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TISSUE SPECIFICITY OF MITOCHONDRIAL MONOOXYGENASE SYSTEM FOR AFLATOXIN B_1 ACTIVATION

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SUMMARY: Rat liver mitoplasts free of detectable microsomal contamination can activate significant amounts of aflatoxin B_1 (AFB_1) into electrophilic reactive forms. The activated carcinogen binds to mitochondrial macromolecules and inhibits mitochondrial RNA and protein biosynthesis. The mitochondrial monooxygenase system for AFB_1 activation is restricted to liver and kidney as tested by enzyme assays and effects on mitochondrial RNA and protein synthesis.

Mammalian mitochondria (mt*) are unique because of their remarkable heterogeneity with respect to both structure and function. In addition to their primary role in electron transfer and oxidative phosphorylation, they also carry out several metabolic functions. One of these specialized functions is steroid hydroxylation by a unique mitochondrially located cytochrome P-450 in various steroidogenic tissues (1,2). Existence of cytochrome P-450 for 26-cholesterol hydroxylation and 25-vitamin D_3 hydroxylation in liver mitochondria has also been reported (3,4). It is now well known that almost all of the carcinogens require activation by cytochrome P-450 type monooxygenase system before they can covalently bind to cellular nucleophilic sites (5,6). Enzymes for carcinogen activation have been reported to occur in the cell nucleus (7) and in the microsomal fraction (8). Recent work in our laboratory has demonstrated the presence of a unique cytochrome P-450 type monooxygenase system in rat liver mt for the activation of hepatic carcinogen AFB_1 into electrophilic reactive form (9). The activated components bind to mt circular DNA, mt RNA and protein (9). In this paper we show that the enzymatic transformation of AFB_1 by the mt enzyme system leads to marked inhibition of RNA and protein synthesis in rat liver

*Abbreviations: mt, mitochondria; AFB_1, aflatoxin B_1; SDS, sodium dodecyl sulfate

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mt particles. The AFB_1 activation enzyme is present only in liver and kidney mt. Similarly, the carcinogen mediated inhibition of mt macromolecular synthesis is restricted to liver and kidney mt and mitoplasts from brain, spleen and heart are refractory to treatment with AFB_1.

MATERIALS AND METHODS

Isolation of Mitochondria: Mitochondria were isolated from livers of male Sprague Dawley rats (235 to 260 g) after overnight starvation. Minced livers were homogenized in mt isolation buffer (2 mM Hepes (pH 7.4), 222 mM mannitol, 70 mM sucrose, 1 mM EDTA) with 3-4 strokes of a loose fitted teflon homogenizer and made to 15% (w/v) with mt isolation buffer. The crude mt fraction was separated by differential centrifugation (10). Mitoplasts were prepared by the digitonin procedure of Greenawalt (11), washed twice with mt isolation buffer and used for carcinogen activation as described elsewhere (9). Mitoplasts from kidney and heart were prepared essentially by the same procedure. In the case of brain, the purification step included sedimentation through a layer of ficol (12).

Mt Protein and RNA Synthesis: The incorporation of ^{35}S methionine into mitoplasts was essentially as described before (13). Mitoplasts were suspended in a buffer containing 0.18 M sucrose, 60 mM KCl, 10 mM $mg(CH_3COO)_2$ at a concentration of 6-10 mg protein/ml and supplemented with 2 mM ATP, 2 mM GTP, 2 mM creatine phosphate, 16 µg/ml creatine phosphokinase, 40 µM each of 19 amino acids excepting methionine and 300 µg/ml cycloheximide. The mixture was pre-incubated for 5 min at 37°C with gentle shaking before initiating the incorporation with 150 µci/ml ^{35}S methionine (650 Ci/mmol). The amount of incorporation was determined by hot CCl_3COOH precipitation. Incorporation of ^3H CTP was carried out in the same buffer system, using conditions described before (14).

