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Suramin: An Inhibitor of the Final Steps of the Mineralocorticoid Pathway?

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The authors used incubated adrenal mitochondria to study the *in vitro* effect of suramin, an antiparasitic drug, on the transformation of corticosterone and 18-hydroxycorticosterone into aldosterone. The results show that, under conditions preserving membrane integrity, the "impermeance" of suramin meant that concentrations similar to the plasma-levels reached in treated patients induced only slight inhibition of the final intramitochondrial steps in aldosterone synthesis. However, suramin strongly inhibited mitochondrial respiration. The inhibition of two intramitochondrial mechanisms (respiration and steroid synthesis) suggests that the effect of suramin involves partial inhibition of metabolic intermediate carriers. The inhibition of the activity of various extramitochondrial enzymes involved in intermediate metabolism, suggests that the inhibition of steroid biosynthesis can be explained only on the basis of an extramitochondrial action of suramin. The action of suramin must, therefore, primarily and directly affect extramitochondrial steroid synthesis and only indirectly affect intramitochondrial steroid synthesis as a result of an impact on the reducing equivalent supply. However, even if suramin does not bind to cytochrome *P*450 11*β* which catalyzes the final steps of aldosterone biosynthesis pathway, this does not imply that suramin has no direct effect on steroid synthesis within the mitochondria, in addition to its toxic effects, particularly if the cell structure is disrupted (as is often the case in tumor tissues).

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INTRODUCTION

The adrenolytic effect of suramin, a drug originally used to treat parasitic diseases [1, 2], has been observed since it was first used. This effect has recently been confirmed in studies in animal models [3–5] and in clinical trials [6–9], notably in the treatment of AIDS [10]. This adrenolytic effect led to suramin being considered for use in the treatment of adrenal carcinoma [11–13] in order to reduce the tumor mass. However, in adrenal carcinoma, chemotherapy is intended not only to reduce the tumor mass, but also to reduce hormone synthesis, which must rapidly be brought under control. What has to be determined, therefore, is whether suramin has an inhibitory effect on steroid synthesis in general as soon as treatment is begun and before it has had any impact on tumor mass. As far as we are aware, no *in-vivo* investigation of this aspect has been carried out to date. The inhibitory effect of suramin on steroid synthesis has been demonstrated *in vitro* using mitochondria after freezing to disrupt the mitochondrial membranes [14] and using cultured adrenal carcinoma cells [5]. However, due to the inability of suramin to cross membranes [15–17] we still did not know whether the steroid synthesis steps which actually take place within the mitochondria can be controlled by this drug.

The purpose of the research reported here was to find out whether, under conditions in which the mitochondrial membrane is intact, suramin has any immediate direct or indirect inhibitory effect on intramitochondrial steroid synthesis. In an attempt to find out, the authors chose a model which had never previously been used: the final steps of the mineralocorticosteroid biosynthesis pathway (from corticosterone to aldosterone) investigated *in-vitro* in the presence of suramin using mitochondria with intact membranes, which act as a barrier to the entry of suramin.

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EXPERIMENTAL

Chemicals

Suramin was a generous gift from Bayer Pharma. Sodium L-malate, sodium succinate, rotenone and antimycin A were supplied by Sigma, KCN by Prolabo, ADP by Boerhinger Inc., [4-^{14}C]aldosterone (11β,21-dihydroxy-4-pregnene-3,18,20-trione) by New England Nuclear Corp., [1,2-^{3}H]corticosterone (11β,21-dihydroxy-4-pregnene-3,20-dione) and 18-hydroxy-[1,2-^{3}H]corticosterone (11β,18,21-trihydroxy-4-pregnene-3,20-dione) by Amersham. The other reagents were commercial products of analytical reagent grade.

