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DESTRUCTION OF TESTICULAR CYTOCHROME *P*-450 BY 7α-THIOSPIRONOLACTONE IS CATALYZED BY THE 17α-HYDROXYLASE

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Summary—Studies were done to determine the role of the 17α-hydroxylase in the conversion of 7α-thiospironolactone (7α-thio-SL) to a reactive metabolite causing the degradation of testicular cytochrome *P*-450. Incubation of guinea pig testicular microsomes with 7α-thio-SL plus NADPH resulted in an approx. 70% decline in cytochrome *P*-450 content and even greater loss of 17α-hydroxylase activity. Addition of the 17α-hydroxylase inhibitor, SU-10'603, to the incubation medium prevented the degradation of *P*-450 by 7α-thio-SL. Similarly, preincubation of testicular microsomes with anti-$P\text{-}450_{17\alpha,lyase}$ IgG to inhibit 17α-hydroxylation, diminished the subsequent loss of *P*-450 caused by 7α-thio-SL. The results indicate that the 17α-hydroxylase catalyzes the conversion of 7α-thio-SL to the reactive metabolite responsible for *P*-450 destruction. The accompanying loss of 17α-hydroxylase activity supports the hypothesis that suicide inhibition is the mechanism involved.

INTRODUCTION

Prior investigations have demonstrated that the mineralocorticoid antagonist, spironolactone (SL), causes degradation of cytochrome(s) *P*-450 in the adrenal cortex and testis, resulting in decreases in steroid hydroxylase activities [1–11]. As a result, adrenal corticosteroid production and testicular androgen secretion are decreased in SL-treated animals [1, 2]. Similarly, a number of clinical reports indicate that adrenocortical and testicular steroidogenesis are comprised in some patients receiving SL [7–11]. *In vitro* studies have demonstrated that the degradation of adrenal or testicular *P*-450 by SL requires microsomal metabolism of the drug to a reactive metabolite that has yet to be identified [5, 6, 12]. However, it has been established that the first step in the activation pathway is the deacetylation of SL to 7α-thiospironolactone (7α-thio-SL) [12]. The subsequent oxidation of 7α-thio-SL generates the metabolite responsible for *P*-450 degradation.

A number of observations have provided indirect evidence that the 17α-hydroxylase is involved in the activation of 7α-thio-SL by adrenal and testicular microsomes [13, 14]. In addition, since a rapid decline in 17α-hydroxylase activity follows 7α-thio-SL activation, it has been proposed that 7α-thio-SL is a suicide inhibitor of the 17α-hydroxylase [5, 6, 13]. We have recently demonstrated that metabolism of 7α-thio-SL by a purified and reconstituted 17α-hydroxylase preparation causes inactivation of the enzyme [15], supporting the suicide inhibition hypothesis. The studies described in this communication were done to directly assess the role of the 17α-hydroxylase in the activation of 7α-thio-SL by testicular microsomes.

METHODS

Adult male English Short Hair guinea pigs (800–1000 g) were obtained from Camm Research Institute (Wayne, NJ), and maintained under standardized conditions of light (6 a.m.–6 p.m.) and temperature (22°C) on a diet of Purina laboratory chow and water *ad libitum*. All animals were allowed at least 7 days to become acclimated to the housing conditions prior to use in experiments. Animals were sacrificed by CO_2 inhalation and testes were quickly removed and placed in cold 1.15% KCl–0.05M Tris–HCl (pH 7.4) on ice. All steps in the preparation of subcellular fractions were carried out at 0–4°C. Tissues were homogenized with a

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motor-driven glass–Teflon homogenizer in KCl–Tris buffer. The homogenates were first centrifuged at 900 *g* for 10 min followed by 9000 *g* for 20 min. The 9000 *g* pellet was washed once and recentrifuged at 9000 *g* for 20 min. The supernatants of the 9000 *g* spins were further centrifuged at 105,000 *g* for 75 min in a Beckman L8-60M ultracentrifuge to obtain the microsomal pellets.

