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INHIBITION OF MICROSOMAL DRUG METABOLISM BY MITOCHONDRIA AND CYTOCHROME *c* OXIDATION OF EXTRAMITOCHONDRIAL NADPH

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Abstract—Microsomal aniline *p*-hydroxylase and aminopyrine *N*-demethylase activities were inhibited by mitochondria. The magnitude of the inhibition increased in parallel with the amount of added mitochondria. The inhibition was reverted by 0.2 mM KCN. Marked inhibition of these microsomal enzyme activities was observed also in the presence of cytochrome *c* and low amounts of mitochondria causing negligible inhibition in themselves. The inhibition increased with the concentration of cytochrome *c* and it was reverted by KCN. Microsome-free mitochondria did not oxidize NADPH even in the presence of cytochrome *c*, although NADH oxidation has been demonstrated under these circumstances [Sottocasa *et al.*, *J. Cell Biol.* **32**, 415, (1967)]. However, completion of the system by addition of microsomes resulted in the oxidation of NADPH, which was inhibited by KCN. These findings may indicate the cooperation of the microsomal and mitochondrial compartments in the regulation of drug metabolism.

Cooperation of different cellular compartments has been demonstrated in several metabolic and regulatory processes. However, the inhibition of the microsomal monooxygenase system by mitochondria [1–3] is not well understood. The extent of inhibition is dependent on the amount and quality (integrity) of mitochondria. Recently developed techniques for the preparation of microsome-free mitochondria and microsomes allowed a more exact investigation of the interaction of microsomes and mitochondria. Our finding that small amounts of mitochondria became inhibitory in the presence of cytochrome *c* has led us to assemble an electron transport system from microsomes, microsome-free mitochondria and cytochrome *c* which is capable of oxidizing extramitochondrial NADPH, i.e. one of the substrates of the microsomal drug metabolizing system. An attempt to demonstrate the relationship of these phenomena is presented in this paper.

MATERIALS AND METHODS

Preparation of microsomes and mitochondria were performed essentially as described previously [4]. Wistar rats weighing 150–180 g were used, and liver mitochondria were isolated according to Schneider [5] in 5 mM Tris–HCl buffer, pH 7.2, containing 0.25 M sucrose and 1 mM ethyleneglycol bis(2-aminoethyl ether)-*N,N* tetraacetic acid (EGTA). The mitochondria were washed once then purified by centrifugation in a nonlinear sucrose gradient [6]; microsomal contamination was about 2% [4]. The microsome fraction was obtained by ultracentrifugation of the postmitochondrial supernatant at 105,000 *g* for 60 min.

Assay system for aniline *p*-hydroxylase and aminopyrine *N*-demethylase enzymes contained 5 mM $MgCl_2$, 0.1 M Tris–HCl (pH 7.5), 0.2 mM NADPH and an NADPH regenerating system consisting of 2 U/ml glucose-6-phosphate dehydrogenase (Boehringer, Mannheim, West Germany) and 10 mM glucose-6-phosphate [4]. Formaldehyde, *p*-aminophenol and protein were determined by standard procedures as described by Nash [7], Imai *et al.* [8] and Lowry *et al.* [9], respectively.

Mitochondrial oxidation of NADH and NADPH was measured polarographically using a Clark oxygen electrode. The reaction mixture contained 0.1 M Tris–HCl (pH 7.5) and 1 mM EGTA.

Crystalline horse cytochrome *c* was obtained from Boehringer, Mannheim.

RESULTS

The activities of microsomal aminopyrine *N*-demethylase and aniline *p*-hydroxylase were inhibited by purified, microsome-free mitochondria (Table 1). This inhibition was dependent on the concentration of mitochondria. The inhibition of aniline hydroxylation was more expressed. Elevation of the concentration of NADPH slightly moderated the inhibition of *N*-demethylase, but increasing the concentration of the other substrate(s) had no similar effect (not shown). The inhibitory effect of mitochondria was almost completely reverted by 0.2 mM KCN. This dose of KCN does not interfere with the function of the microsomal cytochrome P450, however it almost completely blocks mitochondrial cytochrome oxidase activity.

