EFFECT OF CYTOCHROME C ON CARBON TETRACHLORIDE DAMAGE TO THE LIVER

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Abstract—Intramuscular or oral administration of cytochrome c provided a defense against carbon tetrachloride-induced hepatic impairment characterized by accumulation of triglycerides in the liver and damaged mitochondria. The mechanism may involve activation of the mitochondrial function by cytochrome c with the result that ATP production is increased, hence a normal metabolism is regained.

As a major factor involved in cell respiration, cytochrome c has been applied to the treatment of various diseases related to tissue anoxia. Thus far it has been used exclusively by the parenteral route.

The authors previously reported on the pharmacological activity of enteric-coated granules of cytochrome c given orally (1). It was also reported that the heme protein improves spontaneously, lowered functions of the liver and the lipid metabolism in aged rats (2).

A study has been made herein of the effect of cytochrome c on abnormal sugar and lipid metabolism, associated with acute hepatic damage, which had been experimentally induced by carbon tetrachloride.

MATERIALS AND METHODS

The cytochrome c used was taken from fresh horse heart and refined according to the method of Hagihara et al. (3). It has an OD_{550}/OD_{280} ratio of 1:2 in the reduced form. There were oxidized and reduced forms available in a ratio of approx. 1:9. Cytochrome c was given either i.m. in a 0.9% aqueous solution of sodium chloride, or orally as enteric-coated granules, having a diameter of 0.5 mm, containing gelatin as a bulking agent, and coated with cellulose acetate phthalate. The granules complied with the requirements of the Japanese Pharmacopoeia VIII disintegration test.

Groups of 10 male Donryu rats weighing approx. 250 gm were administered 1 ml/kg/day i.p. of a 20% solution of carbon tetrachloride in olive oil for 3 consecutive days to induce hepatic impairment. One hundred mg/kg/day or 200 mg/kg/day of cytochrome c was given i.m. into the thigh, or orally as enteric-coated granules, for 4 days starting from the day prior to the start of the carbon tetrachloride treatment. Control animals on carbon tetrachloride were administered orally enteric-coated granules containing not cytochrome c but gelatin exclusively. It was confirmed beforehand that this treatment was no different from i.m. injection of 0.9% aqueous solution of sodium chloride with
respect to biochemical characteristics. In addition there was no effect exerted on such characteristics when 1 ml/kg/day of olive oil was given i.p. for 3 consecutive days. The day after completion of the treatment, the animals were exsanguinated and promptly laparotomized to perfuse the liver with a 0.9% aqueous solution of sodium chloride infused into the portal vein. The blood and liver thus obtained were subjected to various biochemical investigations.

The next experiment in the series was then carried out to study the short term effect of treatment with cytochrome c on the quantity of lipid peroxides soon after the administration of carbon tetrachloride. Animals were injected i.p. with 0.2 ml/kg of carbon tetrachloride following intramuscular or oral administration of 100 mg/kg/day or 200 mg/kg/day of cytochrome c for 3 consecutive days. Six hr later they were exsanguinated to determine the amount of lipid peroxides in the liver and serum.

Methods of lipid determinations: Total serum cholesterol was estimated by Zarkowski’s method (4); serum phospholipid by the technique of Hoefmayer and Fried (5); free fatty acid by Doncombe’s procedure (6); serum triglyceride by Van Handel’s technique (7); and β-lipoprotein by the heparin calcium chloride precipitation method (8). Determination of liver lipids was made by extraction with a chloroform-methanol (2:1) mixture from a homogenate prepared by adding 9 ml of 0.9% aqueous solution of sodium chloride to 1 gm of the liver, and estimating cholesterol, phospholipid and triglyceride fractions using a method similar to that used for serum.

Procedures for glucose estimation: Blood sugar was determined using the technique of Somogyi & Nelson (9); liver glycogen by the anthron method (10); pyruvate by the technique of Friedmann & Shimizu (11); citrate by the pentobromoacetone method (12); lactate by the method of Barker & Summerson (13); and 2-ketoglutarate by the technique of Bergmeyer and Erich (14).

