Increase of Ceramide in the Liver and Plasma after Carbon Tetrachloride Intoxication in the Rat

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Summary In fulminant hepatic failure, various toxins causing multi-organ failure increase in plasma. As a novel toxin, levels of ceramide, a well-studied lipid mediator of apoptosis, were determined by LC-MS/MS in the liver and plasma of carbon tetrachloride (CCl4)-intoxicated rats. After 6 h of oral administration of CCl4 (4 mL/kg body weight as a 1:1 mixture of CCl4 and mineral oil) to rats, extensive hepatic failure occurred as evidenced by a severe elevation in plasma AST and ALT. The liver concentration of major ceramide components (C16:0, C24:0, C24:1, C18:0, C22:0, and C24:2 in decreasing order), and the sum of these ceramides increased significantly 2 h after CCl4 intoxication compared to that in the control group given mineral oil. The total ceramide concentration in the plasma was also increased to 4.1 times that in the control 24 h after administration of CCl4. In conclusion, the early increase in liver ceramides may contribute to hepatic cell death and the increase in plasma ceramides during fulminant hepatic failure may cause damage in other organs including the brain and kidney.

Key Words carbon tetrachloride, CCl4, cell death, ceramide, hepatic failure

The mechanism of liver injury by carbon tetrachloride (CCl4) has received more attention than that of any other chemical (1). CCl4 is known to be metabolized by P450-dependent dehalogenation to trichloromethyl radical, which causes extensive lipid peroxidation in the liver (1, 2). The mitochondrial respiratory chain is also suggested to cause similar reductive activation of CCl4 (3). CCl4 causes massive necrosis of the liver via oxidative stress and also induces apoptosis in the rat liver, involving the activation of caspase-3 (4). Radical reactions are implicated based on increases in lipid hydroperoxides and a decrease in vitamin C (4).

In addition, CCl4 intoxication has been used as an animal model of fulminant hepatic failure to develop artificial liver support (5, 6). In fulminant hepatic failure, toxic substances and cytokines released into the circulation are assumed to cause encephalopathy and renal dysfunction (7). For the development of effective artificial liver support, it is essential to characterize the cytotoxins and metabolic changes of the liver following intoxication with this chemical. Recently we reported that ceramide is a novel toxin in severe liver failure caused by d-galactosamine (8).

It is well documented that ceramides regulate apoptosis in a variety of cells (9). However, their role has mainly been evaluated in cultured cells and studies on their metabolism in animals are limited. In this study we report the changes in ceramides during fulminant hepatic failure caused by CCl4, utilizing LC-MS/MS as in our previous reports (8, 10). Since the maximal liver damage occurred 24 h after CCl4 administration and extensive regenerative response followed to restore the liver damage via α-receptor (11), we focused our study on the change in ceramides during initial 24 h after CCl4 intoxication.

MATERIALS AND METHODS

Animals. This study was approved by the Animal Care Committee of Nara Women’s University. 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, with a 12 h/12 h light-dark cycle. Animals were fed commercial laboratory chow (MF, Oriental Yeast Co., Ltd., Osaka, Japan) and water ad libitum. Eight-week-old rats were administered a mixture of CCl4 and mineral oil (1:1, 4 mL/kg body weight) through an intragastric tube as previously (4). The control rats received mineral oil alone (4 mL/kg body weight).

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. Blood was centrifuged at 8,400 × g for 5 min at 4°C to separate plasma. The activities of plasma aspartate aminotransferase (AST: EC 2.6.1.1)
and alanine aminotransferase (ALT: EC 2.6.1.2) were determined using diagnostic kits (GOT and GPT-UV Test Wako, Wako Pure Chemical Industries, Ltd., Osaka) and expressed as Karmen Units. For determination of ceramide, to the plasma (1 mL), 5 mL of a mixture of chloroform, methanol (2 : 1), and water (1 : 1) was added. After vigorous shaking and centrifugation for 5 min at 3,400 rpm, the chloroform layer was collected. Four milliliters of the chloroform was added to the residue, and the extraction was performed again. Liver extractions were made as previously described (8, 10). Liver extractions and dissolved in chloroform to perform TLC. TLC separation was performed as previously described (8, 10).