Preparation of homogenate and mitochondrial fraction

The duck adrenal gland is the best animal gland for this investigation since the aldosterone biosynthesis pathway is the same as in humans and the conversion yield is sufficient to study all the steps in this pathway, even those with a low yield [18–20]. Adrenal glands were taken from adult male Musk ducks (*Cairina moschata*) weighing 2.5–3.5 kg. The ducks were killed in the laboratory and the adrenal glands rapidly cooled in an ice bath. The following procedures were carried out at +4°C. The mitochondrial fraction was prepared according to the methods of Hogeboom [21]and Sauer and Mulrow [22], with some modifications as described previously [23]. The composition of the buffer used for homogenization was as follows: sucrose 450 mM, Tris 30 mM, EDTA 1 mM, bovine serum albumin 1%, pH 7.4. The mitochondrial fraction was not subjected to any physicochemical treatment which could modify membrane permeability. Mitochondrial protein concentrations were determined by the method of Lowry *et al.* [24] using bovine serum albumin as standard. Respiratory characteristics were determined by polarography with a Clark oxygen electrode at +40°C. The incubation medium (buffer) used both to determine the respiratory characteristics and for incubation had the following composition: sucrose 250 mM, EGTA 0.5 mM, KH_2PO4 10 mM, Tris 10 mM, $MgCl_2$5 mM, pH 7.0. Bovine serum albumin, which is usually included in the composition of this medium, was not added. This was because suramin is known to have very high affinity towards serum albumin [25] and binding could have made it impossible to observe any impact of this drug on the aldosterone biosynthesis pathway. This modification of the composition of the medium did not have any impact on respiratory function or steroid synthesis. The purity of the mitochondrial preparation was determined using enzyme markers (succinate cytochrome-*c*-reductase, cytochrome-*c*-oxidase, NADPH cytochrome-*c*-reductase, glucose-6-phosphate dehydrogenase) as described previously [23, 26].

Incubation conditions

The general incubation conditions were as follows: 1 mg of the mitochondrial fraction in 2 ml of incubation medium, was incubated for 30 min under aerobic conditions in a Dubnoff metabolic shaker at +40°C with either 94 pmol of [1,2-^{3}H]corticosterone (sp. act. 50 Ci/mmol) or 134 pmol of 18-hydroxy[1,2-^{3}H]-corticosterone (sp. act. 37.8 Ci/mmol). Before starting the reaction by adding 15 mM of L-malate or succinate, concentrations of suramin similar to plasma-levels reached in treated patients, were added. The nature and concentration of the reagents used are reported in each table. The reaction was stopped by adding chloroform and the samples were then frozen at −18°C until extraction.

Aldosterone purification and identification

An internal standard "[4-^{14}C]aldosterone" was included in order to determine the yield. The steroids were extracted by chloroform. Aldosterone was purified and identified by paper chromatography as reported previously [23]. The tritiated aldosterone formed was calculated from the dpm (liquid scintillation counter, Tricarb 2000). The results were expressed as pmol of aldosterone formed per mg of mitochondrial protein per 30 min.

Spectrophotometric study

Cytochrome *P*450 11β was prepared according to Katagiri *et al.* [27] with minor modifications [28]. Cytochrome *P*450 11β was diluted in phosphate buffer (50 mM, pH 7.4) containing dithiothreitol (0.1 mM), EDTA (0.1 mM) and Tween 20 (0.3%). Signal-producing ligand binding was determined at the appropriate wavelengths (λ 390–420 nm). Spectra were recorded using a Uvicon Kontron spectrophotometer.

Statistical analysis

Statistical significance was determined using Student's *t*-test.

Table 1. *Enzyme activities in the mitochondrial fraction and homogenate*

Enzyme	Homogenate	Mitochondria
Succinate cytochrome-*c*-reductase (EC 1.3.1.6)	26.1 ± 3.4	88.4 ± 1.2
Cytochrome-*c*-oxidase (EC 1.9.3.1)	376.2 ± 7.5	716 ± 15.3
NADPH cytochrome-*c*-reductase (EC 1.6.2.4)	21.5 ± 1.7	12 ± 0.9
Glucose-6-phosphate dehydrogenase (EC 1.1.1.49)	8.6 ± 0.7	0

Each value is reported as the mean ± SD of 5 different experiments. Specific activities are expressed as nmol/mg protein per min.

