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INDOMETHACIN-SENSITIVE 3α-HYDROXYSTEROID DEHYDROGENASE IN RAT TISSUES

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Abstract—The purified 3α-hydroxysteroid dehydrogenase (EC 1.1.1.50) of rat liver cytosol is potently inhibited by the nonsteroidal anti-inflammatory drugs in rank-order of their therapeutic potency, i.e. by micromolar concentrations that would inhibit cyclooxygenase [T. M. Penning, and P. Talalay, *Proc. natn. Acad. Sci. U.S.A.* **80**, 4504 (1983)]. In the present study, indomethacin-sensitive 3α-hydroxysteroid dehydrogenase is shown to exist in seven rat tissues, including those that require androgens for growth (e.g. prostate) and those that rapidly metabolize prostaglandins (e.g. lung). Thus, the reduction of 5α-dihydrotestosterone catalyzed by prostatic cytosol was potently inhibited by indomethacin (IC_{50} = 10 μM), while the reduction of 5β-dihydrocortisone catalyzed by liver, lung and testis was more sensitive to inhibition by this drug (IC_{50} 1–3 μM). These data suggest that, under conditions in which cyclooxygenase is inhibited, androgen and cortisone metabolism may be affected. A surprising feature is that the specific activity of the indomethacin-sensitive dehydrogenase was higher in the lung than in tissues that are hormonally responsive (e.g. prostate and testis).

3α-Hydroxysteroid dehydrogenase† (EC 1.1.1.50) catalyzes the reversible $NAD(P)^+$-linked oxidation of 3α-hydroxysteroids to 3-ketosteroids [1]. The role of this enzyme in cortisol metabolism was first recognized by Tomkins [2, 3] who described the pyridine nucleotide-dependent oxidoreduction of 5β-dihydrocortisone to tetrahydrocortisone. Since that time, the cytosolic 3α-hydroxysteroid dehydrogenases of liver and prostate have been shown to catalyze the first step in androgen metabolism, namely, the reduction of 5α-dihydrotestosterone to 3α-androstanediol [4, 5]. Recently, the 3α-hydroxysteroid dehydrogenase of rat liver cytosol has been purified to homogeneity and appears to be identical with dihydrodiol dehydrogenase (EC 1.3.1.20) [6] which may detoxify proximate carcinogens [7, 8]. Striking properties of the purified enzyme include its high affinity binding site for prostaglandins, and its sensitivity to inhibition by the nonsteroidal anti-inflammatory drugs (NSAIDs; [6]). This inhibition occurs at concentrations comparable to those reported for the inhibition of preparations of sheep and bovine seminal vesicle cyclooxygenase [6]. If these observations were to hold for all tissues, they would suggest that NSAIDs may alter the metabolism of steroid hormones and carcinogenic *trans*-dihydrodiols. In this study we have measured the ability of indomethacin to inhibit either the oxidation of 3α-hydroxysteroids or the reduction of 3-ketosteroids in seven rat tissues, including those that are responsive to steroid hormones (e.g. prostate and testis), and the lung, which is known to rapidly metabolize prostaglandins [9]. In every tissue examined, steroid dehydrogenation was potently inhibited by this "aspirin-like" drug.

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† Abbreviations: 3α-Hydroxysteroid dehydrogenase, 3α-hydroxysteroid:NAD(P)-oxidoreductase (EC 1.1.1.50); androsterone, 5α-androstan-3α-ol-17-one; 3-α-androstanediol, 5α-androstane-3α,17β-diol; 5β-dihydrocortisone, 5β-pregnane-17α,21-diol-3,11,20-trione; tetrahydrocortisone, 5β-pregnane-3α,17α,21-triol-11,20-dione; 5α-dihydrotestosterone, 5α-androstan-17β-ol-3-one; and indomethacin, 1-(*p*-chlorobenzoyl)-5-methoxy-2-methyl-3-indoleacetic acid.

MATERIALS AND METHODS

Materials. Indomethacin, 5α-dihydrotestosterone, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase (Torula Yeast) were purchased from the Sigma Chemical Co. (St. Louis, MO). Androsterone and 5β-dihydrocortisone were products of Steraloids (Wilton, NH). β-NAD^+ and NADPH (monosodium salts) were purchased from P-L Biochemicals (Milwaukee, WI). [4-^{14}C]-5α-Dihydrotesterone (57.8 mCi/mmole) was obtained from the New England Nuclear Corp. (Boston, MA). Enzyme grade ammonium sulfate and sucrose were obtained from Schwarz/Mann, Inc. (Spring Valley, NY).