**Mass spectrometry.** Quantitative measurements of ceramide species were made using a triple-quadrupole mass spectrometer (Finnigan MAT TSQ 7000). ESI-MS/MS was performed as previously described (8, 10). HPLC was conducted using a Waters 600S system equipped with a µ-Bondasphere column (5 µC18 100A Waters). ESI-MS was performed at a flow rate of 0.2 mL/min with a mixture of 5 mM ammonium formate, methanol, and tetrahydrofuran at a volume ratio of 1 : 2 : 7. The mobile phase stream was connected to the ion source interface of an ESI-MS/MS system. Standards and cellular ceramide extracts were stored at −20°C. Mass analysis was performed in the positive ion mode in a heated capillary tube at 250°C with an electrospray potential of 4.5 kV, a seath gas pressure of 60 psi, and a collision gas pressure of 1.6–2.0 mtorr. Under optimized conditions, monitoring ions were ceramide molecular species [M+H]⁺ for the product ion at m/z 264 (8–10) of the sphingoid base. Standard and sample were injected with 5 µL containing 5 pmol C8:0-ceramide as an internal standard for ESI-MS/MS. The quantity of each ceramide was calculated from each ceramide/C8:0 ceramide ratio, assuming that the calibration curve of ceramides bearing C16–24 acyl chains was similar to that of C16:0-ceramide. Each sample was analyzed in duplicate. Data were expressed as mean±SD. Statistical analysis was carried out with Statcel (Excel 2000). Differences between the group means were considered significant at p<0.05 using Fisher’s protected least significant difference test (PLSD), *p<0.05 and **p<0.01.

**RESULTS AND DISCUSSION**

**Liver injury caused by CCl₄**

A necrogenic dose of CCl₄ (4 mL/kg; as a mixture of CCl₄ : mineral oil = 1 : 1) was orally administered to rats. After 1, 2, 3, 6, 12, and 24 h, plasma AST and ALT activities in CCl₄-treated rats were assayed as described in the text. Control rats received mineral oil (4 mL/kg) and the enzyme activity was determined after 6 h. Values are means±SD for 4 rats and asterisks indicate significant differences from the corresponding control group (ANOVA Fisher’s protected least significant difference test (PLSD), *p<0.05 and **p<0.01).

**Table 1. The plasma AST and ALT activities (Karmen units) in CCl₄-treated rats after 1, 2, 3, 6, 12, and 24 h and in the control rats.**

|          | 1 h     | 2 h     | 3 h     | 6 h     | 12 h    | 24 h    | Control |
|----------|---------|---------|---------|---------|---------|---------|---------|
| AST      | 101.7±51.6 | 315.2±16.8 | 463.3±90.6 | 1147.0±240.1 | 2608.9±448.6** | 9381.4±3174.1** | 109.0±24.3        |
| ALT      | 46.8±19.2 | 121.6±28.6 | 116.3±29.1 | 378.2±61.8** | 1688.8±280.9** | 4406.1±124.5** | 39.2±6.69         |

CCl₄ (4 mL/kg; as a mixture of CCl₄ : mineral oil = 1 : 1) was orally administered to rats. After 1, 2, 3, 6, 12, and 24 h, plasma AST and ALT activities were assayed as described in the text. Control rats received mineral oil (4 mL/kg) and the enzyme activity was determined after 6 h. Values are means±SD for 4 rats and asterisks indicate significant differences from the corresponding control group (ANOVA Fisher’s protected least significant difference test (PLSD), *p<0.05 and **p<0.01).

**Table 2. The ceramide concentration (nmol/g tissue) in the liver of CCl₄-treated rats after 1, 2, 3, 6, 12, and 24 h and in the control group.**

|       | C16:0 | C18:0 | C22:0 | C24:0 | C24:1 | C24:2 | Total |
|-------|-------|-------|-------|-------|-------|-------|-------|
| 1 h   | 37.8±8.2 | 7.4±1.8 | 17.6±2.5 | 113.0±9.3 | 48.7±7.7 | 8.4±1.1 | 232.8±29.2 |
| 2 h   | 57.9±12.6** | 15.1±4.4** | 27.1±1.9** | 152.8±5.2** | 67.5±6.6** | 12.6±0.4** | 333.0±25.0** |
| 3 h   | 51.0±6.6** | 16.6±4.3** | 26.8±5.7** | 147.2±31.2** | 61.6±11.3** | 11.4±2.8** | 314.6±58.4** |
| 6 h   | 60.6±17.5** | 25.9±5.9** | 34.9±8.9** | 196.7±53.7** | 90.1±23.7** | 17.0±4.9** | 425.1±111.1** |
| 12 h  | 45.6±5.4** | 15.3±1.9** | 22.2±3.2 | 142.2±31.6 | 67.9±11.3** | 12.6±3.9** | 305.7±55.8** |
| 24 h  | 71.2±1.0** | 30.0±7.1** | 28.5±2.7** | 162.0±10.2** | 78.2±4.9** | 15.0±1.6** | 384.9±17.3** |
| Control | 26.1±3.7 | 1.7±0.2 | 14.3±2.1 | 91.5±15.0 | 32.4±4.9 | 7.0±0.9 | 172.9±26.1 |