Glucose tolerance test was carried out as follows: rats not ingesting anything other than water for 16 hr were injected with 2 ml/kg of 25% aqueous solution of glucose into a penial vein. Blood glucose level was estimated by the method of Somogyi and Nelson (9) 30 and 60 min later.

Liver ATP was estimated by Holloway’s method (15).

Mitochondrial tissue respiration and oxidation of fatty acids were studied on hepatic mitochondria specimens prepared according to the procedure of Hagihara et al. (16, 17).

Cytochromes were determined by Fukuhara’s technique (18).

Determination was made of lipid peroxides using the TBA method (19). Data were submitted to Student’s t-test of significance.

RESULTS

1) Effects of cytochrome c on lipid metabolism

Table 1 shows the effects of cytochrome c on abnormalities of lipid metabolism. Treatment with carbon tetrachloride was found to bring a significant reduction in serum cholesterol and β-lipoprotein and a significant increase in hepatic cholesterol and triglyceride.
TABLE 1. Effects of cytochrome c on lipid metabolism disturbed by carbon tetrachloride.

|                     | Untreated control animals | Control animals given CCl₄ | Animals given cytochrome c |
|---------------------|---------------------------|----------------------------|---------------------------|
| Serum               |                           |                            |                           |
| Cholesterol         | 69.4±5.9**                 | 46.5±3.5                   | 71.8±8.6*                 |
| Triglycerides       | 49.8±5.7                   | 38.2±3.4                   | 35.9±7.2                 |
| Phospholipids       | 122±10                     | 102±6                      | 123±10                    |
| Free fatty acid     | 6.18±0.75                  | 9.36±1.17                  | 7.22±0.82                 |
| β-Lipoprotein       | 180±10**                   | 120±6                      | 162±14*                   |
| Liver               |                           |                            |                           |
| Cholesterol         | 2.70±0.24*                 | 3.53±0.21                  | 3.71±0.16                 |
| Triglycerides       | 9.50±0.72**                | 38.2±1.5                   | 34.1±3.3                 |
| Phospholipids       | 30.0±1.0                   | 29.0±1.3                   | 28.3±1.5                 |
| Oxidation of mg fatty acid per mg min | 75.8±4.6**                | 49.1±7.5                   | 53.6±4.9                 |

Results are given as means±S.E. for 10 animals. Significant differences from control animals given CCl₄ are marked: * (p<0.05); ** (p<0.01).

Furthermore, serum triglyceride and phospholipid were decreased and serum free fatty acids increased, although not significantly. Such abnormal lipid metabolism caused by carbon tetrachloride was inhibited by i.m. or oral administration of cytochrome c. In animals on the oral treatment, serum cholesterol, β-lipoprotein and liver triglyceride in particular, were improved significantly, and, in those animals given the substance i.m., serum β-lipoprotein, hepatic cholesterol and triglyceride were significantly improved. There was an evident relationship between the dosage level of cytochrome c and its effect, except for a few cases. Oral treatment had a somewhat more favorable effect on serum lipids while hepatic lipids showed this effect with an i.m. injection. The phospholipid content of the liver was hardly affected by administration of carbon tetrachloride or cytochrome c, while the fatty acid oxidizing ability of the mitochondria was significantly reduced by carbon tetrachloride but slightly enhanced by cytochrome c.

2) Effects of cytochrome c on lipid metabolism

Table 2 shows the effects of cytochrome c on blood glucose levels. The significant

TABLE 2. Effects of cytochrome c on blood glucose levels decreased with carbon tetrachloride.

|                     | Untreated control animals | Control animals given CCl₄ | Animals given cytochrome c |
|---------------------|---------------------------|----------------------------|---------------------------|
| Non-fasting         | 161±6**                   | 133±6                      | 140±4                     |
| Fasting             | 128±5**                   | 104±4                      | 108±3                     |
| 30 min after glucose load | 185±10**                | 126±6                      | 148±10*                   |
| 60 min after glucose load | 139±11*                 | 107±4                      | 111±3                     |

Results are given as means±S.E. for 10 animals. Significant differences from control animals given CCl₄ are marked: * (p<0.05); ** (p<0.01).
reduction in blood glucose level due to carbon tetrachloride given to non-fasting animals was inhibited to a significant and comparative extent both by i.m. injection and oral administration of cytochrome c.