Incubation conditions for studies on the degradation of microsomal cytochrome(s) *P*-450 by 7α-thio-SL were essentially the same as described previously [12], except that the temperature was maintained at 4°C. The lower temperature was employed in order to decrease the rate of cytochrome *P*-450 degradation, thereby allowing for better control of experimental conditions. Microsomal suspensions (0.5 mg prot/ml) in 1.15% KCl–0.05 M Tris–HCl containing 3 mM $MgCl_2$ were incubated with 7α-thio-SL (0.1 mM) and/or an NADPH generating system (0.4 mM $NADP^+$, 10 mM sodium isocitrate, 0.3 U/ml isocitrate dehydrogenase) under air with shaking for 30 min. Some incubations were done in the presence of the 17α-hydroxylase inhibitor, SU-10′603 [21]. For each of the incubation conditions employed, one set of flasks served as unincubated or 0-time controls. After incubation, microsomes were recentrifuged and washed twice prior to measurement of cytochrome *P*-450 concentrations or 17α-hydroxylase activities. The washings were necessary to remove residual SU-10′603 and thereby eliminate its inhibitory effect on 17α-hydroxylation.

Anti-$P\text{-}450_{17\alpha,lyase}$ IgG was prepared from rabbit serum and elicited with purified $P\text{-}450_{17\alpha,lyase}$ from guinea pig adrenal microsomes, as described previously [16]. Preparation included ammonium sulfate precipitation, DE-52-column chromatography, and gel-filtration with G-200. Samples were dissolved in 0.9% NaCl containing 20 mM Tris–HCl (pH 8.0). Control IgG was similarly prepared from preimmune serum. For inhibition of microsomal 17α-hydroxylase activity, aliquots of microsomal suspensions were preincubated with anti-$P\text{-}450_{17\alpha,lyase}$ (or control IgG) at a ratio of 2 mg IgG per mg microsomal protein for 2 h at 4°C as described previously [17]. The preincubations had no effects on cytochrome *P*-450 concentrations, and preincubations in the absence of anti-$P\text{-}450_{17\alpha,lyase}$ did not affect steroid hydroxylase activities. After the preincubations, aliquots of the microsomal suspensions were taken for incubations with 7α-thio-SL and/or NADPH, as described above.

Steroid 17α-hydroxylase activity was assayed as the rate of conversion of progesterone to 17α-hydroxyprogesterone. After incubation of testicular microsomes with steroid and/or NADPH, aliquots of the incubation mixtures were diluted 1:2 with enzyme assay buffer [18]. Enzyme assays were done essentially as described previously [18], but were scaled down to accommodate smaller amounts of protein. Microsomal assays were done with 0.025 mg protein in a total volume of 0.625 ml and were incubated for 45 min. In all cases, incubation conditions were employed which ensured linearity of product formation with respect to incubation time and protein concentration. After incubation, steroids were extracted with ethyl acetate and metabolites separated by reverse phase HPLC as described previously [18]; metabolites were quantified by u.v. absorbance at 254 nm.

Cytochrome *P*450 was measured as the dithionite-reduced CO complex by the method of Omura and Sato [19]. Microsomal protein concentrations were determined by the method of Lowry *et al.* [20] with bovine serum albumin as the standard. Data are presented as means ± standard errors. Statistical analyses of differences between group means were done with the Student's *t*-test or the Newman–Keuls multiple range test, as appropriate.