Assay for AFB_1 Activation: The assay procedure was modified from Gurtoo et al. (15) as described earlier (9). The reaction mixture was pre-incubated at 37°C for 15 min to generate NADPH (15). Calf thymus DNA (400 µg/ml of reaction mixture) and ^3H AFB_1 (4 µci/ml=100 p mol) were added and the incubation was continued for 1 hour at 37°C. The DNA was extracted by Phenol-cresol method (16), washed several times with 70% ethanol and dissolved in 15 mM NaCl and 1.5 mM sodium citrate, pH 7.0. Aliquots were counted with 10 ml Cab-O-Cil scintillation mixture in an Intertechnique SI-400 counter.

RESULTS AND DISCUSSION

Recently we have described a method for assaying the enzyme activity for AFB_1 activation in rat liver mt fractions (9). The assay system includes a NADPH generating system (15), excess of calf thymus DNA (400 µg/ml), optimal concentration of AFB_1 (100 p mol/ml) and 0.8 to 1 mg/ml of lysed mt or sub mt fractions. Under optimal conditions the liver mt enzyme shows 61 p mol/mg activity as compared to 19 p mol/mg in the kidney mt (see Table 1). Mitoplasts from other tissues, such as spleen, heart and brain show very low (1.7 p mol/mg) to negligible activity. The inability of brain mt to activate AFB_1 was surprising since the occurrence of a cytochrome P-450 for steroid hydroxylation in these organelles is now well established (1,2). Furthermore, the ability of liver, brain and adrenal cortex

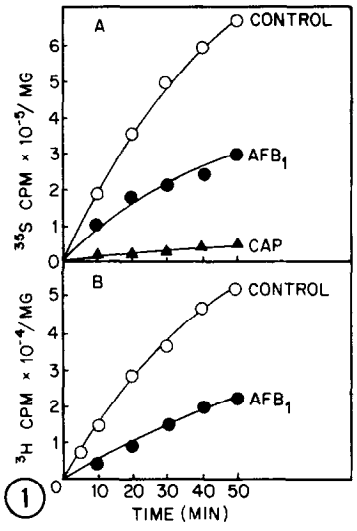
Table 1. Tissue specificity of mitochondrial AFB_1 activation enzyme system.

Source of Mitochondria	p mole ^3H AFB_1 Bound to DNA/mg Protein	Relative Activity
Liver	61.0	1
Kidney	19.0	0.32
Spleen	1.7	0.028
Brain	<0.005	Negligible
Heart	<0.005	Negligible

Mitoplasts were isolated from various rat tissues as described in Materials and Methods and sonic disrupted mt fractions were used for AFB_1 binding assays, also as described in Materials and Methods.

mt enzymes to hydroxylate steroid hormones (1-4,24,25) suggested identical substrate specificity of these mt enzymes. Our results, however, show distinct tissues specificity of the AFB_1 activation system - suggesting substantial differences between the brain and liver mt cytochrome P-450.

Metabolic conversion of inactive carcinogen into electrophilic reactive form is an important step in the induction of cancer (5,6). It is well known that the activated components bind to cellular DNA, RNA and proteins and cause inhibition of macromolecular synthesis in the nucleus as well as in the cytoplasm (5,6,8). We have, therefore, verified the effects of AFB_1 activation by the liver mt system on the RNA and protein synthesis by isolated mitoplasts. As shown in Figure 1, incubation of rat liver mt with 50 µg/ml AFB_1 results in 40 to 50% inhibition of both mt RNA and protein synthesis. Although not shown here, the extent of inhibition is dose dependent and at doses of 100 µg/ml, over 90% inhibition is observed. In order to determine if the inhibition is specific or general, the translation products in the control mt and mt preincubated with AFB_1 were analyzed on SDS-polyacrylamide gels. The autoradiogram presented in Figure 2A shows that rat liver mt synthesize 19-22 polypeptide species in the size range of about 8×10^3 daltons to over 10^5 daltons, all of which are sensitive to chloramphenicol (see Figure 2B). In separate experiments reported elsewhere (17), we have shown that these polypeptides are authentic mt products. Incubation with