Treatment with carbon tetrachloride brought a significant reduction in blood glucose in either fasting animals or those loaded with glucose after fasting. This reduction was significantly inhibited by cytochrome c but only 30 minutes after loading with glucose.

Table 3 shows the effects of cytochrome c on abnormalities of sugar metabolism. Treatment with carbon tetrachloride was also followed by a significant reduction in liver glycogen and a significant increase in liver and serum pyruvates and citrates. Liver and serum lactates and α-ketoglutarates were not significantly increased by carbon tetrachloride. These abnormal variations caused by carbon tetrachloride were alleviated by treatment with cytochrome c: abnormal liver glycogen and serum pyruvate levels were obviously corrected by cytochrome c, which exerted, when given i.m., a marked effect on the citrate content of the liver and serum.

Table 3. Effects of cytochrome c on sugar metabolism disturbed by carbon tetrachloride.

| Control animals given CCl₄ | Animals given cytochrome c 100 mg/kg i.m. | Animals given cytochrome c 200 mg/kg i.m. |
|---------------------------|------------------------------------------|------------------------------------------|
| Untreated control animals | 100 mg/kg p.o. | 200 mg/kg p.o. | 100 mg/kg i.m. | 200 mg/kg i.m. |

| Serum | Untreated control animals mg/dl | Control animals given CCl₄ mg/dl | Animals given cytochrome c 100 mg/kg i.m. | Animals given cytochrome c 200 mg/kg i.m. |
|-------|---------------------------------|---------------------------------|------------------------------------------|------------------------------------------|
| Pyruvate | 3.55±0.13** | 4.41±0.13 | 3.86±0.13** | 4.05±0.13 | 4.02±0.20 | 3.95±0.08** |
| Lactate | 19.3±0.8 | 22.4±0.8 | 22.5±1.8 | 20.9±0.8 | 21.0±1.2 | 19.9±0.6 |
| Citrate | 3.85±0.34* | 5.60±0.59 | 5.07±0.33 | 4.58±0.5 | 3.81±0.54* | 2.64±0.40** |
| α-ketoglutarate | 0.99±0.27 | 1.34±0.27 | 1.33±0.39 | 1.31±0.11 | 1.31±0.11 | 0.75±0.17 |

| Liver | Untreated control animals mg/g | Control animals given CCl₄ mg/g | Animals given cytochrome c 100 mg/kg i.m. | Animals given cytochrome c 200 mg/kg i.m. |
|-------|---------------------------------|---------------------------------|------------------------------------------|------------------------------------------|
| Glycogen | 45.1±3.0** | 8.1±2.0 | 9.6±1.7 | 17.3±3.6** | 17.8±2.4** | 20.0±2.7** |
| Pyruvate | 0.28±0.00* | 0.35±0.03 | 0.32±0.02 | 0.33±0.01 | 0.33±0.01 | 0.32±0.01 |
| Lactate | 12.6±0.9 | 18.7±2.4 | 14.3±1.1 | 16.4±0.7 | 16.7±0.7 | 15.3±0.8 |
| Citrate | 0.38±0.07** | 1.36±0.27 | 0.84±0.15 | 0.82±0.25 | 0.81±0.25 | 0.67±0.10* |
| α-ketoglutarate | 0.20±0.05 | 0.31±0.03 | 0.34±0.08 | 0.23±0.03 | 0.21±0.03 | 0.34±0.12 |

Results are given as means±S.E. for 10 animals. Significant differences from control animals given CCl₄ are marked : *(p<0.05) ; **(p<0.01).