RESULTS AND DISCUSSION

As reported previously, incubation of testicular microsomes with 7α-thio-SL plus NADPH caused large declines in cytochrome *P*-450 concentration and 17α-hydroxylase activity. These effects were fully demonstrable even after extensive washing of the microsomal preparations (Table 1), illustrating that the inactivation of the enzyme by 7α-thio-SL is irreversible. In the absence of NADPH, 7α-thio-SL did not affect *P*-450 content or enzyme activity, demonstrating the need for metabolic activation. To determine the role of the 17α-hydroxylase in the activation of 7α-thio-SL, the effects of the 17α-hydroxylase inhibitor, SU-10′603, on the 7α-thio-SL-mediated degradation of *P*-450 were examined (Table 1). The three concentrations of SU-10′603 employed, 0.5, 0.1 and 0.05 mM, inhibited 17α-hydroxylase activity by 76, 32 and 15%, respectively, during the incubation period

(data not shown). However, washing the microsomes after the incubation removed sufficient amounts of the SU-10'603 to restore 17α-hydroxylase activity to control levels (Table 1).

The presence of SU-10'603 during the incubation period decreased the degradation of *P*-450 by 7α-thio-SL in a concentration-dependent manner (Table 1). The 7α-thio-SL-mediated decline in 17α-hydroxylase activity was similarly diminished by including SU-10'603 in the incubation medium. The highest concentration of SU-10'603 prevented any significant decrease in *P*-450 content or in enzyme activity, and the protective effects generally paralleled the degree of inhibition of 17α-hydroxylase by SU-10'603 (Table 1).

An alternative approach used to assess the role of the 17α-hydroxylase in the activation of 7α-thio-SL was to determine the effects of anti-P-$450_{17\alpha,lyase}$ IgG on the degradation of *P*-450 by 7α-thio-SL (Fig. 1). The inhibitory effects of the anti-P-$450_{17\alpha,lyase}$ on testicular 17α-hydroxylase activity were concentration dependent, but limited supplies of the antibody precluded doing complete dose–response studies. In microsomes preincubated without anti-P-$450_{17\alpha,lyase}$ or with control IgG, 7α-thio-SL effected a >50% decline in cytochrome *P*-450 content (Fig. 1). Preincubation of testicular microsomes with anti-P-$450_{17\alpha,lyase}$ at a concentration of 2 mg IgG/mg microsomal protein caused an approx. 60% decline in 17α-hydroxylase activity (0.33 vs 0.86 nmol/min × mg prot). The amount of *P*-450 degradation caused by 7α-thio-SL was decreased, but not completely prevented, by

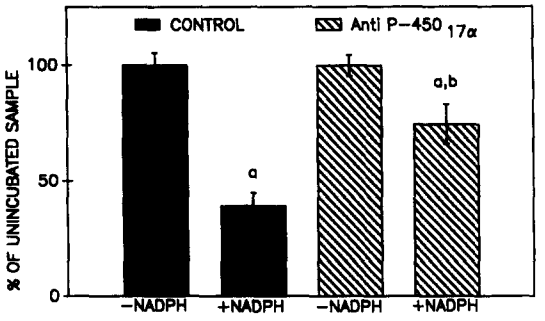

Fig. 1. Effects of anti-P-$450_{17\alpha,lyase}$ IgG on the 7α-thio-SL-mediated degradation of cytochrome *P*-450 in testicular microsomes. Microsomal suspensions were preincubated with or without anti-P-$450_{17\alpha,lyase}$ IgG and then incubated with 7α-thio-SL in the presence or absence of NADPH as described in Methods. Values are means ± SE of 3 experiments and are expressed as % of the corresponding unincubated (0-time) values. 100% equivalent to 0.15 nmol/mg prot. [a] $P < 0.05$ (vs corresponding -NADPH value); [b] $P < 0.05$ (vs corresponding control value).

preincubation of microsomes with the anti-P-$450_{17\alpha,lyase}$ (Fig. 1). The extent of *P*-450 protection by the anti-P-$450_{17\alpha,lyase}$ was proportionally similar to its inhibition of 17α-hydroxylation (~60%).