Enzyme preparation. Male Sprague–Dawley rats were killed by cervical dislocation, and tissues were rapidly removed, frozen in liquid nitrogen, and stored at −80°. Frozen tissues were thawed, homogenized in 3 vol. (w/v) of cold buffer containing 50 mM Tris–HCl, 250 mM sucrose, 1 mM EDTA, and 1 mM dithiothreitol (DTT) of pH 8.6, and centrifuged at 10,000 *g* for 30 min. The resulting supernatant fraction was filtered through non-absorbent cotton, and microsomes were harvested by centrifugation at 100,000 *g* for 1 hr. Three ammonium sulfate fractions of 0–40%, 40–75%, and >75% satu-

ration were prepared from the resulting supernatant fraction. The protein that precipitated in the first two fractions was collected by centrifugation at 15,000 *g* for 30 min, and was redissolved in a minimal volume of dialysis buffer containing 10 mM Tris–HCl, 1 mM EDTA, and 0.5 mM DTT of pH 8.6. All three fractions were then dialyzed overnight against three 1-liter volumes of the same buffer. The dialyzed fractions were then used for enzyme assays.

Spectrophotometric assay of 3α-hydroxysteroid dehydrogenase. Oxidoreductions were monitored by following the change in absorbance of the pyridine nucleotide at 340 nm; $\varepsilon = 6270\ M^{-1}\ cm^{-1}$ in a 1.0 cm light path using a Gilford model 260 UV/Vis recording spectrophotometer. Oxidation of androsterone was measured in a 1.0 ml system containing 100 mM potassium phosphate buffer of pH 7.0, 2.3 mM NAD^+ and 75 μM androsterone. Reduction reactions were also assayed in a 1.0 ml system which contained 100 mM potassium phosphate buffer of pH 6.0, 180 μM NADPH, and either 5α-dihydrotestosterone (30 μM), or 5β-dihydrocortisone (50 μM). Steroids were dissolved in acetonitrile and the final concentration of organic solvent was 4% (v/v). All reactions were initiated by addition of the crude preparation of enzyme. Corrections were made for non-enzymatic rates measured in the presence of nucleotide and steroid. Activities were expressed as nmoles of nucleotide transformed per min per mg protein.

Radiochemical assay of 3α-hydroxysteroid dehydrogenase. Portions (50 μl) of the ammonium sulfate fractions were added to 50 μl of 200 mM potassium phosphate buffer of pH 7.4 containing 20 nmoles EDTA, 3.5 nmoles [4-^{14}C]-5α-dihydrotestosterone (11,485 cpm/nmole), 420 nmoles $NADP^+$, 220 nmoles glucose-6-phosphate, and 1.0 unit of glucose-6-phosphate dehydrogenase. The steroid was dissolved in methanol, and the final concentration of organic solvent was kept constant at 6% (v/v). Assay