CCl₄ (4 mL/kg; as a mixture of CCl₄ : mineral oil = 1 : 1) was orally administered to rats. After 1, 2, 3, 6, 12, and 24 h, concentrations of ceramides in the liver were determined as described in the text. After 6 h of the administration of mineral oil (4 mL/kg), determinations were made for control rats. Values are means±SD for 4 rats and asterisks indicate a significant difference from the control group (ANOVA Fisher’s protected least significant difference test (PLSD), *p<0.05 and **p<0.01).
CCl₄ may contribute to the extensive liver cell apoptosis 2 h) in ceramides with a long acyl chain induced by mitochondrial pathway (ceramide (14)).

The mechanism of apoptosis by increased ceramides in the rat liver remains to be explored. Changes in the plasma level of ceramides in rats treated with CCl₄

In the control plasma, the major ceramides were C24:0, C24:1, C22:0, C24:2, and C18:0 in decreasing order (8). In the liver and plasma, ceramides with 24-carbon atom side chains predominated, consistent with a previous study (8).

Two hours after the administration of CCl₄, significant increases of C16:0, C18:0, C24:1, and the total ceramide in the plasma were observed (Table 3). During 3–12 h, the changes in each ceramide were not uniform. The differing behaviors of each ceramide component remained unexplored. However, 24 h after the CCl₄ intoxication, the plasma concentrations of all ceramides increased significantly compared to the control (Table 3). Consequently the total amount of ceramides significantly increased to 4.1 times that in the control plasma (Table 3).

A 1.6-fold elevation in the plasma concentration was reported (15) in rats administered lipopolysaccharide, which is also a well-known toxin appearing in the plasma of rats with fulminant hepatic failure (15). Since ceramides are a lipid causing death characterized even in cultured cell systems. Therefore the mechanism of apoptosis by increased ceramides in the rat liver remains to be explored.

CONCLUSION

In fulminant hepatic failure, toxins causing multi-organ failure are assumed to be released into the circulation. As a novel toxin, ceramide levels were deter-

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Table 3. The ceramide concentration (nmol/mL plasma) in the plasma of CCl₄-treated rats after 1, 2, 3, 6, 12, and 24 h and the control group.

|       | C16:0 | C18:0 | C22:0 | C24:0 | C24:1 | C24:2 | Total   |
|-------|-------|-------|-------|-------|-------|-------|---------|
| 1 h   | 0.70±0.33 | 0.10±0.04 | 0.72±0.19 | 5.43±1.26 | 1.90±0.46 | 0.33±0.07 | 9.18±2.21 |
| 2 h   | 1.65±0.05** | 0.35±0.08** | 1.27±0.06 | 7.69±0.43 | 3.19±0.25** | 0.49±0.05 | 14.63±0.58* |
| 3 h   | 1.11±0.14* | 0.29±0.06** | 1.01±0.13 | 6.02±0.71 | 2.32±0.27 | 0.36±0.02 | 11.11±1.13 |
| 6 h   | 1.26±0.05* | 0.45±0.06** | 1.18±0.20 | 6.41±0.99 | 2.64±0.46* | 0.42±0.10 | 12.36±1.84 |
| 12 h  | 1.08±0.45 | 0.29±0.09** | 1.30±0.44 | 7.79±3.14 | 2.44±0.94 | 0.44±0.22 | 13.33±5.25 |
| 24 h  | 2.62±0.56** | 1.08±0.29** | 3.31±0.88** | 19.78±5.05** | 5.40±1.38** | 1.16±0.39** | 33.35±8.53** |
| Control | 0.66±0.07 | 0.04±0.00 | 0.71±0.09 | 4.91±0.43 | 1.59±0.20 | 0.30±0.02 | 8.20±0.76 |

CCl₄ (4 mL/kg; as a mixture of CCl₄ : mineral oil = 1 : 1) was orally administered to rats. After 1, 2, 3, 6, 12, and 24 h, concentrations of ceramides in plasma were determined as described in the text. After 6 h of the administration of mineral oil (4 mL/kg), determinations were made for control rats. Values are means±SD for 4 rats and asterisks indicate a significant difference from the control group (ANOVA Fisher’s protected least significant difference test (PLSD), *p<0.05 and **p<0.01).
mined by LC-MS/MS in the liver and plasma of CCl4-intoxicated rats. The total concentration of ceramide in the liver was increased by the administration of CCl4, and that in the plasma was increased to 4.1 times that in the control 24 h after administration of CCl4. Based on these observations we propose that the early increase in liver ceramides may contribute to hepatic cell death, and the increase in plasma ceramides during fulminant hepatic failure may be one of the important toxicities causing damage in other organs including the brain and kidney.

