal preparations from different organs or animals are used for comparison (Liang *et al.*, 1983).

Effects of lipids on microsomal 5α-reductase activity

When the microsomal fraction of rat liver was solubilized with acetic acid and then mixed with methanol, more than 80% of microsomal proteins were removed as precipitates. This procedure inactivated the 5α-reductase activity completely. The soluble fraction, but not the precipitated fraction, contained compounds that inhibited 5α-reductase activity (determined by the enzymic assay or [^{3}H]4-MA-binding) of rat liver microsomes. Sephadex G-50 column chromatography of the methanol-soluble fraction showed separation of the inhibitory activity from the majority of the protein peak, which eluted in the void volume. The inhibitory activity was also found in methylene chloride extracts of rat liver microsomes, suggesting that the inhibitors were lipids.

We therefore tested various lipids for their ability to affect binding of [^{3}H]4-MA to rat liver microsomes. Table 1 shows that only certain unsaturated fatty acids are inhibitory. Among the lipids we have tested, inhibitory fatty acids have 14–22 carbon chains and one to six double bonds. Presence of a double bond was required for inhibitory activity; saturated fatty acids were totally inactive. Only compounds with double bonds in the *cis* configuration were active at low concentrations (< 10 μM), whereas the *trans* isomers were inactive even at high concentrations (> 0.2 mM). The difference in the effects of *cis* and *trans* isomers of fatty acids is obvious when the following sets of fatty acids are compared: oleic acid ($C_{18:1,cis\text{-}9}$) versus elaidic acid ($C_{18:1,trans\text{-}9}$), and linoleic acid ($C_{18:2,cis\text{-}9,12}$) versus linolelaidic acid ($C_{18:2,trans\text{-}9,12}$). The number and the position of the double bonds also affected the potency. For example, the inhibitory potencies of the C_{18} fatty acids were, in decreasing order: γ-linolenic acid (*cis*-6,9,12) > octadecatetraenoic acid (*cis*-6,9,12,15) > α-linolenic acid (*cis*-9,12,15) > linoleic acid (*cis*-9,12) > oleic acid (*cis*-9) > petroselinic acid (*cis*-6). Erucidic acid ($C_{22:1,cis\text{-}13}$) was inactive, whereas *cis*-4,7,10,13,16,19-

Table 1. Inhibition of [^{3}H]4-MA binding to 5α-reductase of rat liver microsomes by lipids

Binding assays were performed as described in the Materials and methods section. Lipids were tested at concentrations ranging from 0.01 to 0.2 mM. Each experiment was carried out in duplicate and several experiments were performed to ensure that the results shown are representative. Compounds that showed less than 10 % inhibition were considered not active (NA). At 200 μM, no significant effect was observed with (*a*) saturated aliphatic fatty acids, including caproic acid, heptanoic acid, caprylic acid, nonanoic acid, capric acid, undecanoic acid, lauric acid, tridecanoic acid, myristic acid, pentadecanoic acid, nonadecanoic acid, arachidic acid, heneicosanoic acid, behenic acid, tricosanoic acid and lignocenic acid, (*b*) fatty acyl esters and alcohols, including stearic acid methyl ester, *S*-stearoyl CoA, palmitic acid methyl ester, *S*-palmitoyl CoA, *cis*-9-tetradecenol and arachidonyl alcohol, and (*c*) vitamin A related compounds, including α- and β-carotenes, retinoic acid, 9-*cis*-retinal, retinal and 13-*cis*-retinal. At this high concentration, some aliphatic lipids showed inhibitory activities that were significantly lower than those of the corresponding unsaturated fatty acids (% inhibition in the parentheses): myristoleic acid methyl ester (27 %), γ-linolenic acid methyl ester (32 %) and *cis*-4,7,10,13,16,19-docosahexaenol (51 %). Retinol, 13-*cis*-retinoic acid and 13-*cis*-retinol showed 58 % stimulation at 200 μM but no stimulation or inhibition at 40 μM. At 200 μM, the percentage inhibition (in parentheses) shown by some prostaglandins were: A_1 (39 %), A_2 (68 %), B_1 (44 %), B_2 (43 %), D_2 (47 %), E_1 (41 %), $F_{1\alpha}$ (37 %), E_2 (0 %), $F_{2\alpha}$ (0 %) and I_2 (0 %). At 40 μM, prostaglandin A_2 showed 50 % inhibition, but other prostaglandins were not active. IC_{50} values for potent fatty acids were: γ-linolenic acid (10 μM), octadecatetraenoic acid (57 μM), α-linolenic acid (60 μM), arachidonic acid (65 μM), palmitoleic acid (108 μM), linoleic acid (117 μM) and oleic acid (128 μM).