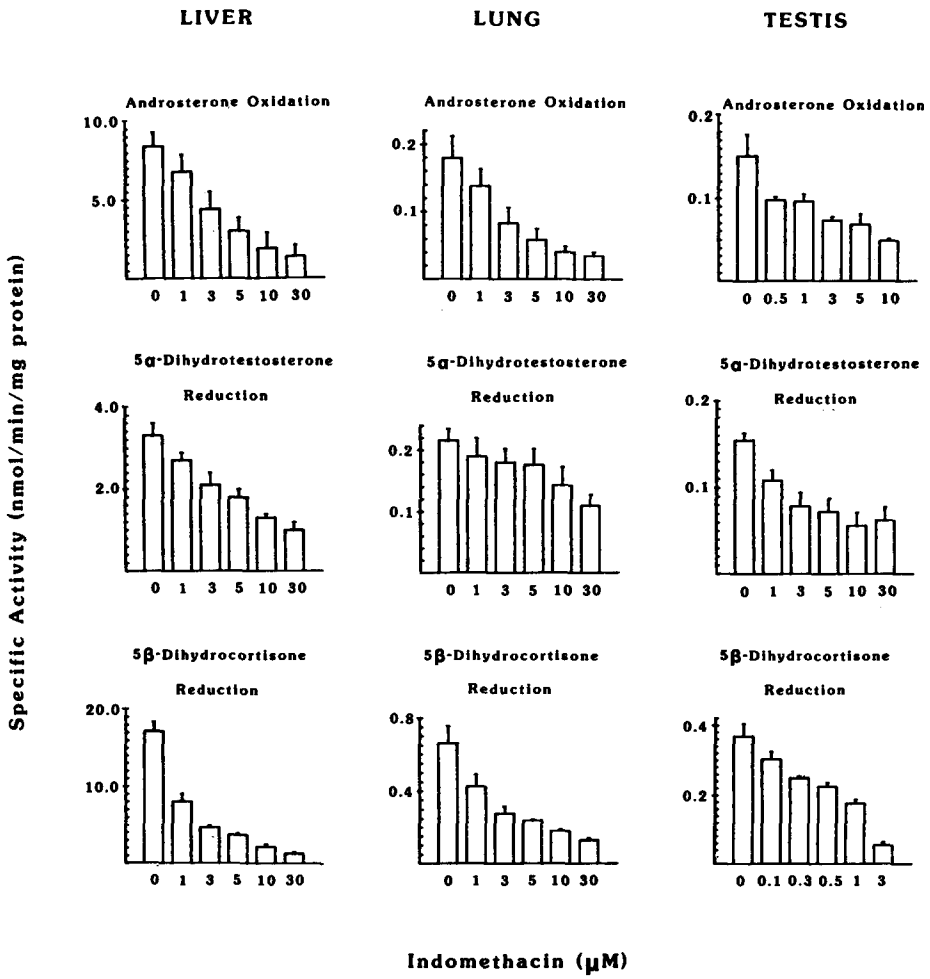

Fig. 1. Inhibition of 3α-hydroxysteroid dehydrogenase in 40–75% ammonium sulfate fractions of rat liver, lung, and testis cytosols by indomethacin. Oxidation of androsterone and reduction of 5α-dihydrotestosterone and 5β-dihydrocortisone were monitored spectrophotometrically. Reactions were run in the presence and absence of indomethacin; the concentration of drug required to produce at least 50% inhibition of each activity was determined. Each bar represents the mean value ± S.E.M. of specific activities measured in tissue cytosols prepared from at least three different animals.

mixtures were incubated at 37° for 15 min and were terminated by the addition of ethyl acetate (200 μl). The resulting extracts were evaporated to dryness, the residues (substrate and product) were redissolved in 40 μl methanol, and 20 μl aliquots were applied to Gelman instant thin-layer chromatography sheets (Gelman Instrument Co., Ann Arbor, MI), which were developed in chloroform–ethyl acetate (4:1). Labeled steroids were localized on the chromatogram by running standards of substrate and product along each edge, and visualizing them with a *p*-anisaldehyde spray. Radioactive 5α-dihydrotesterone ($R_f = 0.46$) and 3α-androstanediol ($R_f = 0.26$) were cut from the paper chromatogram and placed in a toluene-based scintillation fluid containing 4 g PPO (2,5-diphenyloxazole) and 50 mg POPOP (p-*bis*-[2-(5-phenyloxazolyl)]-benzene)/liter. Radioactive steroid was quantified by scintillation counting in a Tracor Analytic model 43 counter whose machine efficiency for ^{14}C was 96%. ^{14}C-Radioactivity was determined as corrected cpm. By using the specific radioactivity of the steroid, enzyme activity was expressed as nmoles of 3α-androstanediol formed per hr per mg protein. With every tissue examined, product formation was linear with respect to both protein concentration and time.

Inhibition by indomethacin. Using either the spectrophotometric or radiochemical assay, increasing amounts of indomethacin were added, while the concentration of organic solvent was kept constant. That concentration of drug required to produce 50% inhibition of the initial velocity was determined (IC_{50} value).

Protein determinations. Protein was measured by the method of Lowry *et al.* [10], using crystalline bovine serum albumin (Armour Pharmaceutical Co., Kankakee, IL) as a standard.

RESULTS

The oxidation of 3α-hydroxysteroids by NAD^+ and the reduction of 3-ketosteroids by NADPH catalyzed by cytosolic fractions prepared from rat liver, lung, and testis was monitored at 340 nm. Steroidal substrates included androsterone, 5α-dihydrotestosterone, and 5β-dihydrocortisone. In every instance, the majority of these activities precipitated in the 40–75% ammonium sulfate fraction. Examination of the specific activities for these reactions indicates that the liver cytosol contained 20- to 60-fold more activity than any other tissue (Fig. 1). Our results also show that these crude preparations of rat liver 3α-hydroxysteroid dehydrogenase retained their sensitivity to inhibition by indomethacin since the IC_{50} values for the oxidation of androsterone were 1–3 μM. These values are in excellent agreement with those seen for the purified enzyme [6]. Our data show for the first time that oxidation reactions of 3α-hydroxysteroids (i.e. androsterone) catalyzed by rat lung and testis cytosols were equally sensitive to inhibition by this drug (IC_{50} = 1–3 μM; Fig. 1).

