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ADRENAL MITOCHONDRIAL METABOLISM OF SPIRONOLACTONE

ABSENCE OF METABOLIC ACTIVATION

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Abstract—Previous investigations have established that spironolactone (SL) administration to guinea pigs decreases adrenal mitochondrial and microsomal cytochrome P-450 content, and that the latter requires microsomal activation of the drug. Studies were carried out to determine if adrenal mitochondrial metabolism (activation) of SL was similarly involved in the effects of the drug on mitochondrial cytochrome P-450 destruction. Incubation of guinea pig adrenal mitochondria with SL in the absence of NADPH resulted in the formation of 7α-thio-SL as the only metabolite. In the presence of an NADPH-generating system, an unknown polar metabolite was also produced. The mass spectrum of the unknown compound suggested that it was a hydroxylated derivative of SL. Incubation of mitochondrial preparations with 7α-thio-SL also resulted in the formation of a polar metabolite, but the latter had a different HPLC retention time than that of the SL metabolite. Formation of the polar SL metabolite was prevented by metyrapone, an 11β-hydroxylase inhibitor, and was greatest in mitochondria from the adrenal zone having the highest 11β-hydroxylase activity. Steroid substrates for 11β-hydroxylation inhibited the production of the SL metabolite. Mitochondrial incubations with SL or with 7α-thio-SL in the presence or absence of an NADPH-generating system did not affect cytochrome P-450 concentrations. The results indicate that, unlike the microsomal effects of SL, local activation of SL is not responsible for the destruction of adrenal mitochondrial cytochromes P-450. The major adrenal mitochondrial metabolites of SL appear to be 11β-hydroxy-SL and 7α-thio-SL.

Spironolactone (SL) is a renal mineralocorticoid antagonist that is widely used in the treatment of congestive heart failure, essential hypertension, and various other edematous conditions [1, 2]. Among the side effects of the drug is the inhibition of steroidogenesis in the adrenal cortex and testes [3–10]. The actions of SL on steroid synthesis are the result of effects on cytochrome(s) P-450, the terminal oxidase(s) for various steroidogenic enzymes [5–10]. Administration of SL to experimental animals decreases adrenal and testicular cytochrome P-450 content and lowers steroid hydroxylase activities.

It has been clearly established that one or more metabolites of SL is responsible for the effects of the parent drug on microsomal cytochromes P-450 in steroidogenic organs [5–12]. Formation of the active metabolite(s) is catalyzed by adrenal and testicular microsomal preparations *in vitro*, resulting in the destruction of cytochromes P-450. Accordingly, the microsomal metabolism of SL has been under investigation [12] in an attempt to delineate the activation pathway. However, SL administration to guinea pigs decreases adrenal mitochondrial as well as microsomal cytochrome P-450 concentrations [6–8], but little has been done to determine the mechanism of action of SL on mitochondrial monooxygenases. In particular, it is not known if mitochondrial activation of SL is required for the effects. The studies presented in this report were done to define the pathway involved in adrenal mitochondrial metabolism of SL and to determine its relationship to the destruction of mitochondrial cytochrome(s) P-450.

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METHODS

Adult male English Short Hair guinea pigs, weighing approximately 800–1000 g, were obtained from Camm Research Institute (Wayne, NJ) and used in all experiments. Animals were maintained under standardized conditions of light (6:00 a.m. to 6:00 p.m.) and temperature (22°) on Wayne Guinea Pig Diet and water *ad lib*. Animals were killed by decapitation between 8:00 and 9:00 a.m. Adrenal glands were quickly removed and placed in cold 1.15% KCl containing 0.05 M Tris–HCl (pH 7.4). In some experiments, adrenals were bisected longitudinally, and the dark-brown inner zone, consisting primarily of zona reticularis, was gently dissected from the tan outer zone, which was comprised of the zona glomerulosa and zona fasciculata [13]. Whole adrenal glands or tissue from each zone were homogenized in 0.25 M sucrose containing 0.05 M Tris–HCl (pH 7.4), and mitochondria were obtained by differential centrifugation as previously described [14].

Mitochondrial 11β-hydroxylase activity was assayed as the rate of conversion of 11-deoxycortisol to cortisol, as described previously [15]. Cortisol was measured fluorometrically [16]. For studies on the metabolism of spironolactone (SL) or 7α-thio-SL, incubation medium contained 0.25 M sucrose–0.05 M Tris–HCl (pH 7.4), 5.0 mM $MgCl_2$, and adrenal mitochondrial protein (0.5 to 2.0 mg/ml) in a total volume of 2.5 ml. Where indicated, sodium isocitrate (10 mM) was included in the incubation medium as a source of intramitochondrial NADPH. 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. In some experiments [1,2-^{3}H]SL (100 μM, 1.9 Ci/mmol) or [1,2-^{3}H]7α-thio-SL (100 μM, 1.9 Ci/mmol) was used as substrate. Incubation times varied from 10 to 30 min, depending upon the substrate employed. For all incubations, conditions were employed that ensured linearity of product formation with respect to incubation time and mitochondrial 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 mitochondrial suspensions were recentrifuged, washed with sucrose–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 [17]. Microsomal protein concentrations were determined by the method of Lowry et al. [18].

