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Species differences in adrenal spironolactone metabolism: relationship to cytochrome P-450 destruction

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Spironolactone (SL) has been widely used as a potassium-sparing diuretic for approximately two decades [1, 2]. The mechanism of action of the drug involves competitive binding to renal mineralocorticoid receptors, resulting in sodium excretion and potassium retention. Among the side effects noted for SL is the inhibition of steroidogenesis in several tissues, including the adrenal cortex and testes [3–6]. The decline in steroidogenesis caused by SL is attributable, at least in part, to destruction of adrenal and testicular cytochromes P-450 [5–10], the terminal oxidases for several essential steroidogenic enzymes. The destruction of testicular cytochrome(s) P-450 by SL has been observed in all species studied, but effects on adrenal cytochrome(s) P-450 seem to be limited to those species that secrete cortisol as their major adrenal glucocorticoid, that is, species having an adrenal 17α-hydroxylase enzyme system [9, 10]. The mechanism(s) responsible for the species differences in the adrenal effects of SL has not yet been resolved.

The results of numerous studies indicate that most, if not all, of the actions of SL are mediated by metabolites of the drug [7, 8, 11–13]. Recent investigations suggest that 7α-thiomethyl-SL is the therapeutically active metabolite of SL [14, 15], but a different metabolite appears to be responsible for the destruction of adrenal and testicular cytochromes P-450 [16, 17]. It has been demonstrated that 7α-thio-SL is an obligatory intermediate in the actions of SL on cytochromes P-450 [17], but further metabolism of the intermediate within target tissues is also required. Because of the apparent importance of local metabolism in the actions of SL, studies were carried out to determine if species differences in the effects of SL on adrenal cytochromes P-450 [9, 10] are related to differences in adrenal metabolism of the drug. The results presented in this communication indicate that such a relationship does indeed exist.

Materials and methods

Adult male English Short Hair guinea pigs (800–1000 g), Sprague–Dawley rats (250–300 g), New Zealand White rabbits (1.5–2.5 kg), or mongrel dogs (17–22 kg) were used in all experiments. All animals were maintained under standardized conditions of light (6:00 a.m to 6:00 p.m) and temperature (22°), and received food and water *ad lib.* Animals were killed between 8:00 and 9:00 a.m.; adrenal glands were collected and microsomal fractions obtained by differential centrifugation as described previously [17].

For studies on the metabolism of SL or 7α-thio-SL, incubation medium contained 0.05 M Tris–HCl (pH 7.4), 5.0 mM $MgCl_2$, and adrenal microsomal protein (0.2 to 2.0 mg) in a total volume of 2.5 ml. Where indicated, an NADPH-generating system consisting of NADP (0.5 mM), sodium isocitrate (10 mM), and isocitrate dehydrogenase (0.15 units) was included in the incubation medium. Incubations were done in 25-ml Erlenmeyer flasks at 37° under air in a Dubnoff Metabolic Incubator. The reaction was initiated by the addition of SL (100 μM) or 7α-thio-SL (100 μM) in small volumes (5–10 μl) of ethanol. Incubation times varied from 10 to 60 min, depending upon the amount of microsomal enzyme activity in adrenals from the different species studied. Conditions which resulted in approximately 20–30% depletion of substrate (50–75 nmol metabolized) were employed in all experiments. The HPLC analyses used for measurement of SL and of 7α-thio-SL are sensitive to approximately 2 nmol of each compound. For all microsomal preparations, the incubation conditions employed ensured linearity of product formation or substrate (7α-thio-SL) metabolism with respect to incubation time and microsomal protein concentration. After incubation, 40 μg of progesterone was added to each flask to serve as an internal standard. The incubation media were extracted twice with 4 ml of ethyl acetate (HPLC grade) and the extracts were combined, filtered, and evaporated. Samples were then reconstituted in small volumes of HPLC grade acetonitrile for subsequent HPLC analyses.

