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INDOMETHACIN AND GLUCOCORTICOID METABOLISM IN RAT LIVER CYTOSOL

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Abstract—3α-Hydroxysteroid dehydrogenase (EC 1.1.1.50) of rat liver cytosol catalyzes the second step in glucocorticoid metabolism, namely the NADPH-dependent reduction of 5β-dihydrocortisol to tetrahydrocortisol. The purified enzyme is potently inhibited by the nonsteroidal anti-inflammatory drugs [Penning and Talalay, *Proc. natn. Acad. Sci. U.S.A.* **80**, 4504 (1983)], and the consequences of this inhibition on hepatic glucocorticoid metabolism are now examined. Analyses of the specific activities for the reduction of 5β-dihydrocortisone catalyzed by homogenous preparations of 3α- and 3β-hydroxysteroid dehydrogenases of rat liver cytosol indicated that this steroid was predominantly reduced to the 3α-hydroxysteroid (tetrahydrocortisone). Whether this reaction was catalyzed by the purified 3α-hydroxysteroid dehydrogenase or unprocessed cytosol, it was potently inhibited by indomethacin (IC_{50} = 0.6 μM). In the presence of 30 μM indomethacin, the rate of reduction of 5β-dihydro-glucocorticoids was 16 times smaller than in the absence of drug. Measurement of 5β-reductase activity in rat liver cytosol indicated that it was 25–30 times less active than the 3α-hydroxysteroid dehydrogenase. In the presence of 30 μM indomethacin, enough residual dehydrogenase may exist to reduce 5β-dihydro-glucocorticoids as they are formed. This was confirmed by incubating the supernatant fraction obtained from the 10,000 *g* centrifugation of a rat liver homogenate, with [1,2-^{3}H]cortisol plus NADPH in the presence and absence of drug. At the end of the incubation, cortisol metabolites were analyzed by TLC and the results expressed as CL:CM ratio (cortisol left:cortisol metabolized). It was found that no change in this ratio occurred in the presence of the drug. Moreover, the addition of trapping quantities of 5β-dihydrocortisol to incubations containing 30 μM indomethacin were insufficient to promote an increase in cortisol levels as reflected by a rather small increase in the CL:CM ratio. Thus, nonsteroidal anti-inflammatory drugs cannot effect a rise in hepatic glucocorticoid levels *in vitro*, and do not promote a "glucocorticoid saving effect" in this organ.

Indomethacin is a nonsteroidal anti-inflammatory drug which prevents prostaglandin synthesis [1–3] by inhibiting fatty acid cyclo-oxygenase [4]. We have purified to homogeneity the 3α-hydroxysteroid dehydrogenase† from rat liver cytosol, and a surprising property of this enzyme is its potent inhibition by the "aspirin-like" drugs [5, 6]. Thus, comparable concentrations of indomethacin inhibit both the dehydrogenase and the fatty acid cyclo-oxygenase. Upon further examination, it appears that the inhibition of the dehydrogenase by nonsteroidal anti-inflammatory drugs displays several correlates that suggest it may be an alternative target enzyme for these agents. First, the rank order of inhibitory potency of the "aspirin-like" drugs for the dehydrogenase parallels that observed for cyclo-oxygenase [5]. Second, the IC_{50}, values of nonsteroidal anti-inflammatory drugs for the dehydrogenase are substantially lower than their peak plasma concentrations [5]. Third, the indomethacin-sensitive dehydrogenase is widely distributed in rat tissues, being present in tissues that require androgens for growth (e.g. prostate) and in those that rapidly metabolize prostaglandins (e.g. lung and spleen) [7]. Fourth, the dehydrogenase binds prostaglandins at the active site with an affinity in the low micromolar range [5, 6]. These findings have led us to investigate possible mechanisms by which inhibition of the dehydrogenase by the nonsteroidal anti-inflammatory drugs could lead to an anti-inflammatory response.