Tables 2 and 3 demonstrate the interaction of mitochondria and cytochrome *c* in the inhibition of microsomal enzyme activities. Mitochondria were applied in small quantities, hardly affecting the microsomal enzymes. However, when 0.2–2.0 μM

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Table 1. Inhibition of aniline *p*-hydroxylase and aminopyrine *N*-demethylase by mitochondria

Mitochondrial protein (mg/ml)	Aniline *p*-hydroxylase activity (nmole *p*-aminophenol/min × mg protein)		*N*-Demethylase activity (nmole HCHO/min × mg protein)	
	—	0.2 mM KCN	—	0.2 mM KCN
—	0.85 ± 0.11	0.98 ± 0.15	4.90 ± 0.27	4.64 ± 0.37
0.16	0.71 ± 0.18	0.74 ± 0.15		
0.40	0.60 ± 0.15	0.85 ± 0.17	4.60 ± 0.25	4.50 ± 0.41
0.80	0.20 ± 0.12	0.95 ± 0.20	3.20 ± 0.27	4.40 ± 0.45
1.60			2.85 ± 0.18	3.90 ± 0.24
3.20			2.10 ± 0.26	3.80 ± 0.24

Results represent mean ± S.D. of at least three independent experiments with different preparations of microsomes and mitochondria. Concentration of microsomal protein was 0.8 mg/ml. NADPH was applied at a concentration of 0.2 mM; an NADPH regenerating system was also added to the reaction mixtures (cf. Materials and Methods).

Table 2. Effect of mitochondria and cytochrome *c* on the activity of aniline *p*-hydroxylase

	Cytochrome *c* (μM)			
	—	0.2	0.4	2.0
Microsomes	0.88 ± 0.05	0.90 ± 0.10	0.80 ± 0.13	0.85 ± 0.07
Microsomes + mitochondria	0.71 ± 0.09	0.56 ± 0.12	0.40 ± 0.17	0.18 ± 0.05
Microsomes + mitochondria + KCN	0.90 ± 0.15	0.85 ± 0.15	0.81 ± 0.14	0.79 ± 0.19

Results represent mean ± S.D. of at least three independent experiments with different preparations of microsomes and mitochondria. Enzyme activity is expressed as nmole *p*-aminophenol/min × mg protein. The following concentrations were applied: 0.8 mg/ml microsomal protein, 0.2 mg/ml mitochondrial protein, 0.2 mM NADPH, and 0.2 mM KCN. The assay mixtures contained an NADPH regenerating system (cf. Materials and Methods).

Table 3. Effect of mitochondria and cytochrome *c* on the activity of aminopyrine *N*-demethylase

	Cytochrome *c* (μM)			
	—	0.2	0.4	2.0
Microsomes	4.7 ± 0.16	4.9 ± 0.23	4.8 ± 0.11	5.1 ± 0.33
Microsomes + mitochondria	4.3 ± 0.20	2.8 ± 0.18	2.2 ± 0.14	1.5 ± 0.26
Microsomes + mitochondria + KCN	4.9 ± 0.23	5.0 ± 0.26	4.7 ± 0.22	4.8 ± 0.15

Results represent mean ± S.D. of at least three independent experiments with different preparations of microsomes and mitochondria. Enzyme activity is expressed as nmole formaldehyde/min × mg protein. The following concentrations were applied: 0.8 mg/ml microsomal protein, 0.5 mg/ml mitochondrial protein, 0.2 mM NADPH, and 0.2 mM KCN. The reaction mixtures contained an NADPH regenerating system (cf. Materials and Methods).

cytochrome *c* was added to the system, marked inhibition of both *N*-demethylase and aniline *p*-hydroxylase appeared. The inhibitory effect increased in parallel with the concentration of cytochrome *c*. In these cases KCN also prevented the inhibition, indicating the involvement of mitochondrial cytochrome oxidase.