Test compound		Inhibition of [^{3}H]4-MA binding (%)		
Name	Numeric symbol	Concn. (μM)... 10	40	200
Undecylenic acid	$C_{11:1.10}$		NA	13±2
Myristoleic acid	$C_{14:1.cis\text{-}9}$	NA	25±4	43±1
Palmitic acid	$C_{16:0}$			NA
Palmitoleic acid	$C_{16:1.cis\text{-}9}$	NA	16±5	73±7
Palmitoleic acid methyl ester			NA	NA
Palmitoleyl alcohol			NA	16±4
Stearic acid	$C_{18:0}$	NA	NA	NA
Petroselinic acid	$C_{18:1.cis\text{-}6}$		NA	52±9
Oleic acid	$C_{18:1.cis\text{-}9}$	NA	16±6	63±12
Elaidic acid	$C_{18:1.trans\text{-}9}$	NA	NA	NA
Oleic acid methyl ester			NA	NA
Oleyl alcohol			NA	NA
Linoleic acid	$C_{18:2.cis\text{-}9.12}$	NA	12±3	86±4
Linolelaidic acid	$C_{18:2.trans\text{-}9.12}$		NA	19±5
Linoleic acid methyl ester			NA	NA
Linoleyl alcohol		NA	NA	25±5
α-Linolenic acid	$C_{18:3.cis\text{-}9.12.15}$	19±3	27±7	84±6
α-Linolenic acid methyl ester		NA	NA	NA
α-Linolenyl alcohol		NA	NA	24±1
γ-Linolenic acid	$C_{18:3.cis\text{-}6.9.12}$	50±2	83±12	96±2
Octadecatetraenoic acid	$C_{18:4.cis\text{-}6.9.12.15}$	NA	40±6	88±2
Arachidonic acid	$C_{20:4.cis\text{-}5.8.11.14}$	NA	30±10	88±5
Docosahexaenoic acid	$C_{22:6.cis\text{-}4.7.10.13.16.19}$	NA	27±1	87±6
Erucic acid	$C_{22:1.cis\text{-}13}$		NA	NA
Nervonic acid	$C_{24:1.cis\text{-}15}$		NA	NA

docosahexaenoic acid was a potent inhibitor. Undecylenic acid ($C_{11:1.10}$) and nervonic acid ($C_{24:1.cis\text{-}15}$) were also inactive. A free carboxyl group is important, since the methyl ester and alcohol analogues of these inhibitory unsaturated fatty acids were either inactive or only slightly active. Prostaglandin E_2, $F_{2\alpha}$ and I_2 were also not active, whereas the A_1, A_2, B_1, B_2, D_2, E_1 and $F_{1\alpha}$ forms were somewhat active at 0.2 mM. Carotenes, retinals and retinoic acid were also inactive. Phosphatidylcholine, phosphatidylethanolamine, 3-diolein, retinol, 13-*cis*-retinoic acid, and 13-*cis*-retinol were slightly stimulatory.

The inhibitory effects of fatty acids were also tested by the enzymic assay. The relative potencies of these fatty acids were in agreement with those obtained by the [^{3}H]4-MA-binding assay (Table 1) whether rat liver microsomes or prostate microsomes were used as the source of the enzyme. The *trans* isomers, elaidic acid ($C_{18:1.trans\text{-}9}$) and linolelaidic acid ($C_{18:2.trans\text{-}9.12}$) were much less inhibitory than their *cis* isomers, oleic acid ($C_{18:1.cis\text{-}9}$) and linoleic acid ($C_{18:2.cis9.12}$), in the [^{3}H]4-MA binding assay (Table 1 and Fig. 1*a*), but they were as potent as their *cis* isomers in the enzymic assay using either liver microsomes (Fig. 1*b*) or prostate microsomes (results not shown). Fig. 2 also shows that the extent of inhibition with a given concentration of oleic acid or linoleic acid was greater in the enzymic assay than in the binding assay. This was apparently due to the fact that a larger amount of microsomes was used for the binding assay than for the enzymic assay.