Table 4. Effects of cytochrome c on ATP level decreased with carbon tetrachloride.

| Control animals given CCl₄ | Animals given cytochrome c 100 mg/kg i.m. | Animals given cytochrome c 200 mg/kg i.m. |
|---------------------------|------------------------------------------|------------------------------------------|
| Untreated control animals | 100 mg/kg p.o. | 200 mg/kg p.o. | 100 mg/kg i.m. | 200 mg/kg i.m. |

| Liver | ATP mg/g | Untreated control animals | Control animals given CCl₄ | Animals given cytochrome c 100 mg/kg i.m. | Animals given cytochrome c 200 mg/kg i.m. |
|-------|---------|---------------------------|---------------------------------|------------------------------------------|------------------------------------------|
| Glycogen | 0.658±0.025** | 0.400±0.030 | 0.454±0.027 | 0.489±0.032 | 0.475±0.023 | 0.480±0.033 |

Results are given as means±S.E. for 10 animals. Significant differences from control animals given CCl₄ are marked : **(p<0.01).

3) Effect of cytochrome c on liver ATP

In Table 4, the effects of cytochrome c on liver ATP level are summarized. Liver ATP level, which was significantly lowered by carbon tetrachloride, was elevated by cytochrome c but not significantly.
4) Effects of cytochrome c on mitochondria

Table 5 shows the effects of cytochrome c on respiratory function of mitochondria. Carbon tetrachloride significantly reduced the respiratory function of hepatic mitochondria: the index of respiratory control, P/O ratio and the oxygen consumption in state 3 was decreased significantly, whereas oxygen consumption in state 4 was increased significantly. Cytochrome c was generally effective in inhibiting such a reduction in respiratory function, although the effect was significant only with respect to the index of respiratory control in animals given 200 mg/kg orally. The substance appeared to be somewhat more effective when given orally rather than i.m..

In Table 6, the effects of cytochrome c on the amount of cytochrome a, b and c are summarized. The levels of cytochrome a, b and c was evidently decreased by carbon tetrachloride administration and restored following administration cytochrome c.

| Table 5. Effects of cytochrome c on mitochondrial respiration disturbed by carbon tetrachloride. |
|-------------------------------------------------------------|---------------------------------|----------|----------|----------|----------|
| Untreated control animals | Control animals given CCl₄ | Animals given cytochrome c |
| Respiratory control (R.I.) | 4.35 ±0.14*** | 1.24 ±0.03** | 1.74 ±0.22 | 2.09 ±0.30* | 1.87 ±0.38 | 2.18 ±0.42 |
| P/O | 1.84 ±0.29 | 0.88 ±0.29 | 1.30 ±0.29 | 1.39 ±0.23 | 0.78 ±0.31 | 1.17 ±0.29 |
| O₂ uptake at state 4 | 29.5 ±1.2 ** | 61.9 ±10.0 | 50.7 ±7.2 | 51.6 ±8.3 | 49.1 ±6.0 | 48.8 ±6.7 |
| O₂ uptake at state 3 | 128.0 ±6.3 ** | 77.8 ±9.2 | 89.7 ±9.8 | 95.1 ±4.8 | 76.5 ±8.7 | 90.4 ±9.7 |

Results are given as means±S.E. for 10 animals. Significant differences from control animals given CCl₄ are marked : * (p<0.05) ; **(p<0.01).

5) Effect of cytochrome c on lipid peroxides

In Table 7, the effects of cytochrome c on lipid peroxides are summarized. Lipid peroxides in the liver were significantly increased and those in the serum were also increased by carbon tetrachloride. Such an increase in lipid peroxides was reversed by cytochrome c.
TABLE 7. Effects of cytochrome c on lipid peroxides increased with carbon tetrachloride.

|                | Untreated control animals | Control animals given CCl₄ | Animals given cytochrome c |
|----------------|---------------------------|----------------------------|----------------------------|
|                |                           | 100 mg/kg                  | 100 mg/kg                  |
|                |                           | p.o.                       | l.m.                       |
| Liver O.D. x 10⁶ | 19.5 ± 2.3*               | 17.1 ± 1.3*                | 20.0 ± 1.4*                |
| Serum O.D. x 10⁶ | 12.0 ± 2.5                | 10.8 ± 1.4                 | 8.0 ± 1.2*                 |

Results are given as means ± S.E. for 10 animals. Significant differences from control animals given CCl₄ are marked : *(p<0.05).