Incubation conditions for the preincubation experiments were similar to those described above. However, after the preincubation period, the microsomal suspensions were recentrifuged at 105,000 *g* for 60 min, washed with KCl–Tris buffer, and centrifuged again. The final pellets were resuspended in buffer and used for cytochrome P-450 determinations. Cytochrome P-450 was measured as the dithionite-reduced CO complex as described by Omura and Sato

[18]. Microsomal protein concentrations were determined by the method of Lowry *et al.* [19]. Data were analyzed for differences between group means by ANOVA and the Newman–Keuls test. A value of $P < 0.05$ was considered significant.

The analyses of spironolactone and its metabolites were carried out using high performance liquid chromatography (HPLC) as descibed by Sherry *et al.* [14]. The HPLC analyses were done with a Waters 5 μ-C_{18} radial-PAK cartridge. Compounds were separated using a 30-min concave gradient (curve 9) of 65–100% methanol–water. The solvent flow rate was 1.0 ml/min, and the chromatographic system was operated at ambient temperature. The eluent was monitored by absorbance at 254 nm, and progesterone was used as an internal standard.

Results and discussion

Incubation of guinea pig or dog adrenal microsomal preparations with SL in the presence of NADPH decreased cytochrome P-450 levels (Table 1). The effect was somewhat greater with guinea pig than with dog microsomes. By contrast, cytochrome P-450 concentrations in adrenal microsomes from rats or rabbits were not affected by incubation with SL + NADPH. In the absence of NADPH, SL had no effect on adrenal cytochrome P-450 in any of the species studied.

7α-thio-SL, the compound which is an obligatory intermediate in the actions of SL on adrenal cytochromes P-450 [17], effected a greater decrease in guinea pig and dog cytochrome P-450 levels than did SL (Table 1). The effects of 7α-thio-SL, like those of SL, were demonstrable only when microsomal preincubations were done in the presence of NADPH. Rat and rabbit adrenal microsomal cytochrome P-450 concentrations were not affected by 7α-thio-SL, in the presence or absence of NADPH.

We recently reported that the effects of SL on guinea pig adrenal microsomal cytochrome(s) P-450 destruction require deacetylation of the parent compound to 7α-thio-SL and the subsequent NADPH-dependent oxidation of 7α-thio-SL [17]. As indicated in Table 2, both guinea pig and dog adrenal microsomal preparations had the capacity to convert SL to 7α-thio-SL, and in the presence of NADPH, further catalyzed the metabolism of 7α-thio-SL. The rate of metabolism of 7α-thio-SL, as indicated by the disappearance of substrate, was greater with microsomal preparations from guinea pigs than from dogs. Adrenal microsomal preparations from rats and rabbits also effected the conversion of SL to 7α-thio-SL, but at somewhat lower rates than guinea pig and dog adrenal microsomes (Table 2). However, the metabolism of 7α-thio-SL was virtually undetectable with rat or rabbit adrenal microsomal preparations.

The results suggest that the species differences in the effects of SL on adrenocortical cytochromes P-450 previously noted by Menard and coworkers [9, 10] are the result of differences in adrenal metabolism of the drug. We recently demonstrated that the "activation" of SL by adrenal microsomes requires both deacetylation of the parent compound to 7α-thio-SL and the subsequent NADPH-dependent metabolism of 7α-thio-SL [17]. It is

Table 1. Species differences in the effects of preincubating adrenal microsomes with spironolactone (SL) or 7α-thio-SL on cytochrome P-450 concentrations*

Preincubation conditions	Cytochrome P-450 concentrations (% of control)†			
	Guinea pig	Dog	Rabbit	Rat
Control	100	100	100	100
NADPH	96 ± 3‡	98 ± 6	97 ± 4	94 ± 6
SL	97 ± 3	95 ± 6	95 ± 5	95 ± 5
SL + NADPH	69 ± 5§	81 ± 5§	102 ± 6	95 ± 4
7α-thio-SL	97 ± 3	96 ± 5	96 ± 5	103 ± 5
7α-thio-SL + NADPH	52 ± 5§‖	57 ± 4§‖	94 ± 5	95 ± 5

* Preincubations were done for 30 min at 37° as described in the Materials and Methods.
† Absolute cytochrome P-450 concentrations (nmol/mg protein) corresponding to 100% were the following: guinea pig, 1.6 ± 0.3; dog 0.9 ± 0.2; rabbit, 0.4 ± 0.1; rat, 0.5 ± 0.1.
‡ Values are expressed as mean percent of control values ± SE of four to six experiments.
§ $P < 0.05$ (vs corresponding control).
‖ $P < 0.05$ (vs SL + NADPH group).