One reaction which the 3α-hydroxysteroid dehydrogenase catalyzes is the second step in glucocorticoid metabolism, namely the NADPH-linked reduction of 5β-dihydrocortisone (a 3-ketosteroid) to tetrahydrocortisone (a 3α-hydroxysteroid) (see Fig. 1). Inhibition of glucocorticoid metabolism by indomethacin at the level of the dehydrogenase could promote a "glucocorticoid-saving effect" which would be anti-inflammatory. It is of interest that

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† Abbreviations: 3α-hydroxysteroid dehydrogenase, 3α-hydroxysteroid: NAD(P)†-oxidoreductase (EC 1.1.1.50); 3β-hydroxysteroid dehydrogenase, 3β-hydroxysteroid: NAD(P)†-oxidoreductase (EC 1.1.1.51) [in rat liver cytosol this enzyme is identical to alcohol dehydrogenase (EC 1.1.1.1)]; cortisol, 11β,17α-21-trihydroxy-pregn-4-ene-3,20-dione; cortisone, 17α,21-dihydroxypregn-4-ene-3,11,20-trione; 5β-dihydrocortisol, 11β,17α,21-trihydroxy-5β-pregnane-3,20-dione; 5β-dihydrocortisone, 17α,21-dihydroxy-5β-pregnane-3,11,20-trione; tetrahydrocortisol, 3α,11β,17α,21-tetrahydroxy-5β-pregnan-20-one; tetrahydrocortisone, 3α,17α,21-trihydroxy-5β-pregnane-11,20-dione; and indomethacin, 1-(*p*-chlorobenzoyl)-5-methoxy-2-methyl-3-indoleacetic acid.

Mathies [8] reported that therapeutic doses of indomethacin could enhance plasma prednisolone levels in patients with rheumatoid arthritis. In addition, chronic *in vivo* treatment of rats with indomethacin has been found to substantially reduce hepatic corticosterone metabolism [9]. The effect of indomethacin on glucocorticoid metabolism in male rat liver cytosol is the subject of the present paper.

MATERIALS AND METHODS

Chemicals

Glucocorticoids and their metabolites were products of Steraloids (Wilton, NH). [1,2-^{3}H]Cortisol (sp. act. 41.0 Ci/mmole) was purchased from Amersham (Arlington Heights, IL). Polysilicic acid impregnated glass fiber chromatography sheets (Gelman ITLC) were obtained from Fischer Scientific (King of Prussia, PA). Indomethacin, nicotinamide and glucose-6-phosphate dehydrogenase (Torula yeast) were purchased from the Sigma Chemical Co. (St. Louis, MO). NADH and NADPH were products of Pharmacia-P.L. Biochemicals (Piscataway, NJ).

Enzymes

Purified hydroxysteroid dehydrogenase. 3α-Hydroxysteroid dehydrogenase was purified to homogeneity by the procedure described by Penning *et al.* [5, 6]; the final specific activity of this enzyme was 3.58 μmoles of 5β-dihydrocortisone reduced/min/mg protein. Homogeneous 3β-hydroxysteroid dehydrogenase of rat liver cytosol was purified in the laboratory of Dr. Paul Talalay, The Johns Hopkins University School of Medicine, Baltimore, MD. The final specific activity of this enzyme was 2.20 μmoles of 5α-androstan-3β-ol-17-one oxidized/min/mg protein.

Crude preparations of 3α-hydroxysteroid dehydrogenase and 5β-reductase. Livers from adult male Sprague–Dawley rats (150–200 g) were excised and homogenized in 3 vol. (w/v) of cold buffer containing 50 mM Tris–HCl, 250 mM sucrose, 1 mM EDTA and 1 mM dithiothreitol, of pH 8.6. Following differential centrifugation, the cytosol was subjected to three ammonium sulfate fractions of 0–40%, 40–75% and greater than 75% saturation. In each case, the precipitated protein was collected by centrifugation, dissolved in a minimal volume of dialysis buffer, and dialyzed over night. Dialysis buffer contained 10 mM Tris–HCl, 1 mM EDTA and 0.5% dithiothreitol, of pH 8.6. All three fractions were assayed for 3α-hydroxysteroid dehydrogenase and 5β-reductase activity.