The analyses of spironolactone and its metabolites were carried out using high performance liquid chromatography (HPLC) as described by Sherry et al. [19]. The HPLC analyses were done with a Waters 5μ-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. When [^{3}H]SL or [^{3}H]7α-thio-SL was used as the substrate, fractions collected every 20 sec were added to 10 ml of Scintiverse II (Fisher Scientific Co.) scintillation fluid, and the amount of radioactivity in each was determined by liquid scintillation spectrometry with a Beckman LS-9000 scintillation counter. Mass spectral analyses of mitochondrial spironolactone metabolites and authentic standards were done on a Finnigan model 4021 automated GC/MS equipped with an INCOS automatic data system. Samples were run at 20 eV by direct probe in the EI mode.

RESULTS AND DISCUSSION

Incubation of adrenal mitochondria with spironolactone (SL) in the absence of isocitrate (a source of mitochondrial NADPH) resulted in the production of 7α-thio-SL as the only metabolite (Fig. 1). Identification of the 7α-thio-SL was confirmed by mass spectroscopy as described previously [11]. The rate of production of 7α-thio-SL was 1.9 ± 0.2 nmol · min^{-1} · (mg protein)$^{-1}$ (mean ± SE

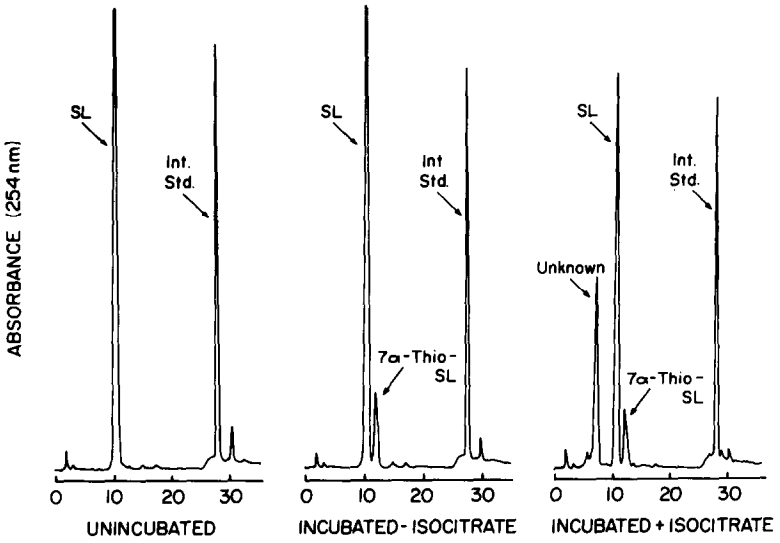

Fig. 1. Metabolism of spironolactone (SL) by adrenal mitochondria in the presence and absence of sodium isocitrate (10 mM). Adrenal mitochondria (0.5 mg protein/ml) were incubated with SL (100 μM) for 60 min at 37° as described under Methods. After incubation, 40 μg of the internal standard (Int. Std), progesterone, was added to each flask, and the samples were processed for HPLC analyses as described under Methods. HPLC retention times (min) are indicated on the abscissa.

Table 1. Mass spectra of spironolactone (SL), 7α-thio-SL, and the mitochondrial metabolite of SL

	Mitochondrial metabolite	SL	7α-Thio-SL
Apparent parent ion (*m/e*)	390	416	374
Fragment ions (*m/e*)	374	374	359
	357	359	341
	356	341	340
	338	340	325
	323	325	301
	265	301	267
		267	

of five experiments). In the presence of isocitrate, 7α-thio-SL and an unknown polar metabolite were produced (Fig. 1). Incubation with [^{3}H]SL as substrate and subsequent determination of the amount of radioactivity in each HPLC fraction indicated that 7α-thio-SL and the polar metabolite fully accounted for the amount of SL that was metabolized. On the basis of the specific activity of the [^{3}H]SL and the amount of radioactivity corresponding to the polar metabolite peak, the rate of formation of the unknown metabolite was 2.2 ± 0.3 nmol · min^{-1} · (mg protein)$^{-1}$ (mean ± SE of four experiments).