No definite dose-response relationship was demonstrated, although cytochrome c given orally proved to have a significant effect on lipid peroxides in the liver. Thus, cytochrome c displayed a more potent effect on lipid peroxides when given per os.

DISCUSSION

1) The effect of cytochrome c on abnormalities of lipid metabolism

Carbon tetrachloride-induced accumulation of triglycerides in the liver has been attributed to disturbed transport of lipids from the liver to the blood, as suggested by simultaneous manifestation of lowered β-lipoprotein level. A major contribution, may also be the reduction in mitochondrial oxidation of fatty acids. Increased serum free fatty acids are related to a lowered ability of the liver to dispose of fatty acids (20, 21, 22).

Cytochrome c has an inhibiting effect on such abnormalities of lipid metabolism, caused by carbon tetrachloride, presumably by preventing the disturbance of lipoprotein formation and increasing oxidation of fatty acids. It was found that cytochrome c can change the carbon tetrachloride-induced increase in lipid peroxides into a decrease; substances responsible for the disturbance of lipoprotein formation (21).

ATP content of the liver is decreased by carbon tetrachloride (23). This decrease brings about a reduction in proteosynthesis in the liver (24), so that it is likely to encourage lipid accumulation due to the resultant decrease in proteins which would combine with lipids. The fact that cytochrome c caused an increase in hepatic ATP may indicate a usefulness in preventing disturbance of lipoprotein formation.

The improvement of fatty acid oxidation by cytochrome c may be explained by the increased supply of ATP required for this purpose.

2) The effect of cytochrome c on abnormalities of sugar metabolism

The decrease in liver glycogen due to carbon tetrachloride may be ascribed to accelerated decomposition of glycogen (25) as well as decreased food intake. At the same time, there is an apparent disturbance in the glyconeogenic system (26), along with damage to the TCA cycle due to increased organic acid and diminished ATP. It follows that liver glycogen is metabolized into glycerol to participate in the synthesis of triglyceride. There was a significantly low blood glucose level in animals treated with carbon tetrachloride. This may be due to decreased liver glycogen or impairment of the glyconeogenic system. No difference existed, however, in the extent of rise in the blood glucose level following super loading and the rate at which blood glucose thus increased was reduced to normal.
It appeared therefore that blood glucose was normally absorbed into various tissues.

Such abnormalities of sugar metabolism caused by carbon tetrachloride were controlled by cytochrome c. The mechanism involved may be the activation of the TCA cycle by cytochrome c, resulting in improved lipid metabolism, which, in turn, may favorably affect sugar metabolism.

3) The effect of cytochrome c on mitochondrial functions

Cytochrome c tended to inhibit the reduction in tissue respiration caused by carbon tetrachloride and the amount of cytochromes a, b and c contained in mitochondria (27). Mitochondria is closely related to energy production and its behavior corresponds to variations in the level of ATP in the liver. The effect of cytochrome c on mitochondria may be based on a defense against carbon tetrachloride damage to mitochondria and activation of the functions of the damaged mitochondria. The former may be related to the action of cytochrome c on lipid peroxides, while the latter may be related to the fact that cytochrome c constitutes an essential element of the electron transport system.

Carbon tetrachloride damage to mitochondria has not been considered to be of primary significance, but mitochondria plays such an important role in energy metabolism that the activity of mitochondria cannot be disregarded in hepatic impairment due to carbon tetrachloride. It is hypothesized that in order to recover from hepatic damage, mitochondrial functions are activated to accentuate the production of ATP.

4) Effect of cytochrome c as affected by different methods of administration

The authors reported that cytochrome c is absorbed at a rate of approx. 10-20% when administered through the digestive tract (28, 29). A comparison, however, of the effect of cytochrome c when given i.m. and orally, revealed that the oral route is as useful or frequently more useful than the i.m. route. This is attributed not only to the high blood level of cytochrome c maintained over a period of a few hr (28, 29) but to the fact that the substance, when administered through the digestive tract, passes through the portal vein directly into the organ where it exerts its effect. In addition, substances of low molecular weight resulting from digestion or metabolism in vivo may be involved. Further investigation should be done on these factors.

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