The oxidation of extramitochondrial NADH by isolated mitochondria in the presence of cytochrome *c* (Fig. 1a) has been described [10]. However, as shown by polarographic measurement of mitochondrial respiration (Fig. 1b), NADPH was not oxidized under the same circumstances. NADPH was readily oxidized when microsomes were also present beside mitochondria and cytochrome *c* (Fig. 1c, d). Oxidation of NADPH was inhibited by KCN but not by rotenone. No oxygen consumption was measured when the mitochondria were omitted. Mitochondrial

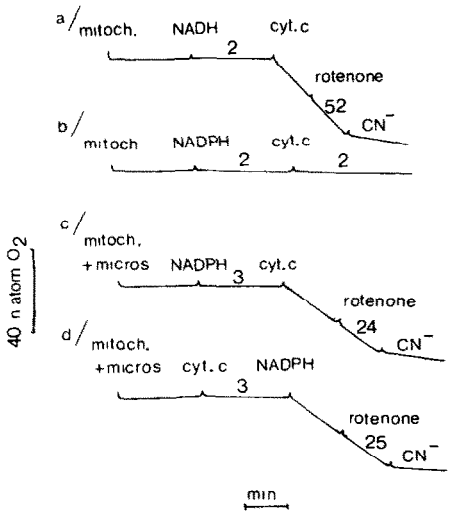

Fig. 1. Oxidation of extramitochondrial NADH and NADPH by purified mitochondria. Oxygen uptake was measured polarographically. The following concentrations were used: 1 mM NADH or NADPH, mitochondrial and microsomal protein each 0.8 mg/ml, 10 μm cytochrome *c*, 3 μm rotenone, and 1 mM KCN. Numbers indicate the rate of oxygen consumption expressed as natom O_2/min × mg mitochondrial protein.

respiration was not stimulated by either NAD or NADP in these systems.

DISCUSSION

Inhibition of microsomal aminopyrine *N*-demethylase and aniline *p*-hydroxylase activities by purified mitochondria confirms earlier results obtained with crude mitochondria [1–3]. Reversal of the inhibition by KCN indicates the participation of mitochondrial cytochrome oxidase in the inhibitory mechanism. In the following discussion we examine the possibility that the inhibition of microsomal drug metabolism is due to the draining of the reduced coenzyme NADPH by mitochondria.

An electron transport system associated with the outer membrane of liver mitochondria has been described by Sottocasa *et al.* [10], but this 'external' pathway performs the oxidation of extramitochondrial NADH only, and requires exogenous cytochrome *c*. Evidence has been presented that NADH-cytochrome *c* reductase activity is present in the outer mitochondrial membrane, but NADPH-cytochrome *c* reductase activity resides in the microsomal fraction only [10]. This is in agreement with our finding that microsome-free mitochondria did not oxidize extramitochondrial NADPH even in the presence of cytochrome *c*. However, oxidation of NADPH occurred after the addition of microsomes which contained the NADPH-cytochrome *c* reductase activity (Fig. 1). Accordingly, cytochrome *c* greatly enhanced the inhibition of drug metabolizing enzymes by the mitochondria (Tables 2 and 3). The oxidation of extramitochondrial NADH in the system of Sottocasa *et al.* [10] and of NADPH in the present experiments proved to be similar with respect to two important features: both processes are inhibited by KCN but not by rotenone. Therefore, cytochrome oxidase is involved and the mainstream NADH dehydrogenase is by-passed in these oxidative mechanisms. However, many details of these 'external' pathways of NADH and NADPH oxidation are still obscure.

It should be pointed out that relatively high concentrations of mitochondria inhibit microsomal drug metabolism without the addition of cytochrome *c*, and the inhibition can be reverted by KCN. This finding suggests that in this interaction of the microsomal and mitochondrial compartments endogenous cytochrome *c* is able to perform, at least to some extent, the same role which is played by exogenous cytochrome *c* in the oxidation of extramitochondrial pyridine nucleotides.

At present, it is difficult to assess the significance of the inhibition of microsomal drug metabolism by mitochondria in physiological or pathological states. Such an interaction may occur after carbontetrachloride poisoning, when the amount of cytoplasmic cytochrome *c* is elevated [11]. Further, desorption of cytochrome *c* from the inner mitochondrial membrane has been supposed to occur in cold-adapted rats, since extramitochondrial NADH is rapidly oxidized by liver mitochondria of these animals [12].

We suppose that the cause of the inhibition of microsomal drug metabolism is that reducing equivalents are drained from cytochrome P450 by cytochrome *c* and channelled into the mitochondria to the cytochrome oxidase.

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