Spectrophotometric assays

3α- and 3β-Hydroxysteroid dehydrogenase. Assays were conducted in 1.0-ml systems containing 100 mM potassium phosphate buffer (pH 6.0), 50 μM steroid (5β-dihydro-glucocorticoid) and 180 μM pyridine nucleotide in the presence of 4% acetonitrile. The reaction was initiated by the addition of enzyme, and the decrease in absorbance of the reduced pyridine nucleotide was monitored at 340 nm at 25°. In every instance, the non-enzymatic rate observed in the presence of steroid and nucleotide was subtracted. This rate did not exceed 10% of the enzyme-catalyzed reactions. When the reactions were initiated with either cytosol or an ammonium sulfate fraction as the source of enzyme, the ability of these preparations to catalyze a reduction of the pyridine nucleotide in the absence of steroid was also determined. In these instances, no rates of reduction were observed.

5β-Reductase. Assays were conducted exactly as those described above except that the steroidal substrates were either cortisol or cortisone.

Cortisol metabolism in rat liver homogenates

Adult male Sprague–Dawley rats were killed by cervical dislocation, and their livers were excised and homogenized in 2 vol. (w/v) of cold buffer containing 100 mM potassium phosphate, 30 mM nicotinamide and 4 mM $MgCl_2$, of pH 7.3. The supernatant fraction that resulted from centrifugation of the homogenate at 10,000 *g* for 30 min (S10) was filtered through non-adsorbent cotton and used for subsequent studies.

Cortisol metabolism was followed in 1.0-ml incubations consisting of 0.9 ml S10 plus 0.1 ml of a mixture containing 5.0 nmoles [1,2-^{3}H]cortisol (0.1 μCi), 5.0 μmoles glucose-6-phosphate, 1.25 μmoles NADP and 1.0 unit Torula yeast glucose-6-phosphate dehydrogenase. Incubations were conducted at 37° for 10 min, and reactions were stopped by the addition of 1.0 ml water and 2.0 ml ethyl acetate. The aqueous phase was extracted twice with 2.0 ml of ethyl acetate, and the extracts were pooled and evaporated to dryness. The residues were redissolved in 40 μl of ethanol and 20-μl aliquots were applied to polysilicic acid impregnated glass fiber sheets (20 × 20) in lanes 1.5 cm wide. Along the edge of each sheet, standards of cortisol, 5β-dihydrocortisol and tetrahydrocortisol were applied. The chromatograms were then developed in dichloroethane–*t*-butanol (17:3, v/v) and the position of the standards was detected by the distinctive colors which form after spraying in para-anisaldehyde: tetrahydrocortisone, $R_f = 0.40$ (blue-gray); cortisol, $R_f = 0.60$ (orange); and 5β-dihydrocortisone, $R_f = 0.76$, (red). Each lane which corresponded to one assay was cut into 1.0-cm horizontal strips, and each strip was placed into 5 ml of a toluene-based scintillation fluid containing 4.0 g PPO (2,5-diphenyloxazole) and 50 mg POPOP (*p*-bis-[2-(5-phenyloxazolyl)]benzene)/litre. Radioactive steroid was then quantified by scintillation counting in a Tracor Analytical model 43 counter whose machine efficiency for ^{3}H was 53%. ^{3}H-Radioactivity was determined as corrected cpm.

Protein determinations

Protein concentrations were determined by the method of Lowry *et al.* [10] using crystalline bovine serum albumin (Armour Pharmaceutical, Kanakee, IL) as a standard.