These observations provide direct evidence that the 17α-hydroxylase is responsible for the activation of 7α-thio-SL by testicular microsomes. Thus, the activation mechanism is similar to that in the adrenal cortex [15]. Since the 17α-hydroxylase is inactivated as a consequence of 7α-thio-SL activation in both organs, the data lend additional support to the hypothesis that suicide inhibition of the P-$450_{17\alpha,lase}$ is the mechanism involved. However, 7α-thio-SL also catalyzes the degradation of *P*-450 in rat liver microsomes [22, 23], a preparation that does have 17α-hydroxylase activity. Decker *et al.* [22, 23] have implicated *P*-450p in the activation of 7α-thio-SL by hepatic microsomes, and have proposed that oxidation of the thiol moiety generates the reactive thiyl radical or sulfenic acid derivative which causes *P*-450 degradation. Further studies are now needed to determine if a similar reactive metabolite of 7α-thio-SL is produced by the 17α-hydroxylase and to investigate the role of still other *P*-450 isozymes in the activation of 7α-thio-SL. Such studies should contribute to a fuller understanding of the mechanisms involved in some of the adverse effects of SL.

Table 1. Effects of incubating testicular microsomes with 7α-thio-SL in the presence and absence of SU-10'603 on cytochrome *P*-450 concentration and 17α-hydroxylase activity[a]

Incubation conditions	*P*-450 (% of control)	17α-Hydroxylase activity (% of control)
Control	100[b]	100[c]
7α-Thio-SL	96 ± 8	105 ± 10
7α-Thio-SL + NADPH	31 ± 5[d]	15 ± 3[d]
SU-10'603 (0.5 mM)	95 ± 8	96 ± 7
SU-10'603 (0.1 mM)	102 ± 6	99 ± 8
SU-10'603 (0.05 mM)	96 ± 8	105 ± 9
7α-Thio-SL + NADPH + SU-10'603 (0.5 mM)	87 ± 7	82 ± 7
7α-Thio-SL + NADPH + SU-10'603 (0.1 mM)	62 ± 5[d]	53 ± 6[d]
7α-Thio-SL + NADPH + SU-10'603 (0.05 mM)	43 ± 5[d]	27 ± 4[d]

[a] Microsomes were washed twice after the incubation period and prior to determining *P*-450 content and 17α-hydroxylase activities; values are expressed as mean percent of control values ± SE of 4–6 experiments.
[b] 100% equivalent to 0.17 nmol/mg prot.
[c] 100% equivalent to 0.93 nmol/min × mg prot.
[d] $P < 0.05$ (vs corresponding control value).

