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FATTY ACID SYNTHESIS IN THE HEPATIC SOLUBLE FRACTION OF RAT RECOVERED FROM CCl_4 INTOXICATION

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It has been reported previously (1) that a soluble protein, extracted from liver cytoplasm of male rats that were intoxicated with CCl_4, stimulated microsomal NADPH oxidation and that its stimulation was inhibited by carbon monoxide, suggesting that the soluble protein had a carbon monoxide sensitive component such as hemoprotein. If this carbon monoxide sensitive component was a cytochrome P-450 (P-450) like hemoprotein, the soluble fraction would show some characteristic reactions dependent on P-450. That is, the inhibitory effect on the fatty acid synthesis could be observed in hepatic soluble fraction from rats pre-intoxicated with CCl_4 and this phenomenon could be inhibited by CO. These phenomena were observed, as was the unexpected result that soluble P-450 was present in the hepatic 204,000 g supernatant fraction extracted from rats pre-intoxicated with CCl_4; this P-450 was not contaminated by microsomal P-450. This communication describes these results.

MATERIALS AND METHODS

CCl_4 (1 ml/kg of body weight) was injected into male Wistar rats (300-350 g body weight) intraperitoneally. Twelve days after the CCl_4 injection, the rats were decapitated. Livers were perfused with ice-cold 0.9% NaCl, and then a 10% homogenate was made in 0.25 M sucrose, 4 mM Tris-HCl buffer (pH 7.5). The homogenate was centrifuged at 600 g for 10 min, and the resulting supernatant fraction was then centrifuged at 6000 g for 10 min to prepare the mitochondrial fraction. To avoid contamination by free ribosomes, soluble fraction was prepared from this mitochondrial supernatant fraction by centrifuging it at 204,000 g for 240 min. The resulting supernatant fraction was saved as the CCl_4 soluble fraction, and the sedimented microsomes were resuspended in 0.25 M sucrose, 4 mM Tris-HCl buffer (pH 7.5). A normal 204,000 g supernatant fraction was also prepared from normal rat liver (control).

Mitochondrial respiratory activity was measured as follows. The reaction medium was composed of 0.15 M KCl, 10 mM Tris-HCl buffer (pH 7.5), 2.5 mM potassium phosphate buffer (pH 7.5), 5 mM sodium succinate, 0.3 mM sodium ADP and mitochondria (2.5 mg protein/ml) in 3.5 ml at 25°. Dissolved oxygen was measured with an oxygen electrode (Galvanic type). A portion of the soluble fraction was incubated for 1 hr at 37°. After incubation, this soluble fraction was added to the reaction medium. Bovine albumin was purchased from Sigma.

Fatty acid synthesis was measured as follows. The reaction medium was composed of 2 mM ATP, 0.6 mM $MgCl_2$, 0.4 mM acetyl CoA, 1 mM NADPH, 8 mM $NaHCO_3$ and soluble fraction in 1 ml (normal fraction, 15.4 mg protein/ml; CCl_4 soluble fraction, 10.2 mg protein/ml). After the soluble fraction was incubated for 1

hr at 37°, fatty acid was measured by the method described by Shimizu et al. (2). Protein concentration was measured by the biuret method.

P-450 was determined from the carbon monoxide-induced difference spectrum of dithionite-reduced 204,000 g supernatant fraction, assuming a molar extinction coefficient of 91 $mM^{-1}cm^{-1}$ between 450 and 490 nm (3).

Difference spectra of hexobarbital with the 204,000 g supernatant fraction and with the microsomes were measured as follows. The 204,000 g supernatant fraction was divided equally into sample and reference cuvettes and scanned between 500 and 350 nm to produced a baseline, using a Shimazu UV-190 double beam spectrophotometer. Hexobarbital (1.5 μmoles) was added to the sample cuvette, while the reference cuvette received solvent in appropriate amounts. In the case of microsomes, microsomes were suspended in 0.15 M KCl, 10 mM Tris-HCl buffer (pH 7.5) to a concentration of 1.5 to 2.2 mg protein/ml. Reaction temperature was 22°.

RESULTS AND DISCUSSION

One of the reactions sensitive to fatty acid is the uncoupling of mitochondrial oxidative phosphorylation (4,5). We therefore studied the interaction between the soluble fraction and the mitochondrial fraction, with specific reference to mitochondrial respiration.

Table 1 shows the effect of the soluble fraction on mitochondrial respiratory activity. In isolated normal mitochondria (control), state 4 and state 3 respiratory activities were 23 and 118 natoms O per mg protein per min, and the respiratory control index (RCI) was 5.1, indicating that these mitochondria were well coupled. When the CCl_4 soluble fraction was added to the reaction medium (as indicated by the O line), the RCI increased due to suppression of state 4 respiration. When the normal fraction was added, there was slight uncoupling due to the activation of the state 4 respiration. To elucidate whether this difference in effect between the normal fraction and the CCl_4 fraction might have been due to a discrepancy between the metabolic properties of the two fractions, each fraction was incubated at 37° for 1 hr before the addition of the fraction to the reaction medium. The result is shown in the incubation line of Table 1. The discrepancy between the effects of the two fractions was observed more clearly, suggesting that the difference in the metabolic properties of the fractions was stimulated by incubation. The RCI was decreased in both fractions. The uncoupling, brought about by incubating normal and CCl_4 fractions, was suppressed in the presence of 1% albumin. Heat treatment (100° for 10 min) of these incubated fractions had no effect on the uncoupling activity of either fraction. These results indicate that the uncoupling factors bind to albumin and also are heat stable, suggesting that one of the uncoupling factors may be a fatty acid (4,5) and that fatty acid synthesis in the CCl_4 soluble fraction was inhibited. Hence, we measured fatty acid synthesis in the normal and CCl_4 soluble fractions; the results are shown in Table 2.