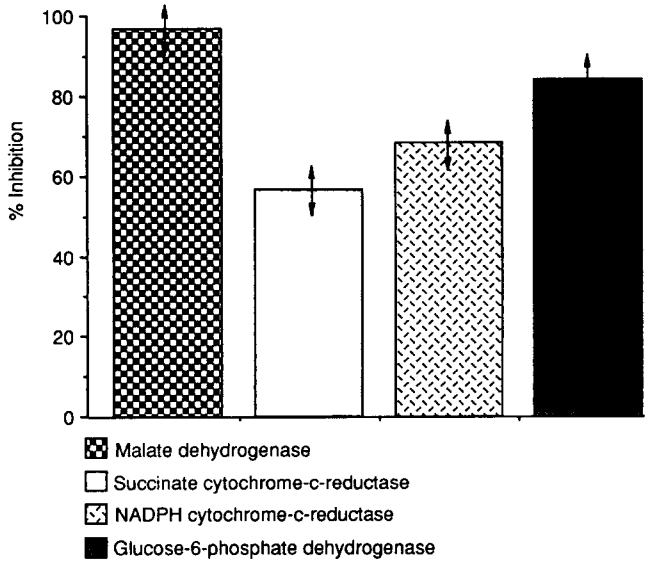

Fig. 1. Suramin (0.1 mM) inhibits various enzyme activities of the intermediate metabolism. Each value is reported as the mean ± SD of 5 different experiments. Concentrations of the reagents were as follows; L-malate 15 mM and succinate 15 mM.

RESULTS

The activities of the various enzymes measured to determine the purity of mitochondrial fraction are shown in Table 1. Levels of the mitochondria-associated enzymes, cytochrome-*c*-oxidase, and succinate cytochrome-*c*-reductase, were increased in the mitochondrial fraction, whereas those of the microsomal marker, NADPH cytochrome-*c*-reductase, were reduced and the cytosol enzyme "glucose-6-phosphate dehydrogenase" was absent.

As shown in Fig. 1, suramin at a concentration of 0.1 mM markedly inhibited the activity of various enzymes involved in intermediate metabolism (malate dehydrogenase, succinate cytochrome-*c*-reductase, NADPH cytochrome-*c*-reductase, glucose-6-phosphate dehydrogenase).

Respiratory characteristics are reported in Table 2. Respiratory intensity (RI) represents oxygen consumption; Respiratory control (RC) and phosphorylation capacity (P/O) provide a true indication of the efficacy of oxidative phosphorylation. The oxidative phosphorylation chain functioned normally (rotenone did not inhibit respiration in the presence of succinate, but did so in the presence of L-malate). The respiratory control ratio was lower than that usually found in other tissues as reported previously [29–31]. As shown in Fig. 2, suramin (0.15 and 0.5 mM) strongly inhibited mitochondrial respiration in the presence of L-malate or succinate. The respiratory control ratio was reduced by 80% by suramin (0.15 and 0.5 mM) in the presence of ADP 0.75 mM.

Tables 3 and 4 show the effects of suramin at various concentrations on the final steps of the aldosterone biosynthesis pathway. The findings show that the percentage inhibition of the conversion of corticosterone into aldosterone induced by suramin at concentrations of 0.15 and 0.5 mM was found to be 3.29 and 20.74%, respectively in the presence of L-malate and 8.39 and 26.30% in the presence of succinate (Table 3). The percentage inhibition of the conversion of 18-hydroxycorticosterone into aldosterone by the same concentrations of suramin was 4.18 and 32.5% in the presence of L-malate and 2.03 and 27.08% in the presence of succinate (Table 4).

Our findings demonstrate that suramin did not bind to reconstituted cytochrome *P*450 11β, which catalyzes the conversion of corticosterone and 18-hydroxycorticosterone into aldosterone (Fig. 3). The change in optical density caused by the binding of metopirone to cytochrome *P*450 11β was not observed with 0.5 mM suramin.

