Evaluation of oxidative stress during apoptosis and necrosis caused by carbon tetrachloride in rat liver

Fang Sun, Eri Hamagawa, Chihiro Tsutsui, Yoshiko Ono, Yukako Ogiri, Shosuke Kojo *

Department of Food Science and Nutrition, Nara Women’s University, Nara 630-8506, Japan

Received 12 September 2000; received in revised form 16 November 2000; accepted 22 November 2000

Abstract

After 12, 18, and 24 h of oral administration of carbon tetrachloride (as a 1:1 mixture with mineral oil: 4 ml/kg body weight) to rats, the activity of caspase-3-like protease in the liver increased significantly compared to that in the control group that was given mineral oil (4 ml/kg). In plasma, the activity of caspase-3 was barely detectable in the control rat, but increased significantly 24 h after drug administration along with a dramatic increase in glutamate oxaloacetate transaminase. These results indicate that carbon tetrachloride causes apoptosis in the liver by activating caspase-3, which is released to plasma by secondary necrosis. After 18 and 24 h of carbon tetrachloride administration, the liver concentration of hydrophilic vitamin C was decreased significantly, while that of hydrophobic vitamin E was not affected. The plasma concentration of vitamins C and E was not influenced significantly. These results suggest that carbon tetrachloride induces oxidative stress mainly in the aqueous phase of the liver cell. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Apoptosis; Carbon tetrachloride; Caspase-3; Necrosis; Vitamin C; Vitamin E

1. Introduction

It is well established that carbon tetrachloride (CCl₄) is a typical hepatotoxin causing centrilobular necrosis [1]. As a result of extensive studies, the initial event in the rat given CCl₄ has been believed to be lipid peroxidation of the endoplasmic reticulum of the liver cell initiated by trichloromethyl radical generated by the reaction between CCl₄ and cytochrome P₄₅₀ [1,2]. Based on these results, CCl₄ has been assumed to be a typical poison causing severe oxidative stress. However, we reported [3] that the level of specifically determined lipid hydroperoxides [4], which was shown to be a good indicator of oxidative stress in typical pathologic conditions such as vitamin E deficiency [5], iron overload [6], diabetes [7], and thioacetamide intoxication [8], was increased by CCl₄ only in mitochondria. It has long been known [3,9,10] that thiobarbituric acid-reactive substances (TBARS), which were widely used index of lipid peroxidation, does not increase significantly in the liver of rats treated with CCl₄. These observations suggest that CCl₄ does not cause extensive lipid peroxidation as widely accepted. To evaluate the oxidative stress caused by CCl₄, changes in the concentration of antioxidative vitamins C and E in the liver and plasma were measured in this study.

In the previous study [3], we also demonstrated that CCl₄ inactivated cytochrome oxidase in mito-
chondria. Recent studies show the key role of mitochondria in apoptosis [11]. Histological study [12] suggests that apoptosis is induced by CCl₄ in the liver. To elucidate the mechanism of apoptosis caused by CCl₄, caspase-3 activity was measured. As a result, we have found that the apoptosis caused by CCl₄ involves the activation of caspase-3 as in the case of thioacetamide [8], a well-known hepatotoxin causing hepatocellular necrosis as does CCl₄ [1,13].

2. Materials and methods

2.1. Animals

Guidelines from the Prime Minister’s Office of Japan (No. 6 of 27 March, 1980) for the care and use of laboratory animals were followed. Male rats (SLC: Wistar strain) were obtained from Japan SLC Co. (Hamamatsu, Shizuoka, Japan). The animals were housed in a room at 24 ± 2°C, and a 12:12-h light/dark cycle. Animals were fed commercial laboratory chow (MF, Oriental Yeast Co., Osaka, Japan) and water ad libitum. Six-week-old rats were administered a mixture of CCl₄ and mineral oil (1:1, 4 ml/kg body weight) through an intragastric tube. The control rats received mineral oil alone (4 ml/kg body weight).