The mass spectrum of the mitochondrial SL metabolite (Table 1) indicated an apparent parent ion at *m/e* 390, suggesting a hydroxylated metabolite of 7α-thio-SL (parent ion at *m/e* 374). However, SL standards yielded a very weak parent ion at 416 (~1% of the base ion at *m/e* 341) but a prominent ion at *m/e* 374 resulting from loss of the acetyl function at the C-7 position (see Refs. 20–22 for discussion of SL mass spectra). Thus, the distinction between hydroxylated metabolites of SL and 7α-thio-SL is very difficult to establish by mass spectroscopy alone. However, the ions at *m/e* 390 and at *m/e* 356 and 357 (replacing those at *m/e* 340 and 341 for SL and 7α-thio-SL) suggested that the unknown compound was a hydroxylated derivative of SL or 7α-thio-SL.

To determine if the unknown polar metabolite was derived from 7α-thio-SL rather than from SL directly, incubations were done with 7α-thio-SL as the substrate (Fig. 2). In the absence of isocitrate, there was no demonstrable metabolism of 7α-thio-SL by adrenal mitochondria. In the presence of isocitrate, very small amounts of a polar metabolite were produced; the retention time of this metabolite was consistently about 1 min greater than that of the metabolite produced from SL. Incubations with [^{3}H]7α-thio-SL indicated that the rate of production of the metabolite was 0.16 ± 0.02 nmol · min^{-1} · (mg protein)$^{-1}$ (mean ± SE of three experiments), far less than the rate of formation of the metabolite resulting from incubations with SL. Thus, the latter is apparently not derived from 7α-thio-SL and is probably a hydroxylated metabolite of SL.

The results of additional experiments suggested that the mitochondrial SL metabolite was hydroxylated at the C-11 position. Addition of metyrapone

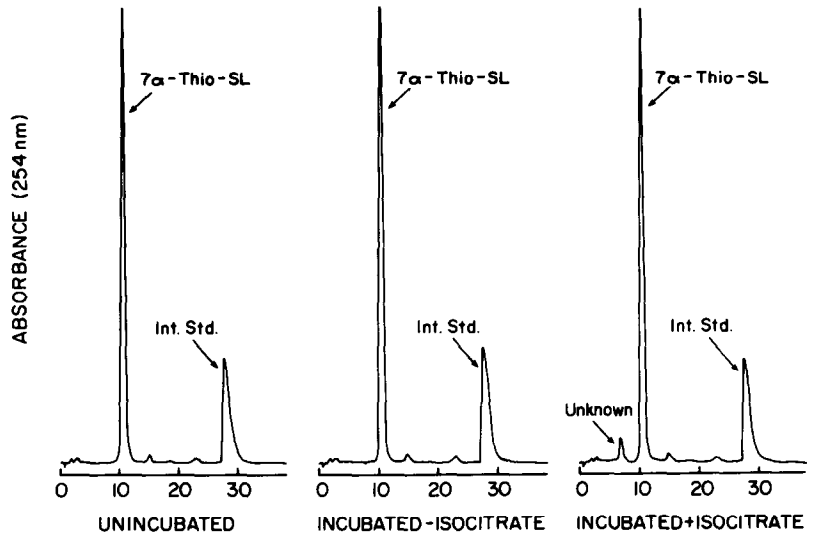

Fig. 2. Metabolism of 7α-thiospironolactone (7α-thio-SL) by adrenal mitochondria in the presence and absence of sodium isocitrate. Experimental conditions were identical to those described in the legend for Fig. 1 except that 7α-thio-SL (100 μM) was used as the substrate.

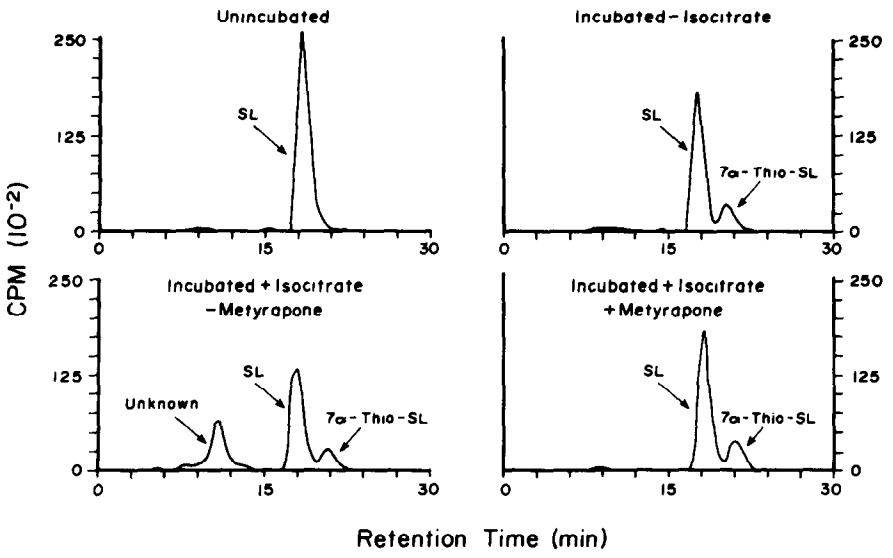

Fig. 3. Effects of metyrapone (1.0 mM) on the metabolism of spironolactone (SL) by adrenal mitochondria. Experimental conditions were identical to those described in the legend for Fig. 1 except that [^{3}H]SL was used as the substrate. Fractions were collected, and the amount of radioactivity in each was determined by liquid scintillation spectrometry as described under Methods.