DISCUSSION AND CONCLUSION

Our results show that *in vitro*, at concentrations similar to those found in the plasma of treated subjects, suramin only slightly inhibited the intramitochondrial steps of the steroid biosynthesis pathway, i.e. the final steps in the mineralocorticosteroid biosynthesis pathway, which are catalyzed by cytochrome *P*450 11β. Since these experimental conditions preserved the integrity of the mitochondrial membrane, the limited

Table 2. Respiratory characteristics of the mitochondrial fraction

Substrate	Inhibitors	RI (nmol O_2/mg protein per min)	RC (ADP 0.75 mM)	P/O
Succinate	0	32.0 ± 1.8	1.67	1.10
	Rotenone	31.6 ± 1.4		
	Antimycin A	0		
	KCN	0		
L-malate	0	26.0 ± 2.4	1.71	1.04
	Rotenone	1.50 ± 0.8		
	Antimycin A	0		
	KCN	0		

Each value of respiratory intensity (RI) is reported as the mean ± SD of 6 different experiments. Concentrations (mM) used were as follows: L-malate and succinate 15, rotenone 25×10^{-3}, antimycin A 9×10^{-5}, KCN 10^{-1}. RI, respiratory intensity; RC, respiratory control (ratio of RI in the presence and without ADP 0,75 mM); P/O, oxidative phosphorylation capacity.

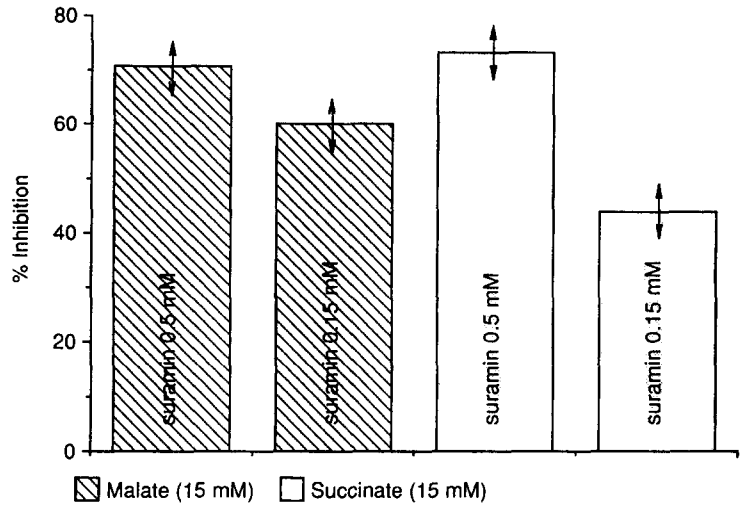

Fig. 2. Suramin induces a strong inhibition of mitochondrial respiratory intensity. Each value is reported as the mean ± SD of 5 different experiments. Concentrations of the reagents were as follows: L-malate and succinate 15 mM, suramin 0.5 or 0.15 mM.

inhibition observed can be attributed to the "impermeance" of suramin.

The apparent conflict between these findings and those of Ashby *et al.* [14], who found major inhibition of the synthesis of androgens and glucocorticosteroids, is probably misleading and attributable to a major methodological difference between the two studies, viz. whether or not the integrity of the mitochondrial membranes was preserved.

However, even when the membranes were ruptured, inhibition of the last steps in the aldosterone biosynthesis pathway could not have been due to an action of suramin on cytochrome *P*450 11β, since suramin is not bound to this cytochrome.

The inhibition of aldosterone biosynthesis which we observed can, however, probably be explained as a consequence of the action of suramin on metabolic intermediate carriers required for hydroxylation reactions.

Our findings demonstrate that suramin strongly inhibits mitochondrial respiration. This inhibition cannot be due to a direct impact on intramitochondrial electron transfer systems (since suramin does not cross the mitochondrial barrier), it is reasonable to attribute this effect to an inhibition of the replenishment of reducing equivalents. This inhibition can be explained by an extramitochondrial action of suramin on metabolic intermediate carriers. In addition, the inhibition of aldosterone biosynthesis induced by suramin is only partial; it depends on the supply of reducing equivalents; it can therefore be concluded that suramin only partially blocks the metabolic intermediate transportation systems. The difference in inhibition by suramin of two intramitochondrial mechanisms (respiration and steroid synthesis), both of which require reducing equivalents could be explained by competition within the adrenal mitochondria between the respiratory pathway and the hydroxylation pathway for these reducing equivalents [32].