Kinetic studies of γ-linolenic acid inhibition of 5α-reductase

With either the enzymic assay (results not shown) or the [^{3}H]4-MA binding assay (Fig. 2), inhibition was observed within 1 min after γ-linolenic acid was mixed with the microsomal enzyme preparation, and was observed with both intact and detergent (polyoxyethylene ether)-solubilized rat liver microsomes (Fig. 3). As the concentrations of protein increased from 2 to 20 μg, the extent of inhibition by 10 μM-γ-linolenic acid decreased from 93 % to 52 % for intact microsomes and from 96 % to 88 % for solubilized microsomes. When [^{3}H]4-MA was allowed to bind to microsomes in the presence of NADPH first and then γ-linolenic acid was added to 10 μM, about 60 % of the microsome-bound [^{3}H]4-MA dissociated from the microsomes within 2 min. The remaining microsome-bound [^{3}H]4-MA dissociated at a much lower rate over the next 60 min. To determine whether γ-linolenic acid inhibition is reversible, we incubated microsomes with γ-linolenic acid and then re-isolated the microsomes to

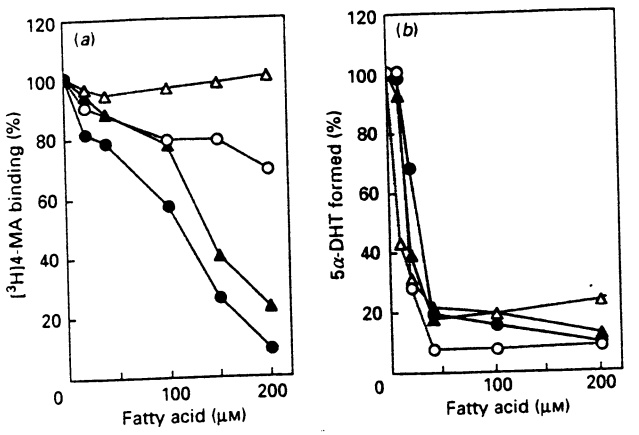

Fig. 1. Effects of oleic acid (▲), linoleic acid (●), elaidic acid (△) and linolelaidic acid (○) on rat liver microsomal 5α-reductase activity as determined by [^{3}H]4-MA binding assay (*a*) or the enzymic assay (*b*)

The amount of rat liver microsomes was 10 μg of protein in the binding assay and 2 μg of protein in the enzymic assay. In the absence of lipid, the control value for the [^{3}H]4-MA binding assay was 30618±975 d.p.m. The control value for the enzymic assay was 9.0±0.9 nmol of 5α-DHT formed/15 min using 0.5 μM-testosterone as substrate. These control values were taken as 100% activity.

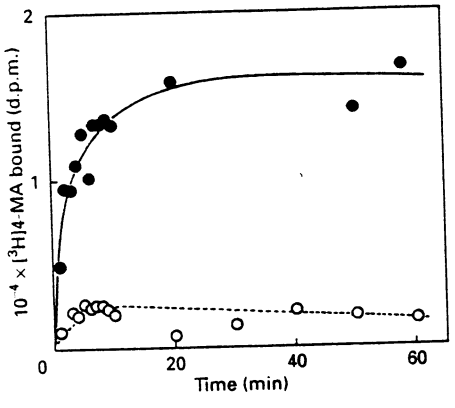

Fig. 2. Time course of γ-linolenic acid inhibition of [^{3}H]4-MA-binding to rat liver microsomes (5 μg of protein)

The experiment was carried out as described in the Materials and methods section. The concentration of γ-linolenic acid was 5 μM. ○, Control; ●, γ-linolenic acid.

remove free γ-linolenic acid. The results showed that the inhibition was only partially reversed (reduced from 78% to 63% inhibition). It was possible that γ-linolenic acid was bound tightly to microsomes and/or irreversibly inactivated components which were essential for the reductase activity.

By either the enzymic or the [^{3}H]4-MA binding assay, the inhibition could not be overcome by increasing the level of NADPH or testosterone (Fig. 4). γ-Linolenic acid did not appear to compete with testosterone or NADPH for their binding to the microsomal reductase. Double reciprocal plots of the data showed that 5 μM-γ-linolenic acid increased the apparent K_m values for NADPH (from 2.0 to 3.1 mM) and testosterone (from 2.4 to 4.5 μM), and decreased the $V_{max.}$ from 7.5 to 2.8 pmol of 5α-DHT formed/15 min per μg of protein. γ-Linolenic acid at 5 and 10 μM increased the apparent K_i values for [^{3}H]4-MA from 13 to 20 and 40 nM respectively, and decreased the maximal binding from 0.56 to 0.45 and 0.40 pmol/10 μg of protein respectively.