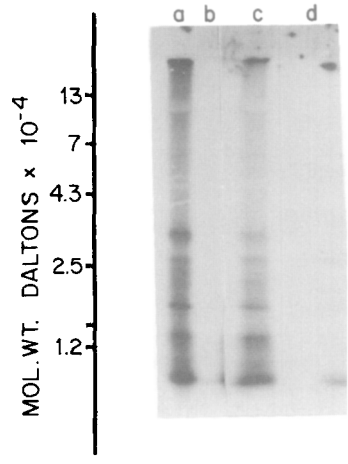


Fig. 1. Effects of AFB_1 on RNA and protein synthesis by rat liver mitoplasts. (A) Protein synthesis was carried out as described in the Materials and Methods using 150 µci/ml ^{35}S methionine. AFB_1 in 50 µl dimethyl sulfoxide was added 10 min before the initiation of protein synthesis. Equivalent volumes of dimethyl sulfoxide were also added to all other tubes (both control and CAP). Chloramphenicol when added was at 500 µg/ml level. (B) RNA synthesis was carried out with 6 mg/ml mitoplasts as described before (14) using 25 µci/ml ^{3}H CTP (20 Ci/mmol).

Fig. 2. Electrophoretic analysis of mt translation products. Samples containing 200 µg mt proteins were electrophoresed on 8 to 16% gradient SDS-polyacrylamide slab gels as described by Laemmli (23). The gels were processed for fluorography as described elsewhere (17). (A) Control mt; (B) Effects of 500 µg/ml chloramphenicol, (C) Effects of 50 µg/ml and (D) 100 µg/ml AFB_1. Protein synthesis was carried out as described in Materials and Methods and in Fig. 2A.

50 µg/ml AFB_1 results in a general inhibition affecting almost all of the products to a similar extent (figure 2C). Addition of 100 µg/ml AFB_1 causes almost total inhibition (Figure 2D). In keeping with the results on metabolic conversion presented in Table 1, AFB_1 causes the highest level of inhibition of RNA and protein synthesis in liver mt and a somewhat reduced level of inhibition in kidney mt (Table 2). The extent of RNA and protein synthesis by brain, spleen and heart mt are not significantly affected by AFB_1 (Table 2). These results show that AFB_1 activating enzyme system is localized in liver and to some extent in kidney mt, suggesting that mt biogenetic system may be one of the direct targets of hepatic carcinogen AFB_1.

Involvement of mt system in cancer has been a subject of argument ever since Warburg's discovery (18) on altered mt oxidative metabolism in tumor cells. Over

Table 2. Comparison of the effects of AFB_1 on mt RNA and protein synthesis.

Source of Mitochondria	RNA Synthesis ^3H CPM x 10^{-4}/mg		Protein Synthesis ^{35}S CPM x 10^{-5}/mg	
	control	+AFB_1	control	+AFB_1
Liver	6.0	0.49	7.0	0.62
Kidney	1.1	0.19	0.97	0.06
Spleen	1.3	1.15	0.98	0.90
Brain	8.1	8.0	7.9	8.0
Heart	7.2	7.2	7.5	7.4

Mitochondrial RNA and protein synthesis were carried out for 45 min as described in Figure 1 and aliquots were used for determining C Cl_3COOH insoluble CPM.

the years, a number of studies have demonstrated differential properties of mt in tumor cells with respect to membrane structure, function and biogenesis. Experimental carcinogenesis and chemotherapy studies have shown that significant levels of nitrosamines (19) and nitrogen mustard (20) are transported to mt compartment which form adducts with mt DNA. Similarly, it has been shown that AFB_1 administration leads to inhibition of mt oxidative phosphorylation (21) and also DNA biosynthesis (22). In extension of these observations, our results show that incubation of liver mitoplasts with AFB_1 results in the activation of the carcinogen and marked inhibition of mt RNA and protein synthesis. These results provide direct evidence for the possible involvement of mt genome in carcinogenesis.

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