(1.0 mM), an 11β-hydroxylase inhibitor, to the incubation flasks inhibited the production of the mitochondrial SL metabolite (Fig. 3). Studies with metyrapone were done using [^{3}H]SL because of the UV absorbance of the inhibitor interfering with the UV detection of SL metabolites. Incubations with mitochondria obtained from the inner (zona reticularis) and outer (zona fasciculata + zona glomerulosa) zones of the guinea pig adrenal cortex further illustrated a close correlation between 11-hydroxylase activity and production of the hydroxylated metabolite. Outer zonal adrenal mitochondria have approximately twice the 11-hydroxylase activity found in the inner zone, and the rates of production of the SL metabolite were similarly greater in the outer than the inner zone [2.7 ± 0.4 vs 1.2 ± 0.2 nmol·min^{-1}·(mg protein)$^{-1}$; mean ± SE of four experiments]. In addition, formation of the metabolite was decreased by approximately 90% by the addition of 11-deoxycorticosterone (100 μM), a substrate for 11-hydroxylation, to the incubation medium, but unaffected by corticosterone, the 11-hydroxylated analogue of 11-deoxycorticosterone. Thus, the SL metabolite appears to be 11-hydroxy-SL.

Previous investigations established that incubation of guinea pig adrenal microsomal preparations with SL or 7α-thio-SL in the presence of NADPH results in the destruction of cytochrome(s) P-450 [5–10]. By contrast, mitochondrial incubations with SL or 7α-thio-SL, in the presence or absence of isocitrate as a source of NADPH, had no significant effects on cytochrome P-450 levels (Table 2). Increasing the concentration of isocitrate in the incubation flasks had no effect on cytochrome P-450 levels and did not increase the rates of mitochondrial SL or 7α-thio-SL metabolism (data not shown).

The results suggest that local activation is not responsible for the destruction of adrenal mitochondrial cytochrome(s) P-450 by SL. In adrenal microsomes, SL is deacetylated to 7α-thio-SL which, in turn, is apparently converted to a reactive metabolite that mediates the destruction of cytochrome(s) P-450 [5–12]. The activation and resulting loss of

Table 2. Effects of preincubating adrenal mitochondria with spironolactone (SL) or 7α-thio-SL on cytochrome P-450 concentrations*

Preincubation conditions	Cytochrome P-450 concentrations (nmol/mg protein)	(% of control)
Control	0.57 ± 0.05†	100
Isocitrate (10 mM)	0.52 ± 0.05	91 ± 8
SL (100 μM)	0.56 ± 0.04	98 ± 7
SL + isocitrate	0.56 ± 0.06	98 ± 10
7α-Thio-SL (100 μM)	0.51 ± 0.05	89 ± 9
7α-Thio-SL + isocitrate	0.53 ± 0.04	93 ± 7

* Preincubations were done for 60 min at 37° as described in Methods.
† Values are means ± SE of four to six experiments.

cytochrom(s) P-450 are readily demonstrable with isolated adrenal microsomal preparations *in vitro* [5–12]. Similar activation of SL has been demonstrated recently with rat hepatic microsomes [23]. Although SL administration to guinea pigs decreases adrenal mitochondrial as well as microsomal cytochrome P-450 levels, mitochondrial activation of SL was not demonstrable in our experiments. Adrenal mitochondria are able to catalyze the conversion of SL to 7α-thio-SL, the first step in the microsomal activation of SL, but the subsequent metabolism of 7α-thio-SL differs in mitochondria and microsomes. The major mitochondrial metabolites of both SL and 7α-thio-SL appear to be the respective 11-hydroxylated products. Similar results were obtained previously when canrenone, the dethioacetylated derivative of SL, was used as the substrate for rat and bovine adrenal mitochondrial preparations [24, 25]. These observations suggest that adrenal activation of SL may be limited to the microsomal fraction of the cell, with the reactive metabolite then diffusing into the mitochondria and causing the destruction of mitochondrial cytochromes P-450. Studies with isolated intact adrenocortical cells are now being done to further evaluate this hypothesis.

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