The reduction reactions of 3-ketosteroids in the presence of NADPH, catalyzed by liver, lung and testis cytosols were also inhibited by indomethacin (IC_{50} = 1–30 μM). Although it is conceivable that these indomethacin-sensitive reactions may have been mediated via a 3β-hydroxysteroid dehydrogenase, two features argue against this possibility. First, the oxidation reactions of the 3β-hydroxysteroid (5β-androstan-3β-ol-17-one) catalyzed by these cytosols were insensitive to inhibition by this drug at concentrations (200 μM) that totally abolished 3α-hydroxysteroid dehydrogenase activity (data not shown). Second, in some rat tissue cytosols (e.g. liver) 3β-hydroxysteroid dehydrogenase is known to be an NAD^+-specific oxidoreductase [11].

It is of interest that, in liver, lung and testis, the NADPH-dependent reduction of 5β-dihydrocortisone catalyzed by the 3α-hydroxysteroid dehydrogenase was more sensitive to inhibition by indomethacin (IC_{50} = 1.0 μM or less) than the reduction of 5α-dihydrotesterone (IC_{50} = 5–30 μM). Although this observation could be explained by differences in K_m, it is noteworthy that the inhibition of the initial rate of 5α-dihydrotesterone reduction in lung and testis plateaued at higher drug levels. This suggests

Table 1. Demonstration of 3α-androstanediol as the major product of 5α-dihydrotestosterone reduction in rat spleen, heart, prostate, and seminal vesicle by carrier recrystallization*

Tissue	Recrystallization ([^{14}C]-3α-androstanediol, cpm/mg) 1st	2nd	3rd
Spleen	975	872	901
Heart	1028	1193	997
Prostate	888	921	1139
Seminal vesicle	511	622	587

* The 40–75% ammonium sulfate fractions prepared from the above rat tissue cytosols were incubated in the presence of [4-^{14}C]-5α-dihydrotestosterone and NADPH for 2 hr at 37°. Radioactive substrate and product were separated by TLC, and the product was extracted in ethyl acetate and evaporated to dryness. The total radioactivity present in each product was as follows: spleen, 46,800 cpm; heart, 62,000 cpm; prostate, 47,400 cpm; and seminal vesicle, 32,500 cpm. Unlabeled 3α-androstanediol (50 mg) was added to each radioactive residue and recrystallized three times from methanol. Results are expressed as cpm/mg of crystals produced from each successive recrystallization.

that in these two tissues the reduction of 3-keto-steroids in the presence of indomethacin may be directed down an alternative pathway.

For several tissues (spleen, prostate, heart and seminal vesicle) the spectrophotometric assay was not sensitive enough to detect 3α-hydroxysteroid dehydrogenase. Therefore, a radiochemical assay was devised in which the reduction of [4-^{14}C]-5α-dihydrotestosterone was followed (see Materials and Methods). With this procedure, ammonium sulfate fractions prepared from cytosols of these tissues were assayed for enzyme activity. In every case, the steroid dehydrogenase predominated in those fractions saturated with 40–75% ammonium sulfate. To ensure that this assay was specific for 3α-hydroxysteroid dehydrogenase, the entire radioactive product from each tissue was shown to co-crystallize with unlabeled 3α-androstanediol to constant specific radioactivity (Table 1). In these four tissues, specific activities ranged from a high of 6.96 nmoles 3α-androstanediol formed per hr per mg protein for the heart, to a low of 1.68 nmoles 3α-androstanediol formed per hr per mg protein for the seminal vesicle. It is surprising that the heart showed an approximately 3-fold higher specific activity than that present in either seminal vesicle or prostate, since these two latter tissues are androgen-dependent and are traditionally associated with high levels of 3α-hydroxysteroid dehydrogenase.

In all four tissues, greater than 80% of [4-^{14}C]-5α-dihydrotestosterone reduction was inhibited by low concentrations of indomethacin (IC_{50} values of 10 μM or less, Fig. 2).