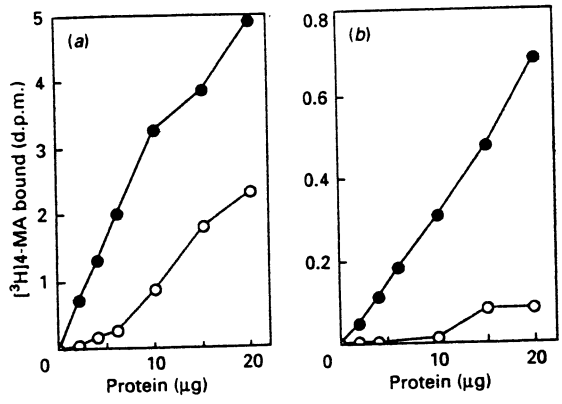

Fig. 3. Inhibition of [^{3}H]4-MA binding to 5α-reductase in intact (*a*) and detergent-solubilized (*b*) rat liver microsomes by γ-linolenic acid

The [^{3}H]4-MA-binding assay was carried out in the absence (●) and presence (○) of 10 μM-γ-linolenic acid and various amounts of microsomal protein.

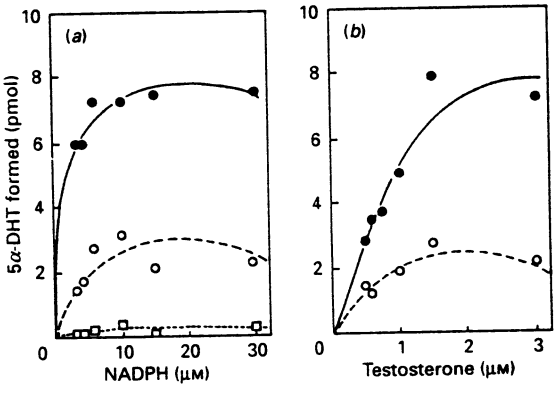

Fig. 4. Inhibition of 5α-reductase activity by γ-linolenic acid at various concentrations of NADPH (*a*) and testosterone (*b*)

The concentrations of γ-linolenic acid were 0 (●), 5 (○) and 15 (□) μM. The conversion of [^{3}H]testosterone to [^{3}H]5α-DHT was determined as described in the Materials and methods section.

Inhibition of 5α-reductase activities of human liver microsomes and human prostate cancer cells by γ-linolenic acid

γ-Linolenic acid also inhibited NADPH-dependent [^{3}H]4-MA binding to human liver microsomes to the same degree as in the experiments with the rat liver microsomes (results not shown). The 5α-reduction of [^{3}H]testosterone by human prostate cancer cells in culture was also selectively affected by γ-linolenic acid. Table 2 shows that γ-linolenic acid, at 5–50 μM, inhibited 5α-reduction of [^{3}H]testosterone in both androgen-sensitive LNCaP cells and androgen-insensitive PC-3 cells. γ-Linolenic acid, however, did not affect the metabolism of testosterone to 4-androstenedione, suggesting that 17β-steroid dehydrogenase was not sensitive to this unsaturated fatty acid. Stearic acid (5–20 μM) did not affect the 5α-reductase or 17β-steroid dehydrogenase activities of PC-3 cells in culture.

Other microsomal enzymes and electron microscopic examination of microsomes

We tested the effect of γ-linolenic acid on the activities of another microsomal reductase and a microsomal enzyme that uses a steroid as a substrate to determine the specificity of the effect of γ-linolenic acid. γ-Linolenic acid at 10–40 μM did not affect the activities of NADH:menadione reductase or UDP-

Table 2. Inhibition of the formation of radioactive 4-androstenedione and 5α-DHT from [^{3}H]testosterone by human prostatic cancer cells in culture by γ-linolenic acid

Values were determined in duplicate as described in the Materials and methods section. The control values for the formation of 4-androstenedione and 5α-DHT by PC-3 cells were 400851±9507 d.p.m. and 12183±74 d.p.m. respectively. The control value for the formation of 5α-DHT by LNCaP cells was 4569±505 d.p.m. No 4-androstenedione formation was detected when LNCaP cells were used. γ-Linolenic acid and stearic acid, at the concentrations tested, did not produce any visible change in cell morphology during the 2 h incubation. IC_{50} values (four experiments) for γ-linolenic acid with the prostate cancer cells were 10±5 μM. ND, not detected.

