Biochemical pathology of fatty liver induced by inhaled carbon tetrachloride, with specific reference to ATP and lipid metabolism in the mouse liver

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Abstract

Cb strain female mice were exposed to 800 p.p.m. of carbon tetrachloride for 3 hours by the use of newly devised gas chamber via constant current of gas. Contents of ATP, triglyceride and total lipid in the liver were measured at appropriate intervals after inhalation of carbon tetrachloride and compared to non-treated controls. And P : 0 ratio of the liver mitochondria was measured by oxymeter and morphological changes of liver mitochondria were observed by electron microscopy. The following results were obtained. 1. ATP content in the liver decreased slightly immediately after inhalation, rapidly decreased until 4 hours after inhalation and gradually decreased until 20 hours after inhalation. 2. Contents of total lipids increased slightly immediately after the exposure and increased gradually until 20 hours later. Contents of triglyceride in the liver increased at almost constant rate during and after the exposure. 3. P : 0 ratio of liver mitochondria did not change immediately after the exposure and gradually increased after the exposure, keeping parallel relation to decrease in ATP content in the liver. Decrease in ATP content in the liver after inhalation of carbon tetrachloride seems to be mainly due to uncoupling of oxidative phosphorylation of liver mitochondria. 4. Morphological changes of liver mitochondria were observed at 4 hours after the exposure by electron microscopy. 5. Decrease in ATP levels of the liver suggested to have a close relation to accumulation of lipid in the liver after the inhalation of carbon tetrachloride.
BIOCHEMICAL PATHOLOGY OF FATTY LIVER INDUCED BY INHALED CARBON TETRACHLORIDE, WITH SPECIFIC REFERENCE TO ATP AND LIPID METABOLISM IN THE MOUSE LIVER

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Received for publication, August 20, 1969

Carbon tetrachloride is widely used in some industries as organic solvent. The threshold limit of value of carbon tetrachloride gas in the air is decided to be 10 p.p.m. (1).

Carbon tetrachloride is being used as experimental hepatotoxic agents by many investigators. It is well known that carbon tetrachloride is toxic to the liver cells and causes fatty necrosis to the liver cells (2, 3). But mechanisms of its hepatotoxicity remain unsolved.

DIANZANII reported that carbon tetrachloride induced the uncoupling of oxidative phosphorylation of liver mitochondria accompanied by the decreasing in the ATP content of the liver of rats 24 hours after injection of carbon tetrachloride (4, 5). RECKNAGEL reported that the activity of Mg-activated ATP-ase was enhanced after injection of carbon tetrachloride (6). However, very little information is available as to the toxic effect of inhaled carbon tetrachloride to the liver.

A newly devided gas chamber via constant current of the air was used for the experiments (7). Mice were exposed to 800 p.p.m. of carbon tetrachloride gas for 3 hours. The contents of total lipid, triglyceride and ATP in the liver were determined at appropriate intervals after exposure to carbon tetrachloride.

In order to clarify the cause of decrease in ATP content in the liver, P;O ratio of the liver mitochondria was studied by oxymeter and morphology of liver mitochondria by electron microscopy.

MATERIALS AND METHODS

Cb strain female mice (weighing 16±2g) were exposed to 800 p.p.m. of carbon tetrachloride for 3 hours.

The mice were sacrificed by decapitation immediately after the inhalation, 4, 8, 20 hours afterwards. By removing the liver, it was washed with ice cold
saline and weighed.

ATP content in the liver was determined by luciferine-luciferase reaction. The principles of luciferine-luciferase reactions.

1) \( \text{ATP} + \text{luciferine} \rightarrow \text{adenyl-luciferine} + \text{pyrophosphate} \)

2) \( \text{Adenyl-luciferine} + \frac{1}{2} \text{O} \rightarrow \text{adenyl-oxyluciferine} + \text{light} \)

The light generated from this reaction was measured by liquid scintillation spectrophotometer (Packard Model 3324) (9).

The preparation of luciferine-luciferase solution (10).

Fifty mg of desicated tails of firefly lanterns (made in Sigma Co) was homogenized with Potter Evelyne type glass homogenizer in the ice cold bath for 5 min, and frozen at \(-20^\circ\text{C}\) as the stock solution.

Standard ATP solution was prepared by 100-fold dilution of stock solution. Liquid scintillation counter was set at gain 50\%, window red, width of the window 50—1000 and preset count 0.1 min.

Distilled water, diluted standard solution and luciferine-luciferase solution (1.0 ml: 1.0 ml; 0.1 ml v/v) were mixed well. Immediately after the start of enzyme reaction, the light generated was measured by liquid scintillation counter.

The standard curve thus obtained was linear at the ATP concentration of \(10^{-6}\) M to \(10^{-8}\) M (as shown in Fig. 1).

Preparation of ATP solution from the mouse liver.

Mouse was killed by decapitation and it's liver excised, washed with ice cold saline, weighed, put in the boiling water, homogenized and quickly chilled in the ice cold water and centrifuged. The supernatant obtained at each time served as the sample solution. ATP concentration in the sample solution was calculated from the standard curve. The interval from sacrifice to boiling the liver was strictly fixed at 2 minutes.