RESULTS

3α-Hydroxysteroid dehydrogenase, the major enzyme responsible for the reduction of 5β-dihydrocortisone in rat liver cytosol

5β-Dihydrocortisone is a 3-ketosteroid and can

Table 1. Comparison of the specific activities for 5β-dihydrocortisone reduction catalyzed by homogeneous 3α- and 3β-hydroxysteroid dehydrogenases of rat liver cytosol

Enzyme	Substrate	Nucleotide	Specific activity (nmoles steroid reduced · min^{-1} · mg^{-1})
3α-Hydroxysteroid dehydrogenase	5β-dihydrocortisone	NADPH	4150
3β-Hydroxysteroid dehydrogenase	5β-dihydrocortisone	NADH	21

Assays were conducted in 1.0-ml systems containing 100 mM potassium phosphate buffer (pH 6.0), 50 μM steroid and 180 μM pyridine nucleotide plus 4% acetonitrile. The change in absorbance of the reduced nucleotide was monitored at 340 nm at 25°.

be reduced by both a 3α- and a 3β-hydroxysteroid dehydrogenase. Rat liver cytosol is known to contain both these enzymes which differ in their nucleotide specificity. Thus, the 3β-enzyme is NADH specific [11], whereas the 3α-enzyme will use either NADH or NADPH, but prefers NADPH [5, 6]. Comparison of the specific activities for the reduction of 5β-dihydrocortisone catalyzed by homogeneous preparations of these two rat liver enzymes, in the presence of the preferred nucleotide (Table 1), indicates that this steroid was predominantly reduced to the 3α-hydroxysteroid (tetrahydrocortisone). Since approximately equal amounts of the two enzymes can be purified from rat liver cytosol (Penning, unpublished observations), this finding may reflect the situation observed in the whole cell. This conclusion would confirm the original observations of Tomkins [12, 13] which showed that 3α-hydroxysteroids are the primary metabolites of glucocorticoids in rat liver cytosol.

Indomethacin inhibition of 5β-dihydro-glucocorticoid reduction in rat liver cytosol

The NADPH-linked reduction of 5β-dihydrocortisone catalyzed by homogeneous 3α-hydroxysteroid dehydrogenase, the 40–75% ammonium sulfate fraction of rat liver cytosol or unprocessed hepatic cytosol, was potently inhibited by indomethacin. Irrespective of the enzyme preparation used, the IC_{50} value for indomethacin (concentration required to produce 50% inhibition of the enzyme-catalyzed reaction) was identical and was found to be 0.6 μM (data not shown). Since this value is submicromolar, and well beneath the peak plasma concentration usually observed after oral administration of the drug [4], the possibility exists that nonsteroidal anti-inflammatory drugs may prevent the hepatic metabolism of glucocorticoids.

5β-Reductase, the rate-limiting step in hepatic glucocorticoid metabolism

The metabolism of glucocorticoids involves two steps: first, the delta-4-ene of the steroid is reduced (a reaction catalyzed by the 5β-reductase), and this is followed by reduction of the 3-ketone group (a reaction catalyzed by the 3α-hydroxysteroid dehydrogenase) (Fig. 1). My studies indicate that all the 5β-reductase and 3α-hydroxysteroid dehydrogenase activities present in rat liver cytosol precipitate following saturation with 40–75% ammonium sulfate. When this fraction was assayed for these enzymes (Table 2), their specific activities indicated that the reduction of cortisone to 5β-dihydrocortisone occurred at a rate that was 25–30 times smaller than the subsequent reduction of 5β-dihydrocortisone to tetrahydrocortisone. These findings