Prostate cell line	Fatty acid added (μM)	Metabolites formed (% of control) 4-Androstendione	5α-DHT
PC-3	None (control)	100	100
	γ-Linolenic acid		
	1	102±6	98±6
	5	110±1	50±3
	20	99±2	2±2
	Stearic acid		
	5	103±2	123±2
	20	106.5	121±5
LNCaP	None (control)	ND	100
	γ-Linolenic acid		
	50	ND	27±0
	100	ND	9±4

Table 3. Selective inhibition of 5α-reductase in rat liver microsomes by γ-linolenic acid

Assays were carried out as described in the Materials and methods section.

Enzyme	γ-Linolenic acid (μM)	Activity (%)
5α-Reductase	0	100±7
	1.8	76±4
	5.4	17±7
Glucuronosyltransferase	0	100±4
	5	100±0
	15	101±14
NADH:menadione reductase	0	100±2
	20	112±11
	30	104±3
	40	90±4

glucuronic acid:5α-DHT glucuronosyltransferase (Table 3). The electron microscopic examination of liver microsomal preparations before and after their incubation in the absence or presence of 10–40 μM of γ-linolenic acid under the experimental conditions did not show any obvious change in organelle structures.

DISCUSSION

Mammalian 5α-reductase is a cellular membrane-bound enzyme. Perturbation of the lipid matrix of the membranes may affect reductase activity non-specifically. The fact that only unsaturated fatty acids with specific configurations were potent inhibitors of 5α-reductase and that two other microsomal enzymes examined were not affected suggests that the inhibition is selective. Since specific inhibition was also observed with intact prostate cells in culture, externally added fatty acids were able to enter cells and exert their inhibitory action on the nuclear or endoplasmic reticulum membrane-bound 5α-reductase *in situ*. Many of the potent unsaturated fatty acids are natural components of mammalian lipids. The acylated unsaturated fatty acids constitute about 50% of total fatty acid in mammalian triacylglycerols and phospholipids. Since these conjugated acids are not inhibitory, and free acids can be generated from these lipids by lipases in the cells (Lands, 1965), the inhibitory activity may be due to non-conjugated unsaturated fatty acids. Further studies are necessary to determine the physiological significance of our findings *in vitro*. Since 5α-DHT stimulates sebum production and promotes acne, it is worth noting that lipids from the scalp of severe acne patients have been found to contain less linoleic acid than that of normal subjects (Morello *et al.*, 1976).

The ability of γ-linolenic acid to inhibit 5α-reductase in solubilized microsomes suggests that the γ-linolenic acid inhibition may not be rigidly dependent on the native structure of endoplasmic reticulum membranes. Whether the fatty acid inhibitors act by interacting with the reductase and/or other components that are vital for reductase activity is not clear. The inhibitory fatty acids may also interact with and potentiate other endogenous inhibitors or lipids.

The proposed mechanism (Brandt *et al.*, 1990) of 5α-reductase (E) reaction includes the following steps:

$$\text{NADPH} + \text{E} \leftrightarrow [\text{NADPH–E}] \xleftarrow{(a)} \xrightarrow{-\text{Testosterone (T)}} [\text{NADPH–E–T}] \xrightarrow{-\text{H}^+}$$

$$[\text{NADP}^+\text{–E–5}\alpha\text{-DHT}] \xrightarrow{(b)}$$

$$5\alpha\text{-DHT} + [\text{NADP}^+\text{–E}] \rightarrow \text{NADP}^+\text{E}$$

It is intriguing that two *trans* isomers of fatty acids tested, elaidic acid and linolelaidic acid, did not have any significant inhibitory activity in the [^{3}H]4-MA binding assay but were as potent as their *cis* isomers, oleic acid and linoleic acid, in the enzymic assay. It is possible that the *cis* unsaturated fatty acids inhibit the formation of [NADPH–E–T] (step *a*), whereas the *trans* isomers act on steps after the formation of the ternary complex (step *b*). However, our present results do not exclude the possibility that *cis* unsaturated fatty acids may also act after formation of this ternary complex. Steroidal inhibitors that can inhibit either step (*a*) or step (*b*) have been found (Liang & Heiss, 1981; Liang *et al.*, 1984*b*, 1985*b*; Brandt *et al.*, 1990).