REFERENCES
1) Bruckner JV, Warren DA. 2001. Toxic effect of solvents and vapors. In: Casarett & Doull’s Toxicology. The Basic Science of Poisons (Klaassen CD, ed), 6th ed, p 869–916. McGraw-Hill, New York.
2) Plaa GL. 2000. Chlorinated methanes and liver injury: Highlights of the past 50 years. Annu Rev Toxicol 40: 43–65.
3) Ikeda K, Toda M, Tanaka K, Tokumaru S, Kojo S. 1998. Increase of lipid hydroperoxides in liver mitochondria and inhibition of cytochrome oxidase by carbon tetrachloride intoxication in rats. Free Radic Res 28: 403–410.
4) Sun F, Tsutsui C, Hamagawa E, Ono Y, Ogiri Y, Kojo S. 2001. Evaluation of oxidative stress during apoptosis and necrosis caused by carbon tetrachloride in rat liver. Biochim Biophys Acta 1535: 186–191.
5) Soloviev V, Hassan AN, Akatov V, Lezhnev E, Ghaffar TY, Ghaffar YA. 2003. A novel bioartificial liver containing small tissue fragments: efficiency in the treatment of acute hepatic failure induced by carbon tetrachloride in rats. Int J Artif Organs 26: 735–742.
6) Shnyra A, Bocharov A, Bochkova N, Spirov V. 1991. Bioartificial liver using hepatocytes on biosilon microcarriers: treatment of chemically induced acute hepatic failure in rats. Artif Organs 15: 189–197.
7) Laleman W, Wilmer A, Evenepoel P, Verslype C, Fevery J. 2006. Review article: non-biological liver support in liver failure. Aliment Pharmacol Ther 23: 351–363.
8) Yamaguchi M, Miyashita Y, Kumagai Y, Kojo S. 2004. Change in liver and plasma ceramides during D-galactosamine-induced acute hepatic injury by LC-MS/MS. Bioorg Med Chem Lett 14: 4061–4064.
9) Pettus BJ, Challiant CE, Hamann YA. 2002. Ceramide in apoptosis: an overview and current perspectives. Biochim Biophys Acta 1585: 114–125.
10) Yamada Y, Kajiwara K, Yano M, Kishida E, Masuzawa Y, Kojo S. 2001. Increase of ceramides and its inhibition by catalase during chemically induced apoptosis of HL-60 cells determined by electrospray ionization tandem mass spectrometry. Biochim Biophys Acta 1532: 115–120.
11) Ochi Y, Yumori Y, Morioka A, Miura K, Tsukamoto I, Kojo S. 1990. Effect of β-blockade on liver regeneration after carbon tetrachloride intoxication in the rat. Biochem Pharmacol 39: 2065–2066.
12) Plaa GL. 1991. Toxic response of the liver. In: Casarett & Doull’s Toxicology. The Basic Science of Poisons (Amidur MO, Doull J, Klaassen CD, ed), 4th ed, p 334–353. Pergamon, New York.
13) Jones BE, Lo CR, Srinivassan A, Valentino KL, Czaia MJ. 1999. Ceramide induces caspase-independent apoptosis in rat hepatocytes sensitized by inhibition of RNA synthesis. Hepatology 30: 215–222.
14) Arora AS, Jones BJ, Patel TC, Brunk SE, Gores GJ. 1997. Ceramide induces hepatocyte cell death though disruption of mitochondrial function in the rat. Hepatology 25: 958–963.
15) Haimovitz-Friedman A, Cordon-Cardo C, Bayoumy S, Garzotto M, McLoughlin M, Gallily R, Edwards III CK, Schuchman EH, Fuks Z, Kolesnick R. 1997. Lipopolysaccharide induces disseminated endothelial apoptosis requiring ceramide generation. J Exp Med 186: 1831–1841.