DISCUSSION

In every tissue examined, indomethacin-sensitive 3α-hydroxysteroid dehydrogenase was detected. It is of interest that the highest specific activities were measured in tissues not generally associated with steroid hormone transformation. For example, the lung maintained the second highest level of enzyme activity (superceded only by the liver), while the heart had activity 3-fold higher than that found in androgen-dependent tissues (prostate and seminal vesicle). As the lung is a major site of both prostaglandin [9] and polycyclic aromatic hydrocarbon metabolism [12], it is conceivable that the lung 3α-hydroxysteroid dehydrogenase may bind prostaglandins and dehydrogenate *trans*-dihydrodiols (e.g. benzenedihydrodiol) in a fashion similar to the liver enzyme [6, 8]. These possibilities are presently being investigated.

Our data indicate that the reduction of 5α-dihydrotestosterone and 5β-dihydrocortisone catalyzed by the various tissues was inhibited by the same low concentrations of indomethacin (IC_{50} values 1–30 μM) that inhibit cyclooxygenase prepared from seminal vesicle microsomes [13, 14]. This suggests that indomethacin may alter androgen and cortisol metabolism in hormonally responsive and unresponsive tissues.

Wilson and his colleagues [5] have shown that the reduction of 5α-dihydrotestosterone to 3α-androstanediol is the major metabolic route for androgens in the prostate and have implicated the 3α-hydroxysteroid dehydrogenase in the regulation of andro-

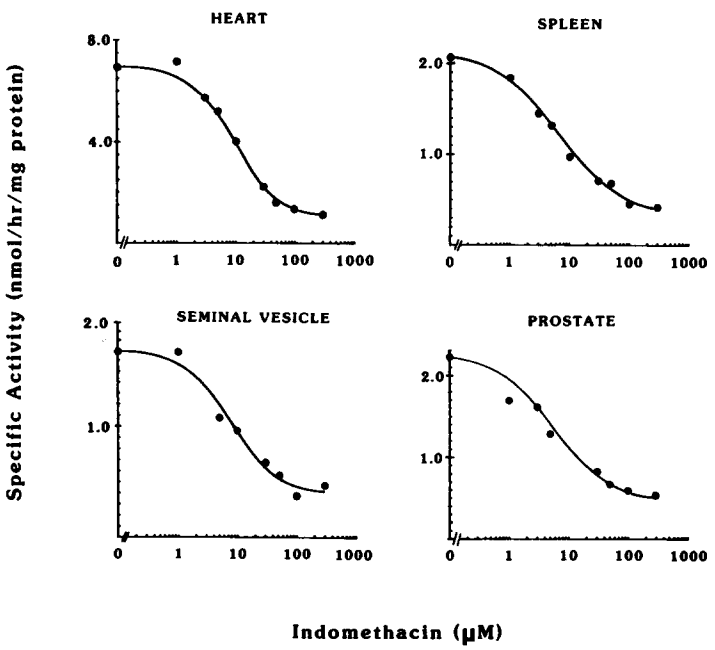

Fig. 2. Inhibition of [4-^{14}C]-5α-dihydrotestosterone reduction in rat heart, spleen, seminal vesicle, and prostatic cytosols by indomethacin. The 40–75% ammonium sulfate fractions were incubated with [4-^{14}C]-5α-dihydrotestosterone and NADPH at 37°, in the absence and presence of 1–300 μM indomethacin. Radioactive product (3α-androstanediol) was separated from unreacted substrate by TLC, and quantified by scintillation counting. Specific activity was expressed as nmoles of 3α-androstanediol formed per hr per mg protein.

gen action. Inhibition of the enzyme by indomethacin suggests that, under certain conditions, this drug may act as a potentiator of androgen action.

It is now accepted that the metabolism of glucocorticoids occurs via two steps: in the first, the Δ^4-3-ketone of the steroid is reduced by 5β-reductase to yield a 5β-dihydroglucocorticoid and, in the second, 3α-hydroxysteroid dehydrogenase reduces the 5β-3-ketosteroid to a tetrahydroglucocorticoid. Inhibition of this second step of glucocorticoid metabolism by low doses of indomethacin may lead to an accumulation of cortisol so that a "glucocorticoid-saving" effect results in a variety of tissues. It is of interest that indomethacin has been shown to enhance plasma prednisolone levels [15], and can reduce the metabolism of 3-ketosteroids (e.g. corticosterone) in rats following chronic *in vivo* treatment with the drug [16].

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