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REFERENCES

1. Menard R. H., Loriaux D. L., Bartter F. C. and Gillette J. R.: The effect of administration of spironolactone on the concentration of plasma testosterone, estradiol, and cortisol in male dogs. *Steroids* **31** (1978) 771–782.
2. Greiner J. W., Kramer R. E., Jarrell J. and Colby H. D.: Mechanism of action of spironolactone on adrenocortical function in guinea pigs. *J. Pharmac. Exp. Ther.* **198** (1976) 709–715.
3. Menard R. H., Bartter F. C. and Gillette J. R.: Spironolactone and cytochrome *P*-450: impairment of steroid 21-hydroxylation in adrenal cortex. *Archs Biochem. Biophys.* **173** (1976) 395–402.
4. Greiner J. W., Rumbaugh R. C., Kramer R. E. and Colby H. D.: Relation of canrenone to the actions of spironolactone on adrenal cytochrome *P*-450-dependent enzymes. *Endocrinology* **103** (1978) 1313–1320.
5. Menard R. J., Guenthner T. M., Taburet A. M., Kon H., Pohl L. R., Gillette J. R., Gelboin H. V. and Trager W. F.: Specificity of the *in vitro* destruction of adrenal and hepatic microsomal steroid hydroxylases by thiosteroids. *Molec. Pharmac.* **16** (1979) 997–1010.
6. Menard R. J., Guenthner T. M., Kon H. and Gillette J. R.: Studies on the destruction of adrenal and testicular cytochrome *P*-450 by spironolactone. *J. Biol. Chem.* **254** (1979) 1726–1733.
7. Walsh P. C. and Siiteri P. K.: Suppression of plasma androgens by spironolactone in castrated men with carcinoma of the prostate. *J. Urol.* **114** (1975) 254–256.
8. Abshagen V., Sporl S., Schoneshofer M. and Oelkers W.: Increased plasma 11-deoxycorticosterone during spironolactone medication. *J. Clin. Endocr. Metab.* **44** (1977) 1190–1193.
9. Tuck M. L., Sowers J. R., Fittingoff D. B., Fisher J. S., Berg G. J., Asp N. D. and Mayes D. M.: Plasma corticosteroid concentrations during spironolactone administration: evidence for adrenal biosynthetic blockage in man. *J. Clin. Endocr. Metab.* **52** (1981) 1057–1061.
10. Serafini P. and Lobo R. A.: The effects of spironolactone on adrenal steroidogenesis in hirsute women. *Fert. Steril.* **44** (1985) 595–599.
11. Sundsfjord J. A., Marton P., Jorgensen H. and Aakvaag A.: Reduced aldosterone secretion during spironolactone treatment in primary aldosteronism: report of a case. *J. Clin. Endocr. Metab.* **39** (1974) 734–739.
12. Sherry J. H., O'Donnell J. P., Flowers L., LaCagnin L. B. and Colby H. D.: Metabolism of spironolactone by adrenocortical and hepatic microsomes: relationship to cytochrome *P*-450 destruction. *J. Pharmac. Exp. Ther.* **236** (1986) 675–680.
13. Sherry J. H., Johnson P. B., Levitt M., Bergstom J. and Colby H. D.: Regional differences in adrenal activation of spironolactone: relationship to steroid 17α-hydroxylase activity. *Drug Metab. Dispos.* **17** (1989) 709–710.
14. Sherry J. H., Johnson P. B. and Colby H. D.: Species differences in adrenal spironolactone metabolism. *Biochem. Pharmac.* **37** (1987) 355–358.
15. Kosser D. C., Kominami S., Takemori S. and Colby H. D.: Role of the steroid 17α-hydroxylase in spironolactone-mediated destruction of adrenal cytochrome *P*-450. *Molec. Pharmac.* **40** (1991) 321–325.
16. Kominami S., Shinzawa K. and Takemori S.: Immunochemical studies on cytochrome *P*-450 in adrenal microsomes. *Biochim. Biophys. Acta* **755** (1983) 163–169.
17. Mochizuki H., Kominami S. and Takemori S.: Examination of differences between benzo(*a*)pyrene and steroid hydroxylases in guinea pig adrenal microsomes. *Biochim. Biophys. Acta* **964** (1988) 83–89.
18. Eacho P. I and Colby H. D.: Differences in microsomal steroid metabolism between the inner and outer zones of the guinea pig adrenal cortex. *Endinocrinology* **116** (1985) 536–541.
19. Omura T. and Sato R.: The carbon monoxide-binding pigment of liver microsomes. *J. Biol. Chem.* **239** (1964) 2370–2378.
20. Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. J.: Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193** (1951) 265–275.
21. Gower D. B.: Modifiers of steroid hormone metabolism: A review of their chemistry, biochemistry, and clinical applications. *J. Steroid Biochem.* **5** (1974) 501–523.
22. Decker C., Sugiyama K., Underwood M. and Correia M. A.: Inactivation of rat hepatic cytochrome *P*-450 by spironolactone. *Biochem. Biophys. Res. Commun.* **136** (1986) 1162–1169.
23. Decker C., Rashed M. S., Baille T. A., Maltby D. and Correia M. A.: Oxidative metabolism of spironolactone: evidence for the involvement of an electrophilic thiosteroid species in drug-mediated destruction of rat hepatic cytochrome *P*-450. *Biochemistry* **28** (1989) 5128–5136.