Effects of the Microsomal and Soluble Fractions from Livers of Rats Poisoned with CCl_4 on Mitochondrial Respiration

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Abstract: The microsomal fraction (pellet: $105,000 \times g$ for 60 min) from the livers of rats poisoned with CCl_4 showed uncoupled normal mitochondrial oxidative phosphorylation in vitro. The supernatant fraction ($105,000 \times g$ for 60 min) antagonized the uncoupling action of the microsomal fraction and stimulated the respiratory control of mitochondria caused by central depression of state 4 respiration.

Keywords: Microsomal and soluble fraction—Mitochondria—Poisoning with CCl_4 .

INTRODUCTION

Carbon tetrachloride (CCl_4) is known to be a potent causative agent of hepatitis. Mitochondria from livers of animals poisoned with this compound are in the uncoupled state^{1,2)}, with some lysosomal enzymes being released into the soluble fraction^{3,4)}. Previously, CCl_4 was thought to attack mitochondria directly, leading to the uncoupled state¹⁾. However, significant microsomal changes due to this poison precede mitochondrial dysfunction and parallel the appearance of fatty liver; hence the subcellular target of CCl_4 is now thought to be the endoplasmic reticulum⁵⁾.

We observed that the microsomal fraction (pellet: $105,000 g \times 60 \text{ min}$) from the livers of rats poisoned with CCl_4 showed uncoupled normal mitochondrial oxidative phosphorylation in vitro. The supernatant fraction ($105,000 g \times 60 \text{ min}$) antagonized the uncoupling action of the microsomal fraction and stimulated the respiratory control of mitochondria caused by central depression of state 4 respiration.

MATERIALS AND METHODS

Male Donryu rats (weighing from 200 to 300 g) fed on a laboratory stock

diet were injected intraperitoneally with CCl_4 (1.5 ml/kg of body weight). They were killed by decapitation 48 and 120 hours after the injection. The liver was immediately immersed in ice-cold 0.25-M sucrose containing 4 mM Tris-HCl buffer (pH 7.5) and then fractionated by differential centrifugation^{6,7)}.

Respiration and oxidative phosphorylation was measured in a reaction mixture of 3.5 ml containing 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 and 0.3 mM sodium ADP at 25°C with a galvanic oxygen electrode (Kysui Kagaku Co. Ltd. Tokyo).

Protein concentration was measured by the biuret method with bovine serum albumin as the standard.

CCl_4 was purchased from Wako Pure Chemical Industry Ltd. All other reagents used in this experiment were of reagent grade.

RESULTS

Isolated normal mitochondria showed the respiratory activity demonstrated in Table 1. State 3 respiratory activity was 116.2 ± 7.2 natoms of O_2 /min/mg of protein, state 4 respiratory activity was 22.3 ± 1.9 natoms of O_2 /min/mg of protein and the respiratory control index (RCI) was 5.24 ± 0.37 , suggesting that these mitochondria were tightly coupled.

When a CCl_4 -poisoned fraction (from the supernatant fraction 10,000 $\text{g} \times 10$ min) was added to the reaction mixture, state 4 respiration was markedly increased, from 22.3 to 34.7 natoms of O_2 /min/mg of protein, and consequently the RCI decreased from 5.24 to 3.0, suggesting that the mitochondria were uncoupled by the CCl_4 -poisoned fraction.

In contrast, when a normal fraction was added to the reaction mixture, almost no change in respiratory activity was observed, suggesting that the normal fraction did not possess the uncoupling activity.

Characterization of the uncoupling action

The uncoupling activity of the CCl_4 -poisoned fraction was not observed in a

Table 1. The effect of the supernatant fraction (10,000 $\text{g} \times 10$ min) from the livers of rats poisoned with CCl_4 on mitochondrial respiration.

	State 3	State 4	RCI (state 3/state 4)
Control	116.2 ± 7.2	22.3 ± 1.9	$5.24 \pm 0.37^*$
+Normal fraction (3.4 mg of protein)	115.0 ± 6.8	21.3 ± 0.47	5.4 ± 0.43
+ CCl_4 fraction (3.6 mg of protein)	109.0 ± 5.2	34.7 ± 4.0	3.0 ± 0.29
+boiled CCl_4 fraction	111.0 ± 6.8	21.3 ± 2.6	5.3 ± 0.85

State 3 and state 4 respiration are expressed as natoms of O_2 /min/mg of protein.

CCl_4 fraction was prepared from the livers of CCl_4 -poisoned rats 2 days after injection.

* (mean $\pm$ S.D. n=6)

boiled CCl_4 -poisoned fraction (Table 1). Bovine serum albumin did not inhibit the uncoupling. The change in the uncoupling activity of the CCl_4 -poisoned fraction with time was examined and the result is shown in Fig. 1. Uncoupling activity 2 days after the injection was greater than that 5 days after the injection.

The CCl_4 -poisoned fraction was centrifuged to separate the microsomal and soluble fractions. As shown in Table 2, the mainly microsomal fraction (pellet: $105,000 \times 60$ min) from CCl_4 -poisoned liver had uncoupling activity; a slight decrease in state 3 respiration from 116 to 110 natoms of $\text{O}_2/\text{min}/\text{mg}$ of protein and an increase in state 4 respiration from 20.7 to 25.4 natoms of $\text{O}_2/\text{min}/\text{mg}$ of protein occurred. The RCI decreased from 5.6 to 4.2.

In contrast, a marked change was brought about by the CCl_4 -supernatant fraction (S fraction: $105,000 \text{ g} \times 60$ min). As shown in Table 2, the CCl_4 -S fraction decreased mainly state 4 respiratory activity from 20.7 to 16.0 natoms of $\text{O}_2/\text{min}/\text{mg}$ of protein and consequently increased the RCI from 5.6 to 7.0, suggesting

Fig. 1. The effect of the supernatant fraction ($10,000 \text{ g} \times 10$ min) on the respiratory control of normal rat liver mitochondria.