The total lipid was measured by Folch method (11) and triglyceride was measured by Van Handels method (12).

Liver mitochondria were prepared.
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pared by a modified method of HOGEBOOM and SCHNEIDER (13).

P : 0 ratio (ADP : 0 ratio) was measured by oxymeter (14). Reaction in oxymeter was performed at 25°C. The respiratory control ratio was obtained by the O₂ consumption of state 2 per minute divided by the O₂ consumption of state 3 per minute (as shown in Fig. 2). ATP contents of the liver was also determined at the same time. After staining the liver mitochondria with 2% permanganate, their morphology after the inhalation of carbon tetrachloride was observed by electron microscopy.

RESULTS

Change in the contents of ATP, triglyceride and total lipids in the liver after inhalation of carbon tetrachloride are shown in Table 1.

Table 1 Change in ATP, total lipids and triglycerides in the liver after inhalation of 800 p. p. m. of CCl₄ for 3 hours

<table>
<thead>
<tr>
<th>Hours after inhalation</th>
<th>No. of cases</th>
<th>ATP in the Liver</th>
<th>Total Lipides in the Liver</th>
<th>Triglycerides in the Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mg/g w. w.</td>
<td>%</td>
<td>mg/g body weight</td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>0.811±0.215*</td>
<td>100</td>
<td>2.13±0.16*</td>
</tr>
<tr>
<td>0</td>
<td>5</td>
<td>0.725±0.196</td>
<td>89</td>
<td>2.39±0.18</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>0.294±0.106</td>
<td>36</td>
<td>3.23±0.29</td>
</tr>
<tr>
<td>8</td>
<td>6</td>
<td>0.163±0.082</td>
<td>20</td>
<td>3.62±0.62</td>
</tr>
<tr>
<td>20</td>
<td>6</td>
<td>0.143±0.065</td>
<td>18</td>
<td>7.03±1.48</td>
</tr>
</tbody>
</table>

* M ± 6

ATP content (mg/g liver wet weight) was 89% of the control, immediately after the exposure, 36% at 4 hours, 20% at 8 hours and 18% at 20

Fig. 3 Change in ATP and triglyceride content in the liver after inhalation of 800 p. p. m. of CCl₄ for 3 hours
hours after the exposure.

Triglyceride content increased to 205% of control immediately after the exposure. Relationship between ATP content and triglyceride content in the liver is illustrated in Fig. 3.

Total lipid content in the liver increased to 112% of control immediately after the exposure, 152% at 4 hours, 193% at 8 hours, and 330% at 20 hours after the exposure. Relationship between ATP content and total lipid content in the liver is illustrated in Fig. 4.

![Fig. 4 Change in ATP and total lipid content in the liver after inhalation of 800 p.p.m. of CCl₄ for 3 hours](image)

Triglyceride: total lipid, ratio in the liver was 16.4% in control immediately after the exposure and 36.3% at 4 hours after inhalation.

These results suggest that the increase in total lipid contents of the liver was mainly due to the increase in triglyceride content of the liver.

In order to clarify the mechanism of decrease in ATP levels of the

<table>
<thead>
<tr>
<th>Hours after Inhalation</th>
<th>Succinate respiratory control ratio</th>
<th>Glutamate respiratory control ratio</th>
<th>ATP in the Liver mg/g liver w.w.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.8</td>
<td>5.4</td>
<td>0.700*</td>
</tr>
<tr>
<td>0</td>
<td>4.5</td>
<td>5.6</td>
<td>0.722</td>
</tr>
<tr>
<td>4</td>
<td>2.8</td>
<td>3.6</td>
<td>0.492</td>
</tr>
<tr>
<td>8</td>
<td>1.3</td>
<td>1.3</td>
<td>0.193</td>
</tr>
</tbody>
</table>

* means of 3 cases
Mitochondria were obtained from the homogenate of the livers of the 3 mice in each.
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2.0 P:o ratio

liver, the P:o ratio of the liver mitochondria was determined by means of oxymeter, using succinate and glutamate as substrate. ATP content of the liver was determined by luciferine-luciferase method at the same time. The results are shown in Fig. 5 and Table 3.

Respiratory control ratio, P:o ratio and ATP content in the liver did not change immediately after inhalation, but moderately decreased at 4 hours and markedly decreased at 8 hours after the exposure.

P:o ratio and respiratory control ratio changed almost parallel to the ATP content in the liver.

These results suggest that the decrease in ATP content of the liver after inhalation of carbon tetrachloride was mainly due to the uncoupling of oxidative phosphorylation of liver mitochondria. Electron micrograph of the liver mitochondria 4 hours after inhalation of carbon tetrachloride showed appearance of giant mitochondria and morphological alteration of mitochondria which suggests the fusion of mitochondria (as shown in Fig. 6)

Decrease in number of mitochondria was observed in treated cases compared to non-treated cases. Increase in number of mitochondrial cristae and elongation of mitochondrial cristae were also observed in treated cases.