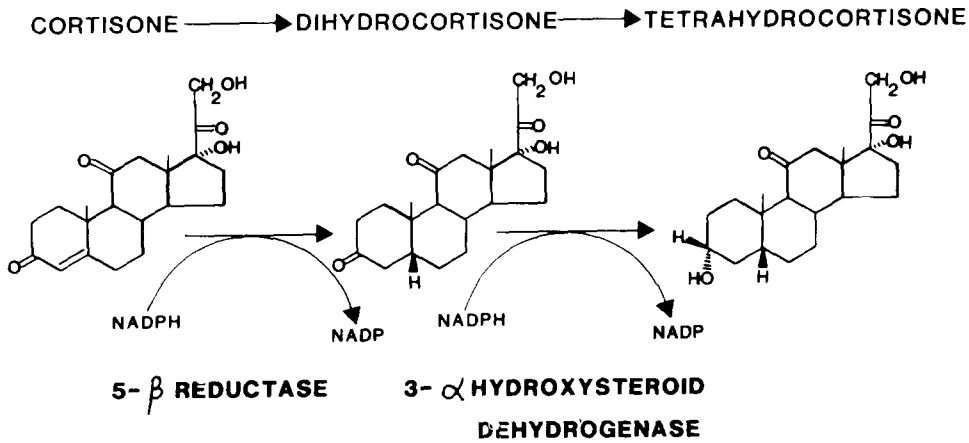

Fig. 1. Glucocorticoid metabolism in hepatic cytosol.

Table 2. Comparison of the specific activities of 3α-hydroxysteroid dehydrogenase and 5β-reductase of rat liver cytosol

Enzyme activity	Substrate	Specific activity (μmoles substrate reduced · min^{-1} · mg^{-1})	Ratio of specific activities (3α-hydroxysteroid dehydrogenase : 5β-reductase)
3α-Hydroxysteroid dehydrogenase	5β-dihydrocortisol	0.0110	26
5β-Reductase	Cortisol	0.00042	
3α-Hydroxysteroid dehydrogenase	5β-dihydrocortisone	0.0216	
5β-Reductase	Cortisone	0.000603	36

Fractionation of male rat liver cytosol with 40–75% ammonium sulfate precipitates all the soluble 5β-reductase and 3α-hydroxysteroid dehydrogenase. This ammonium sulfate fraction was assayed for both of these enzyme activities. Assays contained: 100 mM potassium phosphate (pH 6.0), 50 μM steroid and 180 μM NADPH plus 4% acetonitrile. The change in absorbance of nucleotide was monitored at 340 nm at 25°.

concur with those of Tomkins [12, 13] who showed that the 5β-reductase catalyzes the rate-limiting step in hepatic glucocorticoid metabolism.

Dihydro-glucocorticoid reduction in the presence of indomethacin

When the reduction of either 5β-dihydrocortisone or 5β-dihydrocortisol catalyzed by unprocessed rat liver cytosol was followed to completion in the presence and absence of 30 μM indomethacin (enough drug to inhibit the 3α-hydroxysteroid dehydrogenase by 90–95%; [5, 6]) the rate of formation of tetrahydro-glucocorticoids was 16 times smaller in the presence of drug (Fig. 2). When this change in rate (16-fold) is compared with the ratio of the specific activity of 3α-hydroxysteroid dehydrogenase to that of 5β-reductase (25–30 : 1), it appears that sufficient residual dehydrogenase activity may be present to catalyze the reduction of 5β-dihydro-glucocorticoids as they are formed. Such a situation would prevent indomethacin from causing an accumulation of glucocorticoids.

[^{3}H]*Cortisol metabolism in rat liver homogenates*

To test our hypothesis that nonsteroidal anti-inflammatory drugs cannot prevent glucocorticoid metabolism in rat liver cytosol, [^{3}H]cortisol was incubated with an S10 of a rat liver homogenate (supernatant fraction from a 10,000 *g* centrifugation) and an NADPH-generating system in the presence and absence of indomethacin. At the end of the incubation, radioactive steroids were extracted and analyzed by thin-layer chromatography. Typical radiochromatograms are shown (Fig. 3, A, B and C). In the absence of drug, approximately 20% of the radioactive cortisol was unmetabolized. In the presence of 30 μM indomethacin, the amount of radioactive cortisol was also 20%. Additional of 5β-dihydrocortisone (50 μM) as a trap led to a slight increase in the amount of radioactive cortisol that