2.2. Analytical methods

Rats were anesthetized with diethyl ether and killed by collecting the blood from the inferior vena cava using a syringe containing sodium heparin as an anticoagulant. After perfusion of ice-cooled saline through the portal vein, organs were removed. The excised tissue was homogenized in 5 volumes of phosphate-buffered saline (10 mM, pH 7.4) under ice cooling. All determinations were made in duplicate experiments with 4-6 animals in each group. The determination of vitamin C was made according to a specific and sensitive method [14,15] involving chemical derivatization and HPLC. The concentration of α-tocopherol was determined by HPLC [16]. The conditions of HPLC and fluorescence detection (Shimadzu RF-535, Kyoto, Japan) were reported previously [17]. Blood was centrifuged at 13,000 × g for 5 min at 4°C to separate plasma. The activity of plasma glutamate oxaloacetate transaminase (GOT: EC 2.6.1.1) was determined using the diagnostic kits (GOT-UV Test Wako, Wako Pure Chemicals Co., Osaka, Japan) and expressed as Karmen units. Caspase-3 activity was determined as described previously [8,18].

Protein concentrations were determined according to the method of Lowry et al. [19] using bovine serum albumin as the standard.

Data were expressed as mean ± S.D. and analyzed by analysis of variance using StatView software (Abacus Concepts, Berkeley, CA). Differences between the group means were considered significant at P < 0.05 using the Bonferroni/Dunn Procedure generated by this program.

3. Results and discussion

3.1. Liver necrosis and apoptosis caused by CCl₄

A necrogenic dose [3,20] of CCl₄ as a 1:1 mixture with mineral oil (4 ml/kg) was orally administered to rats. After 12 and 18 h, the activity of plasma GOT was significantly higher than that of the control group which was administered mineral oil alone (4 ml/kg) (Table 1). After 24 h, the plasma GOT activity increased drastically compared to that of the control group. These results showed that the necrotic process had initiated at 12 h and proceeded thereafter, in agreement with the literature [3].

Shi et al. [12] reported that apoptosis in the rat liver was observed histochemically after CCl₄ administration. To elucidate the mechanism of apoptosis, the activity of caspase-3-like protease, a cysteine protease specifically involved in apoptosis [21], was determined. Caspase-3-like protease activity in the liver increased significantly compared to that of the control group. These results showed that the necrotic process had initiated at 12 h and proceeded thereafter, in agreement with the literature [3].

In plasma, the activity of caspase-3 was barely detectable in the control rats and was increased significantly 24 h after drug administration along with a dramatic increase in GOT (Table 1). It is surprising that caspase-3, a protease showed activity in plasma, which has a strong anti-protease activity. These results indicate that the activity of caspase-3 in the liver and plasma is a reliable biochemical indicator...
of apoptosis under pathological conditions. Because apoptosis in animal tissue has been characterized by morphological observations [12,22-24], it is generally assumed that apoptotic cells in animal tissues are phagocytosed rapidly by neighboring cells to prevent inflammation. It is conceivable that the clearing mechanism hampered the histological detection of apoptotic cells induced in the liver by CCl4 [24]. The increase of caspase-3 activity in the liver and plasma was also reported [8,18], when thioacetamide (500 mg/kg), a typical necrogenic toxin, was applied to rats.

These results indicate that CCl4 causes apoptosis in the liver by activating caspase-3 and that the apoptosis and necrosis proceed simultaneously in the liver, from which caspase-3 is released by secondary necrosis taking place around 24 h as shown by the high plasma caspase-3 and GOT activities. It is well documented [25] that procaspase-3 (CPP32) is activated by cytochrome c released from mitochondria. We reported [3] that CCl4 damaged liver mitochondria by inhibition of cytochrome oxidase as well as by enhanced oxidative stress as evidenced by the increase of lipid hydroperoxides. Therefore, it is suggested that mitochondrial damage caused by CCl4 is involved in the apoptotic process.