The respiratory control index of normal rat liver mitochondria was 5.24 ± 0.37 and the concentration of mitochondrial protein was 3.2 mg/ml . Protein concentration of the normal supernatant fraction and of the CCl_4 -supernatant fraction added to the reaction mixture was 2.0 mg .

Table 2. The effects of the microsomal and soluble fractions from livers of rats poisoned with CCl₄ on mitochondrial oxidative phosphorylation.

	State 3	State 4	RCI	% of control
Control	116±8.0*	20.7±1.3	5.6±0.04	100
+ Normal M (2.1 mg of Protein)	115±2.2	21.5±0.09	5.4±0.08	96
+ CCl ₄ M (2.4 mg of protein)	110±5.9	25.9±1.1	4.2±0.2	70
+ Normal S (1.2 mg of protein)	110±3.9	21.6±0.8	5.1±0.04	89
+ CCl ₄ S (1.3 mg of protein)	111±6.1	16.0±1.2	7.0±0.2	130

Normal M refers to the pellet fraction of 105,000 g × 60 min centrifugation from normal liver. CCl₄ M refers to the pellet of 105,000 g × 60 min centrifugation from livers of CCl₄-poisoned rats 5 days after injection. Normal S refers to the supernatant fraction of 105,000 g × 60 min centrifugation from normal liver. CCl₄ S refers to the supernatant fraction of 105,000 g × 60 min centrifugation from livers of CCl₄-poisoned rats 5 days after injection. State 3 and state 4 respiration are expressed as natoms of O₂/min/mg of protein.

* (mean±S.D. n=6)

Table 3. Characterization of the stimulatory activity of the supernatant fraction (105,000 g × 60 min) from the livers of rats poisoned with CCl₄ for respiratory control of mitochondria.

	State 3	State 4	RCI
A: Control	116	20.7	5.6
+ CCl ₄ M fraction	110	24.8	4.43*
+ CCl ₄ M fraction+normal S	118	25.1	4.7**
+ CCl ₄ M fraction+CCl ₄ S	120	17.9	6.7***
B: + Normal S	108	21.5	5.0
+ CCl ₄ S	104	14.4	7.25
+ boiled normal S	124	23.3	5.31
+ boiled CCl ₄ S	113	21.5	5.25

State 3 and state 4 respiration are expressed as natoms of O₂/min/mg of protein. CCl₄ M fraction refers to the microsomal fraction from the livers of CCl₄-poisoned rats 5 days after injection. Normal S refers to the supernatant fraction of 105,000 g × 60 min centrifugation from normal liver and CCl₄ S refers to the supernatant fraction of 105,000 g × 60 min centrifugation from the livers of CCl₄-poisoned rats 5 days after injection.

* (CCl₄ M fraction: 0.5 mg of protein) ** (Normal S: 1.2 mg of protein)

*** (CCl₄ S: 1.3 mg of protein)

that a more tightly coupled state of oxidative phosphorylation is brought about by the CCl₄-S fraction.

Characterization of the stimulatory activity of the CCl₄-S fraction

The stimulatory activity of the CCl₄-S fraction for respiratory control was heat labile (Table 3) and antagonized the uncoupling action of the microsomal fraction (Table 3). The effect of concentration on the stimulatory activity was measured, with the results shown in Fig. 2. The CCl₄-S fraction prepared 5 days after the

Fig. 2. The effect of the supernatant fraction (S fraction: $105,000 \text{ g} \times 60 \text{ min}$) on the respiratory control of normal rat liver mitochondria.

The respiratory control index of normal rat liver mitochondria was 5.60 ± 0.04 and the concentration of mitochondrial protein was 2.0 mg/ml .

injection was more effective than that prepared 2 days after the injection. In other words, the stimulatory activity per milligram of supernatant protein was higher at 5 days than at 2 days.

DISCUSSION

The data presented above show that the microsomal fraction from the livers of rats poisoned with CCl_4 has powerful uncoupling activity. The supernatant fraction ($105,000 \text{ g} \times 60 \text{ min}$) from livers of the poisoned rats antagonized the uncoupling activity of the microsomal fraction and stimulated respiratory control to a degree dependent on the lapse of time after the injection of CCl_4 .

The uncoupling shown by the supernatant fraction obtained by centrifugation at $10,000 \text{ g} \times 10 \text{ min}$ may not be related to the uncoupling action of fatty acids⁸, because the addition of bovine serum albumin to the reaction mixture did not change the uncoupling activity⁸, and the boiled fraction did not have any uncoupling activity. The mechanism of the uncoupling action of the main microsomal fraction is not known.

It is interesting to note that the supernatant fraction ($105,000 \text{ g} \times 60 \text{ min}$) from the livers of rats poisoned with CCl_4 showed significant stimulatory activity

for respiratory control caused by central depression of state 4 respiration and that this stimulatory activity was dependent on time. The time dependency of the stimulatory activity was related to the time course of changes in the uncoupling activity of the CCl_4 -poisoned fraction (data in Fig. 1). The specific stimulatory activity increased with increase in time, and the uncoupling activity decreased with increase in time. The effect of the supernatant fraction ($105,000 \text{ g} \times 60 \text{ min}$) on mitochondrial oxidative phosphorylation may be related to the process of recovery from liver injury. The precise mechanism of the stimulatory action of the supernatant fraction is not known. Perhaps, unknown heat-labile factors (as shown in Table 3) are present in the supernatant fraction from livers of rats poisoned with CCl_4 .

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