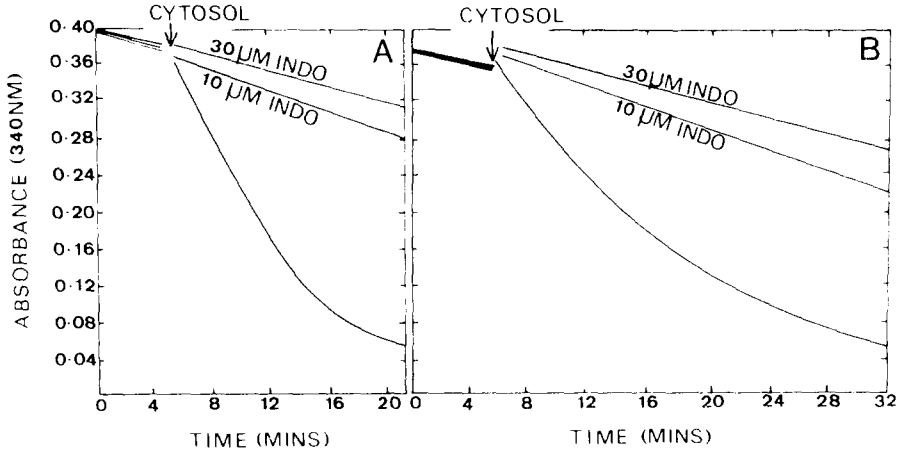

Fig. 2. Reduction of 5β-dihydrocortisone (A) and 5β-dihydrocortisol (B) catalyzed by unprocessed rat liver cytosol, followed to completion in the presence and absence of 10 or 30 μM indomethacin (INDO). Assays contained in a final volume of 1.0 ml: 50 μM steroid, 180 μM NADPH and 100 mM potassium phosphate (pH 6.0). After measurement of the non-enzymatic rate, the reaction was initiated by the addition of 20 μl of unprocessed cytosol.