A reductase gene coding for a 29 kDa polypeptide has been cloned from human (Anderson & Russell, 1990) and rat (Anderson *et al.*, 1989) tissues. These gene sequences have been shown to code for enzymically active 5α-reductase in frog oocytes and in a simian kidney cell line. It is not known whether components other than the 29 kDa polypeptide are essential for reductase function. Previously it was shown that the reductase may be present as a 50 kDa protein (Liang *et al.*, 1985*b*). The relationship between the 29 kDa unit and the 50 kDa form of 5α-reductase is not clear.

The stimulatory effect of certain phospholipids on 5α-reductase activity has been reported previously (Ichihara & Tanaka, 1987; Cooke & Robaire, 1985). We also found that L-α-phosphatidylcholine and L-α-phosphatidylethanolamine can stimulate 5α-reductase. Phospholipids may affect the conformation of 5α-reductase. Whether unsaturated fatty acids can counteract the phospholipid stimulation is not known. Retinoic acid had been previously reported to inhibit 5α-reductase in the human prostate cancer cell PC-3 and in PC-3 cell homogenates (Halgunset *et al.*, 1983); inhibition was shown to be competitive with NADPH. We, however, found that retinoic acid at 40–200 μM had no effect on 5α-reductase activity in rat liver microsomes. The reason for the discrepancy is not clear.

Polyunsaturated fatty acids can correct the effects of fatty acid deficiency that manifests as dermatitis, kidney necrosis, infertility and cardiovascular disease (Phillipson *et al.*, 1985; Herold & Kinsella, 1986; Ziboh & Miller, 1990) and also can exhibit anti-tumour activities (Karmali *et al.*, 1984; Begin, 1990). Many unsaturated fatty acids are essential components of mammalian membranes, most of them are in the acylated form as triacylglycerols and phospholipids (Lands, 1965). Arachidonic acid serves as a specific precursor in the biosynthesis of prostaglandins and leukotrienes (Needleman *et al.*, 1986). Several membrane-associated enzymes (5′-nucleotidase, acetyl-CoA carboxylase, glucose-6-phosphate dehydrogenase, etc.) have been shown to be affected by the polyunsaturated fatty acid content of the dietary fat, which changes the physicochemical properties of cellular membranes (Zuniga *et al.*, 1989; Szepsesi *et al.*, 1989). Treatment of cerebral cortical slices (Baba *et al.*, 1984) or intact retinas (Tesoriere *et al.*, 1988) with unsaturated fatty acids can enhance adenylate cyclase activities, and various types of phospholipases in rat ventricular myocytes are modulated differentially by different unsaturated fatty acids in the culture medium (Nalbone *et al.*, 1990). Very few studies, however, have been carried out on the mode of action of non-esterified fatty acids on enzymes in cell-free systems. Certain *cis*-unsaturated fatty acids, at 50 μM, were shown to stimulate protein kinase C activity (Dell & Severson, 1989) and inhibit steroid binding to receptors for androgens, oestrogens, glucocorticoids and progestins (Vallette *et al.*, 1988; Kato, 1989).

Clinical observations of patients with 5α-reductase deficiency (Imperato-McGinley *et al.*, 1979) and studies of the effects of 5α-reductase inhibitors on experimental animals (Brooks *et al.*, 1982; Liang *et al.*, 1984*a*; George *et al.*, 1989) have indicated that spermatogenesis, maintenance of libido, sex behaviour and feedback inhibition of gonadotropin secretion do not require the conversion of testosterone to 5α-DHT. Thus treatments of androgen-dependent skin and prostatic diseases by 5α-reductase inhibitors would be expected to produce fewer side-effects than the presently available hormonal therapies that involve castration and/or administration of oestrogens, androgen receptor antagonists (e.g. flutamide, spironolactone) or gonadotropin-releasing hormone superagonists (e.g. luprolide). In clinical studies, finasteride (a 4-azasteroid also known as MK906 or Proscar) that inhibits 5α-reductase by competing with testosterone (Liang *et al.*, 1985*a*), has been found to decrease the blood level of 5α-DHT and reduce prostate size (Vermeulen *et al.*, 1989; Rittmaster *et al.*, 1989; Gormley *et al.*, 1990; Imperato-McGinley *et al.*, 1990). Our identification of specific unsaturated fatty acids as potent inhibitors of 5α-reductase may lead to the design of new types of 5α-reductase inhibitors.

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