In the normal soluble fraction, 4.4 nmoles/mg protein of fatty acid was newly synthesized. In contrast, a low synthesis of fatty acid was observed in the CCl_4 soluble fraction; this result confirmed the effect of soluble fraction on mitochondrial respiration. The incubated normal fraction had a greater

Table 1. Effect on mitochondrial respiration of incubation at 37° for 1 hr of normal and CCl_4 soluble fractions*

Treatment	State 4	State 3	RCI
Control	23 ± 4.2	118 ± 6.3	5.1 ± 0.54
Normal fraction			
0	30 ± 6.6	120 ± 4.3	4.0 ± 0.31
Incubation	116 ± 4.0	116 ± 4.0	1.0 ± 0.03
+Albumin	35 ± 5.5	190 ± 7.8	5.4 ± 0.6
CCl_4 fraction			
0	18 ± 2.4	140 ± 5.6	7.8 ± 0.45
Incubation	42 ± 7.0	176 ± 5.8	4.2 ± 0.25
+Albumin	39 ± 3.3	196 ± 5.7	5.0 ± 0.76

*State 3 and 4 respiration is expressed in natoms O per mg protein per min. All values are the average of five experiments ± S.D. The concentrations of normal and CCl_4 soluble fractions were 15.4 and 10.2 mg protein/ml respectively. Albumin was 1%. Other experimental conditions are described in Materials and Methods.

Table 2. Fatty acid synthesis in normal and CCl_4 soluble fractions*

	Fatty acid synthesis	
	Normal fraction	CCl_4 fraction
No treatment	4.43 ± 0.18 (N=4)	3.31 ± 0.083 (N=5)
CO treatment	4.43 ± 0.29 (N=5)	3.84 ± 0.32 (N=6)

*Fatty acid synthesis values are expressed as nmoles per mg protein per 1 hr. CO was bubbled through the reaction mixture for 1 min. Other experimental conditions are described in Materials and Methods.

uncoupling effect than the CCl_4 fraction due to the greater amount of free fatty acid in the normal fraction than in the CCl_4 fraction. Fatty acid synthesis in the CCl_4 fraction was accelerated by carbon monoxide, but the fatty acid synthesis of the normal fraction was not affected by CO. This result suggests that the carbon monoxide sensitive component takes part in diminishing the synthesis of fatty acid. In other words, a carbon monoxide sensitive component might take part in the decomposition of fatty acid. These results might be observed if the CCl_4 soluble fraction was contaminated by microsomal fragments. We therefore measured the activity of glucose-6-phosphatase and the amount of RNA and found that these values were almost zero. If the soluble P-450 was present in the CCl_4 fraction, and its P-450 was not contaminated by microsomal P-450, the CO difference spectrum of the dithionite-reduced CCl_4 fraction would show an absorption maximum at 450 nm, the CCl_4 soluble fraction would give the characteristic substrate binding spectrum of P-450 like that of hexobarbital, and this substrate binding spectrum would not be the same as that of the microsomal one. We have examined these hypotheses. First, the CO difference spectrum of the dithionite-reduced CCl_4 soluble fraction showed an absorbing maximum at 455 nm. Second, when 1.5 μmoles of hexobarbital was added to the CCl_4 soluble fraction, it induced an anomalous spectral change; the absorption maximum and minimum occurred at 410 and 430 nm respectively. The anomalous spectral change induced by hexobarbital has been reported by Vore et al. (6). They reported that P-450, extracted with butanol-acetone (1:2) from microsomes of 3-methylcholanthrene-treated rats, exhibited anomalous spectral changes (410 nm peak, 430 nm trough) induced by hexobarbital in the absence of 25% glycerol. In general, solubilized P-450 is unstable in the absence of glycerol (7), and some parts of solubilized P-450 are converted to P-420 in this condition. P-420 that was induced by mercuric chloride (100 μM) treatment of normal microsomes for 30 min at 37° , however, did not show the same hexobarbital-induced spectral change as the CCl_4 soluble fraction (absorption maximum at 409 nm; absorption minimum did not occur). Therefore, the hexobarbital-induced spectral change of the CCl_4 soluble fraction might have been that of unstable P-450. Third, this hexobarbital binding spectrum of the CCl_4 soluble fraction was not the same as that of the microsomal fraction. In the case of microsomes, the absorption maximum and minimum occurred at 385 and 420 nm. These results suggest that the soluble P-450 was present in the CCl_4 soluble fraction and that its P-450 was not contaminated by microsomal P-450.

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