These morphological changes may give some explanation to the functional changes which were obtained in the experiment of oxymeter.

DISCUSSION

Hepatotoxicity of carbon tetrachloride was studied by many investigators, but most of experiments were performed by intraperitoneal injection or intragastric administration of carbon tetrachloride. However, in the field of industrial hygiene, carbon tetrachloride-gas intoxication is very important.

Kyline stated that carbon tetrachloride inhaled caused fatty liver (15).

In the present experiment, a newly devised gas chamber via constant current of gas was employed. The ATP and lipid metabolisms after inha-
lation of carbon tetrachloride were studied.

Dianzani reported that ATP content decreased 24 hours after injection of carbon tetrachloride, and he demonstrated that uncoupling of oxidative phosphorylation of liver mitochondria (4) and suggested that the decrease in ATP content of the liver induced inhibition of fatty acid decomposition.

We have found that the decrease in ATP content of the liver has a close relation to the accumulation of lipid in the liver after inhalation of tetrachloroethylene, trichloroethylene and carbon tetrachloride (16).

Farber reported that the fall in the ATP levels of liver by ethionine induced accumulation of triglyceride in the liver (17) and he also demonstrated that the decrease in hepatic ATP levels induced inhibition of protein synthesis of the liver (18), and explained that inhibition of the serum lipoprotein synthesis induced accumulation of triglyceride in the liver (19).

Ogata demonstrated that inhibition of the serum albumin and lipoprotein in the liver after injection of carbon tetrachloride by studying the incorporation of C14 glycine into protein part of serum lipoprotein and serum albumin (20, 21).

Recknagel stated that change in microsomal enzyme activity preceded the change in the mitochondrial enzyme activity (23). Sakai reported that activity of cytochrome b 5 or p 450 of microsome decreased earlier than the decrease in P : 0 ratio of liver mitochondria (24).

Slater suggested that free radical metabolite (ClPC°) injured mitochondria and microsome in the liver by lipid peroxidation (25).

This was confirmed by Recknagel (26).

Morphological changes of liver mitochondria and alteration of microsomes in carbon tetrachloride liver injury were confirmed by Bassei and Haba by electron microscopy (27, 28).

In the present experiment, it was suggested that the decrease in the ATP content of the liver and accumulation of triglyceride occurred after inhalation of carbon tetrachloride and decrease in the ATP content of the liver and accumulation of triglyceride occurred after inhalation of carbon tetrachloride and decrease in the ATP content of the liver accompanied by decreased P : 0 ratio of the liver mitochondria was mainly due to the uncoupling of oxidative phosphorylation. Activation of Mg-activated ATPase reported by Recknagel seems to be another cause of decrease in ATP content in the liver. (6).

It was suggested that the decrease in hepatic ATP levels and decreased template activity of liver microsomes caused inhibition of protein part of the very low density lipoprotein synthesis in the liver disturbed the secre-
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Disturbance of secretion of triglyceride from the liver induces accumulation of triglyceride in the liver. The decrease in ATP contents of the liver and impaired function of liver mitochondria seem to take another important role for accumulation of triglyceride in the liver by inhibition of fatty acid oxidation in the liver mitochondria.

CONCLUSION

Cb strain female mice were exposed to 800 p.p.m. of carbon tetrachloride for 3 hours by the use of newly devised gas chamber via constant current of gas.

Contents of ATP, triglyceride and total lipid in the liver were measured at appropriate intervals after inhalation of carbon tetrachloride and compared to non-treated controls. And P:O ratio of the liver mitochondria was measured by oxymeter and morphological changes of liver mitochondria were observed by electron microscopy.

The following results were obtained.

1. ATP content in the liver decreased slightly immediately after inhalation, rapidly decreased until 4 hours after inhalation and gradually decreased until 20 hours after inhalation.

2. Contents of total lipids increased slightly immediately after the exposure and increased gradually until 20 hours later. Contents of triglyceride in the liver increased at almost constant rate during and after the exposure.

3. P:O ratio of liver mitochondria did not change immediately after the exposure and gradually increased after the exposure, keeping parallel relation to decrease in ATP content in the liver. Decrease in ATP content in the liver after inhalation of carbon tetrachloride seems to be mainly due to uncoupling of oxidative phosphorylation of liver mitochondria.

4. Morphological changes of liver mitochondria were observed at 4 hours after the exposure by electron microscopy.

5. Decrease in ATP levels of the liver suggested to have a close relation to accumulation of lipid in the liver after the inhalation of carbon tetrachloride.

ACKNOWLEDGEMENT

The author is indebted much to Professor M. Ogata for invaluable advices and to Mr. K. Tomokuni and Miss S. Ueki for technical assistances.
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1. American Conference of Governmental Industrial Hygienists: Threshold limit values for 1964: *Arch Environ. Health* 2, 545, 1964
Fig. 6 Electron micrograph of the liver mitochondria of the mouse.

1. The liver mitochondria of untreated control
2. The liver mitochondria of the mouse 4 hours after inhalation of (CCl₄)
   M. mitochondria A. accumulation of lipid in the liver cells B. Swelling and fusion of mitochondria