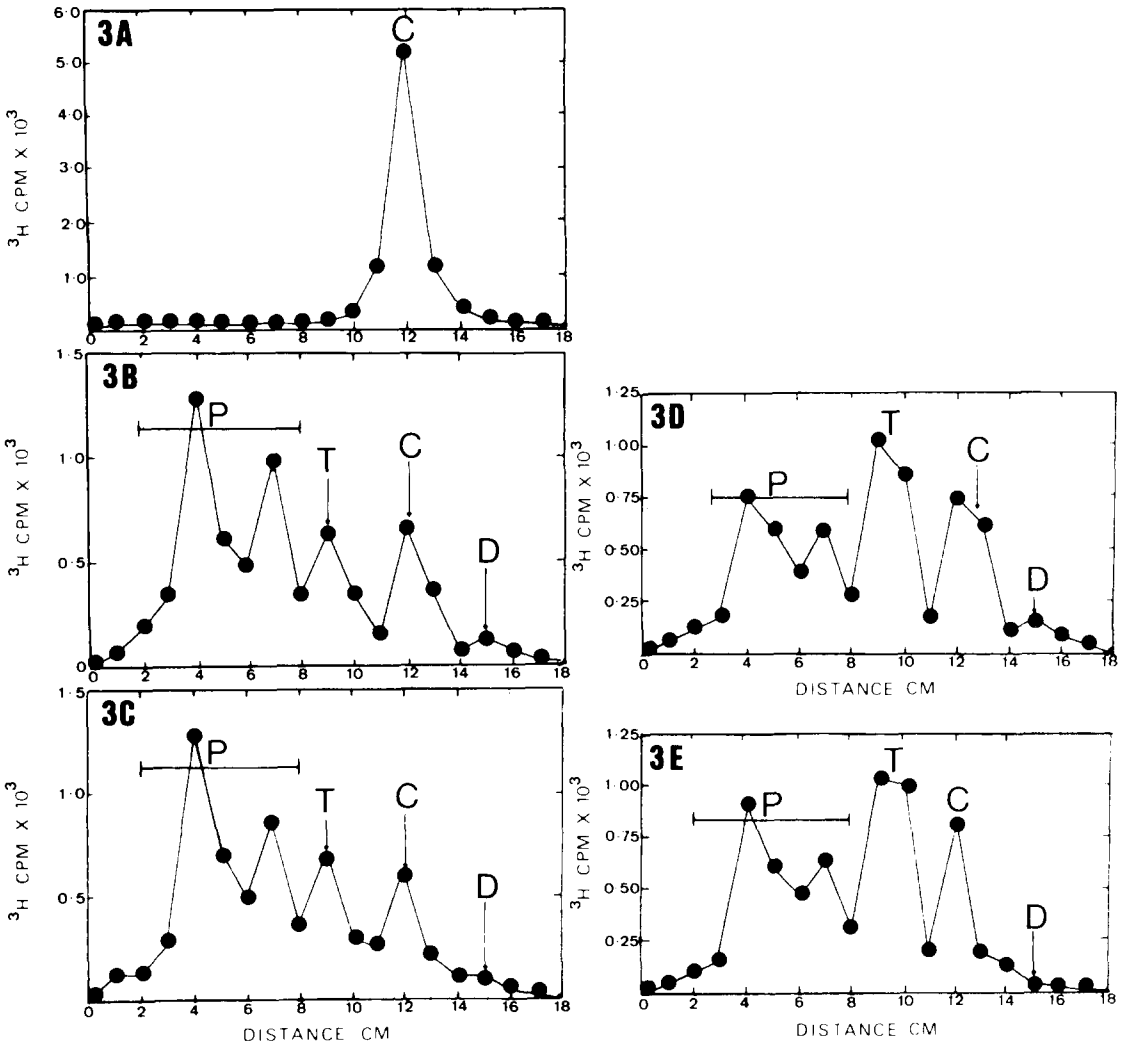

Fig. 3. [^{3}H]Cortisol metabolism in rat liver homogenates. Metabolism was followed as described in Materials and Methods. Ethyl acetate extracts were applied to thin-layer chromatography sheets, and after development they were analyzed for radioactive steroid. Key: zero time (3A); after 10-min incubation (3B); after 10-min incubation in the presence of 30 μM indomethacin (3C); after 10-min incubation in the presence of 50 μM dihydrocortisone (3D); and after 10-min incubation in the presence of 50 μM dihydrocortisone and 30 μM indomethacin (3E). Letters refer to the migration of specific steroids on the chromatogram: cortisol = C; dihydrocortisol = D; tetrahydrocortisol = T; and unidentified polar metabolites = P.

was left unmetabolized (Fig. 3D). When an identical experiment was performed in the presence of 30 μM indomethacin, there was no further decrease in cortisol metabolism (Fig. 3E).

Radiochromatograms from experiments similar to those described in Fig. 3 were analyzed in terms of their CL:CM ratio (cortisol left:cortisol metabolized), and experimental groups were subjected to paired analysis using Student's *t*-test (Table 3). The results clearly indicate that indomethacin has no effect on hepatic cortisol metabolism. Although the addition of trapping quantities of 5β-dihydrocortisone significantly decreased the amount of cortisol metabolized, the inclusion of indomethacin in these experiments did not enhance this effect.

DISCUSSION

This paper tests the hypothesis that inhibition of 3α-hydroxysteroid dehydrogenase by the nonsteroidal anti-inflammatory drugs could promote a cessation in glucocorticoid metabolism which might then cause a local accumulation of cortisol. The results described suggest that this does not take place in male rat liver cytosol and is primarily due to the large difference that exists between the specific activity of the 5β-reductase and the 3α-hydroxysteroid dehydrogenase. Thus, even with high concentrations of drug and trapping quantities of 5β-dihydrocortisone, cortisone metabolism occurs unhindered.