The inhibition of steroid biosynthesis seems therefore to be explicable only on the basis of an extramitochondrial action of suramin, as shown by the inhibition of the activity of various extramitochondrial enzymes involved in intermediate metabolism. These findings,

Table 3. *Inhibitory effect of suramin on the transformation of corticosterone to aldosterone*

Substrate (15 mM)	Suramin (μM)	Aldosterone formed (pmol/mg protein per 30 min)	% Inhibition of the reaction
Malate	0	28.10 ± 3.1	
	150	27.18 ± 2.0	3.29
	300	25.49 ± 1.7	9.28
	500	22.27 ± 1.4	20.74
Succinate	0	28.15 ± 1.1	
	150	25.78 ± 3.2	8.39
	300	24.73 ± 2.1	12.15
	500	20.74 ± 2.2	26.30

Each value is reported as the mean ± SD of 5 different experiments. The reaction was carried out in incubation medium, pH 7.0, 1 mg protein, 94 pmol of [1,2^{3}H]corticosterone and L-malate or succinate 15 mM at 40°C for 30 min.

Table 4. Inhibitory effect of suramin on the transformation of 18-hydroxycorticosterone to aldosterone

Substrate (15 mM)	Suramin (μM)	Aldosterone formed (pmol/mg protein per 30 min)	% Inhibition of the reaction
Malate	0	3.35 ± 0.31	
	150	3.21 ± 0.20	4.18
	300	2.76 ± 0.28	17.83
	500	2.26 ± 0.32	32.56
Succinate	0	2.50 ± 0.19	
	150	2.45 ± 0.36	2.03
	300	2.18 ± 0.12	12.50
	500	1.82 ± 0.02	27.08

Each value is reported as the mean ± SD of 5 different experiments. The reaction was carried out in incubation medium, pH 7.0, 1 mg protein, 134 pmol of 18-hydroxy[1,2^{3}H]corticosterone and L-malate or succinate 15 mM at 40°C for 30 min.

suggesting an extramitochondrial target for suramin, fit in well with its chemical structure of an impermeant substance. The action of suramin must, therefore, primarily and directly affect extramitochondrial steroid synthesis and only indirectly affect intramitochondrial steroid synthesis as a result of an impact on the reducing equivalent supply. Suramin must therefore be able to control the extra- and intramitochondrial steps of steroid synthesis in differing manners under normal physiological conditions.

However, this does not imply that suramin has no effect on steroid synthesis within the mitochondria, particularly under abnormal conditions. It is not unusual for mitochondrial membrane structures to be disrupted in tumor tissues and this would allow suramin to enter these organelles and have a direct effect on steroid synthesis in addition to its cytotoxic effects.

Determination of the plasma levels of the steroids involved in the end steps of the aldosterone biosynthesis pathway could confirm this hypothesis.

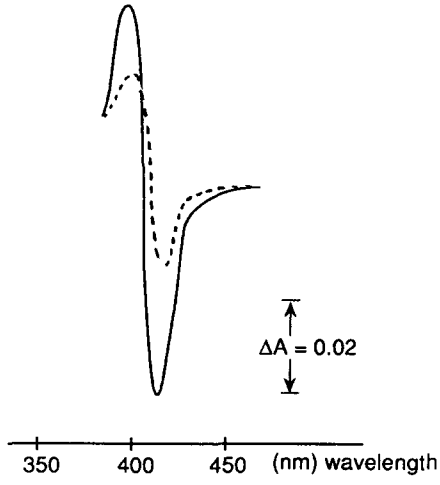

Fig. 3. Suramin is not bound to isolated cytochrome *P*-450 11β. Difference spectra obtained with adrenal mitochondria (proteins 1.5 mg/ml) after adding: ----DOC (5 μM) + metopirone (0.8 μM); ——DOC (5 μM) ± suramin (500 μM).

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