Jaeschke et al. [26] reported that endotoxin, a hepatotoxic leading to necrosis, causes 17-fold activation of caspase-3-like protease in mouse liver after 7 h of administration. The activation caused by endotoxin is much higher than that observed in the present study, although the animal species were different. The extent of apoptosis by CCl4 seems to be much less than that by endotoxin. Therefore, it is suggested that CCl4 mainly causes necrosis and partial apoptosis leading to secondary necrosis, rather than apoptosis and necrosis in a sequential fashion.

Since measurable caspase-3 activity is detected in the control rat liver, it may be argued that the increase in plasma caspase-3 activity is not caused by apoptosis but is the result of leakage of basal levels of caspase-3 from damaged hepatocytes into the plasma in the same way as GOT is increased. To evaluate the relative contribution of apoptosis to plasma caspase-3 activity, we calculated the activity ratio of plasma caspase-3 and GOT based on the data of Table 1. If plasma caspase-3 activity originates solely from necrotic cells and not from apoptotic cells, this ratio is assumed to be constant. The ratios \( \frac{\text{Plasma caspase-3}}{\text{GOT}} \) were calculated to be 6.6, 4.1, 4.8, and 3.4, for 12, 18, and 24 h of CCl4-treated rats and the control rat, respectively. As this ratio for CCl4-intoxicated rats were all higher than that of the control group, it is supported that plasma caspase-3 activity is also derived from apoptotic cells in rats that were given CCl4.

It is well known that CCl4 induces a zonal necrosis [1]. Therefore, in zones of the liver where toxicity of CCl4 is less severe, apoptosis may be induced instead of necrosis and the increase in hepatic and plasma levels of caspase-3 may originate from these cells.

### 3.2. Changes in the levels of vitamins C and E in rats treated with CCl4

After 12 h of CCl4 administration, the hepatic level of hydrophilic vitamin C did not differ from that in the control group (Table 2) and it decreased dras-
tically to 40% of the control group after 18 h (Table 2). After 24 h, vitamin C in the liver still decreased to 32% of the control group (Table 2). Although vitamin C is not a vitamin for Wistar rats, excessive liver damage and oxidative stress caused by CCl₄ may deplete vitamin C. Recently, we reported that the concentration of vitamin C was decreased in the tissues of Wistar rats that were made diabetic [7] with streptozotocin and were administered thioacetamide [8]. These results suggest that the tissue concentration of vitamin C is a good indicator of oxidative stress even in Wistar rats.

The plasma concentration of vitamin C tended to increase 12 h after CCl₄ administration, although there was no significant difference observed. This tendency may be explained on the grounds that vitamin C is released just as GOT from the liver whose content of vitamin C is higher than that of plasma by two orders of magnitude in the control rat (Table 2). Similar results were obtained in the case of thioacetamide intoxication [8].

The level of hydrophobic vitamin E was not affected either in plasma or in the liver 12, 18, and 24 h after CCl₄ administration at this dose (Table 2). Since liver vitamin C is predominantly decreased by CCl₄, these results suggest that extensive lipid phase peroxidation to consume vitamin E did not take place by CCl₄ but that the oxidative stress in the aqueous phase to deplete vitamin C took place exclusively. This result seems to contradict the widely held view that CCl₄ generates trichloromethyl radical with the reaction with cytochrome P450, a redox active hemoprotein [1,2] and causes extensive lipid peroxidation. ESR spectrum [27,28] and in vitro chemical reactions [29,30] clearly show the radical generation from CCl₄ and cytochrome P450. Even the activation of CCl₄ to dichlorocarbene by cytochrome P450 was reported [31]. However, we reported [18] that specifically determined lipid hydroperoxides were not increased in either the whole liver or the microsomal fraction but were increased slightly in mitochondria. Therefore, it is suggested that CCl₄ does not cause extensive lipid peroxidation in microsomes but induces the damage of mitochondria which also contain hemoproteins and generate radicals by the reaction with CCl₄ [32], resulting in the inactivation of cytochrome oxidase [18].