Our *in vitro* studies on the acute effects of indo-

Table 3. Effect of indomethacin on [1,2-^{3}H]cortisol metabolism in male rat liver homogenates

No.	Incubation	Cortisol left: Cortisol metabolized	*t*-Test on paired data (Equal variance) Pair	Result
1.	Control zero time	1.00		
2.	Control + 10 min	0.142 ± 0.051 (N = 6)		
3.	Control + 10 min +30 μM indomethacin	0.183 ± 0.061 (N = 6)	2 vs 3	P = <0.236 (NS)
4.	Control + 10 min +50 μM dihydrocortisol	0.310 ± 0.084 (N = 10)	2 vs 4	P = < 0.01
5.	Control + 10 min +30 μM indomethacin		2 vs 5	P = <0.001
	+50 μM dihydrocortisol	0.340 ± 0.070 (N = 10)	4 vs 5	P = <0.405 (NS)

Radiochromatograms from experiments similar to those described in Fig. 3 were analyzed in terms of their CL:CM ratio (cortisol left:cortisol metabolized), and experimental groups were subjected to paired analysis using Student's *t*-test. NS = not significant.

methacin on male hepatic glucocorticoid metabolism differ from those observed by Vukoson *et al.* [9]. In these earlier studies, the chronic *in vivo* administration of indomethacin to rats, followed by *in vitro* measurements of hepatic corticosterone metabolism, indicated that the metabolism of this steroid is reduced substantially. It is noteworthy that, in the studies of Vukoson *et al.* [9], the greatest effects were observed in female rats, suggesting that a sex difference may exist in corticosteroid metabolism. Our own studies indicate that the activity of 3α-hydroxysteroid dehydrogenase is regulated by ovarian estrogens since administration of 17β-estradiol sulfate to ovariectomized rats leads to a 2-fold increase in specific enzyme activity [14]. Based on these findings, it is predicted that indomethacin would be less efficacious in females since following its administration the residual enzyme activity in the liver of these animals would be greater. In the light of these findings, it is difficult to explain the observations of Vukoson *et al.* However, it should be remembered that in these earlier studies corticosterone metabolism was monitored by following the disappearance of the delta-4-3-ketone. Thus, it cannot be determined whether a decline in the specific activity of 5β-reductase or 3α-hydroxysteroid dehydrogenase was responsible for the decrease in metabolism observed in the presence of indomethacin. In addition, it is conceivable that the effects of indomethacin *in vivo* may be mediated via some more indirect mechanism that may involve one of its metabolites.

We have demonstrated recently that indomethacin-sensitive 3α-hydroxysteroid dehydrogenases are widely distributed in rat tissues [7]. Of the seven rat tissues examined, the liver contained enzyme of the highest specific activity, it being at least 20 times higher than that found in any other tissue. Since the specific activity of the dehydrogenase is substantially lower in other tissues, the levels of 3α-hydroxysteroid dehydrogenase and 5β-reductase may be comparable in these extra-hepatic tissues. Only further experimentation will determine whether the nonsteroidal anti-inflammatory drugs can promote a local accumulation of glucocorticoids in such tissues.

A discussion of the effects of nonsteroidal anti-inflammatory drugs on glucocorticoid levels would be incomplete without reference to the extensive studies on the effects of indomethacin on the neuroendocrine axis. The general consensus would seem to indicate that prostaglandins can restrain corticotropin releasing factor stimulated ACTH secretion at the level of the anterior pituitary [15–18]. Such studies suggest that indomethacin may cause a transient rise in ACTH release. Whether this would increase plasma cortisol levels is difficult to assess since others have shown that indomethacin can prevent the adrenal cortex from responding to ACTH [19, 20]. The studies in the present paper may further complicate the issue since they provide a precedent in which indomethacin may have a direct effect on dihydro-glucocorticoid reduction.

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