Table 2. Species differences in the rates of metabolism of spironolactone (SL) and 7α-thio-SL by adrenal microsomal preparations*

Species	Microsomal protein (mg/g tissue)	Conversion of SL to 7α-thio-SL (nmol/min × mg protein)	Metabolism of 7α-thio-SL (nmol/min × mg protein)
Guinea pig	41.2 ± 3.8	1.2 ± 0.2	20.6 ± 3.3
Dog	28.9 ± 3.3†	1.0 ± 0.1	7.8 ± 1.9†
Rabbit	27.6 ± 3.1†	0.7 ± 0.1†	0.4 ± 0.3†‡
Rat	23.7 ± 2.4†	0.4 ± 0.1†‡	0.3 ± 0.3†‡

* Values are expressed as means ± SE of four to six experiments.
† $P < 0.05$ (vs corresponding guinea pig value).
‡ $P < 0.05$ (vs corresponding dog value).

the latter step which appears to be most deficient in species whose adrenals are not affected by SL. Although some species differences in the rates of SL deacetylation were noted (Table 2), the differences in 7α-thio-SL metabolism were far greater. In addition, it appears to be the latter process which is organ-specific and which accounts for the tissue specificity of SL actions on cytochromes P-450 [17].

Menard *et al.* [9, 10] previously proposed that the steroid 17α-hydroxylase was involved in the activation of SL. They recognized that the destruction of adrenal cytochromes P-450 by SL occurred only in those species whose adrenals produced 17-hydroxylated steroids. Our observations lend support to their hypothesis by demonstrating that the key step in the activation process occurs in adrenals from 17-hydroxylating species (guinea pig, dog) but not in those from other rodents (rat, rabbit). Since the activity of the 17α-hydroxylase decreases as a result of SL activation [9, 10], suicide inhibition may be involved. Further studies utilizing purified 17α-hydroxylase preparations are now needed to unequivocally establish the mechanism(s) involved.

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Facile exchange of the cyano group in highly potent anticancer cyanomorpholinyl anthracyclines

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Recent studies have reported on an unusually potent anthracycline, 3′-deamino-3′-(3-cyano-4-morpholinyl)doxorubicin (CM-DXR), in both animal [1, 2] and human tumor [3, 4] test systems (Fig. 1). It is active in the range of 10^{-9} to 10^{-11} M against doxorubicin (DXR)-sensitive cells and only about 1.5-fold less active in P388 cells resistant to about 160-fold higher amounts of DXR [2]. An essential feature of the unique activity of CM-DXR seems to be the cyano group because the analog, morpholinyl DXR (M-DXR), is about 50-fold less active in DXR-sensitive P388 cells [2]. Previously, Ishiguro *et al.* [5] had shown that the α-cyanoamine moiety of the antibiotic, saframycin A, was essential to the interaction of this compound with calf thymus DNA; and that when [^{14}C]cyanide-labeled saframycin A was reacted with DNA, none of the radioactivity was associated with the DNA. They concluded that an iminium ion or an α-carbinolamine formed by the loss of cyanide was the actual species involved in the interaction with DNA. Lown *et al.* [6] confirmed and extended these studies with saframycin A. Thus, the uniquely high potency of CM-DXR against both DXR-sensitive and -resistant cells may be derived from the ability of CM-DXR to readily form such an alkylating species, which could explain the formation of covalent

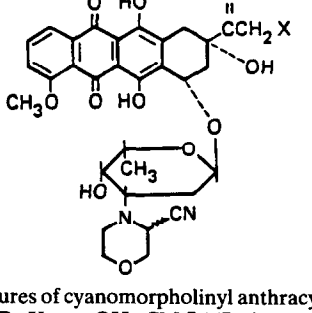

Fig. 1. Structures of cyanomorpholinyl anthracyclines (CM-DXR: X = —OH; CM-DNR: X = —H.