It is worthwhile to note that the inhibition of cytochrome oxidase precedes the decomposition of heme a, a prosthetic group of cytochrome oxidase [18]. This observation suggests that radicals generated by the hemoprotein and CCl₄ initially attack the protein part of the enzyme [18]. We have demonstrated that apolipoprotein B is much more reactive to radicals than other proteins such as albumin and transferrin and is easily decomposed in plasma [33–35]. The high reactivity of apolipoprotein B is explained on the ground that apolipoprotein B forms LDL particle with lipids that are highly reactive to radical species [35]. Cytochrome oxidase is a membrane protein and it also has neighboring lipids such as apolipoprotein B. The decomposition of cytochrome P450 protein, also a membrane-bound protein in CCl₄-treated rat liver was reported [29]. The high reactivity of proteins to radical reaction may explain the reason why vitamin C, a hydrophilic antioxidant, is predominantly consumed in CCl₄ intoxication. It is also possible that trichloromethyl radical generated from CCl₄ and hemoproteins rapidly reacts with oxygen to form trichloromethylhydroper-

Table 2

<table>
<thead>
<tr>
<th>Time</th>
<th>Vitamin C (nmol/g tissue)</th>
<th>Vitamin E (nmol/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plasma</td>
<td>Liver</td>
</tr>
<tr>
<td>After 12 h</td>
<td>74.7 ± 18.5</td>
<td>821 ± 485</td>
</tr>
<tr>
<td>After 18 h</td>
<td>55.8 ± 9.4</td>
<td>473 ± 334*</td>
</tr>
<tr>
<td>After 24 h</td>
<td>67.9 ± 5.0</td>
<td>376 ± 215**</td>
</tr>
<tr>
<td>Control</td>
<td>57.0 ± 35.8</td>
<td>1188 ± 276</td>
</tr>
</tbody>
</table>

A mixture of CCl₄ and mineral oil (1:1, 4 ml/kg) was orally administered to rats. After 12, 18, and 24 h, concentrations of vitamin C and vitamin E in the plasma and liver were determined as described in the text. After 24 h of the administration of mineral oil, determinations were made for control rats. Values are mean ± S.D. of 4–6 rats, and asterisks indicate significant difference from the control group (ANOVA Bonferroni/Dunn procedure, *P < 0.05 and **P < 0.01).
oxy radical, which is much more hydrophilic than trichloromethyl radical. This idea may also explain why hydrophilic antioxidant is consumed predominantly.

3.3. Oxidative stress, apoptosis, and necrosis

Twelve hours after the administration of CCl₄, the activation of caspase-3 took place but the decrease of vitamin C in the liver was not significant. The involvement of reactive oxygen species in apoptosis has been suggested. Recently we reported [36] that hydroxyl radicals were involved in the apoptosis of HL-60 cells caused by anticancer drugs based on systematic evaluation of different kinds of antioxidants. We reported further [36] that the radicals during the apoptotic process did not markedly decrease the cellular concentration of vitamin E. Therefore, the decrease of vitamin C in the liver 18 and 24 h after CCl₄ administration may be induced by necrosis judging from the increase of GOT at 12 h. This is in sharp contrast to the case of thioacetamide (500 mg/kg body weight), which typically increased lipid hydroperoxides and decreased both vitamins C and E in the liver. Previously [8] we did not clarify whether necrosis was caused by radical reaction or radical reaction is a result of necrosis. The present study demonstrates that necrosis does not always involve extensive lipid peroxidation. Therefore, it is suggested that radical reaction is a cause of necrosis caused by thioacetamide.

Along with radical reactions, increase of calcium content of the liver [37,38] and liver mitochondria [39] by CCl₄ was reported. Since it is well documented that cellular calcium concentration transiently augments in cell death [40], it is probable that damage of the respiratory chain in mitochondria [3] and the resulting disorder of calcium metabolism [38,39,41] by CCl₄ are the main cause of apoptosis and necrosis of the liver cell.

References


