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Change in liver and plasma ceramides during D-galactosamine-induced acute hepatic injury by LC–MS/MS

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Abstract—In fulminant hepatic failure, various toxins causing multi-organ failure increase in plasma. As a novel toxin, ceramide, a well-studied lipid mediator of apoptosis, levels were determined by LC–MS/MS in the liver and plasma of D-galactosamine-intoxicated rats. 18 and 24 h after intraperitoneal administration of D-galactosamine (1 g/kg body weight) to rats, fulminant hepatic failure occurred as evidenced by a severe elevation in plasma GOT and GPT. The liver concentration of minor ceramide components (C18:0, C20:0, C22:1, C22:0, and C24:2) increased significantly compared to that in the control group that was given saline. The plasma concentration of major ceramides (C24:0, C24:1, C16:0, C22:0, C22:1, and C18:0) increased 24 h after administration of D-galactosamine and the total ceramide concentration was also increased to 3.6 times that in the control. In conclusion, the increased concentrations of ceramides in plasma during fulminant hepatic failure may be one of important toxins causing damage in other organs including the brain and kidney.

1. Introduction

D-Galactosamine intoxication has been used as an animal model of fulminant hepatic failure to develop artificial liver support.[1] In fulminant hepatic failure, toxic substances and cytokines released into the circulation are assumed to cause encephalopathy[2] and renal dysfunction.[3] For the development of artificial liver support, it is essential to characterize the cytotoxins and metabolic changes of the liver following intoxication with this chemical.

A high dose of D-galactosamine causes massive necrosis of the liver via depletion of UTP and inhibition of protein synthesis.[4] D-Galactosamine also induces apoptosis in rat liver involving the activation of caspase-3.[5] Radical reactions are implicated based on increases in lipid hydroperoxides and a decrease in vitamin C.[5] Recently we reported that anticancer drugs cause apoptosis in HL-60 cells via a radical reaction,[6] the activation of caspase-3,[7] and an increase in ceramides at a later stage.[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 change in ceramides, novel cytotoxins in fulminant hepatic failure, utilizing LC–MS/MS as in our previous report.[8]

2. Results and discussion

2.1. Liver injury caused by D-galactosamine

A necrogenic dose of D-galactosamine (1 g/kg)[5] was intraperitoneally administered to rats. After 18 h, the plasma GOT and GPT levels were significantly higher than those of the control group (Table 1). After 24 h, the plasma GOT and GPT activities reached the maximal level (Table 1). These results are consistent with our previous study[5] and showed that cell death including apoptosis and necrosis was initiated at around 18 h and proceeded extensively thereafter. After 24 h, GOT and GPT levels elevated to 18,480 and 7280 Karmen units,

Abbreviations: GOT, glutamate-oxaloacetate transaminase; GPT, glutamate-pyruvate transaminase; PBS, phosphate-buffered saline.

Keywords: Apoptosis; Ceramide; Galactosamine; Fulminant hepatic failure; Necrosis.

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Table 1. The plasma GOT and GPT activities (Karmen units) in D-galactosamine-treated rats after 18 and 24 h and in the control rats

	18 h	24 h	Control
GOT	$2516 \pm 765^{**}$	$18{,}480 \pm 7440^{**}$	69.9 ± 32.0
GPT	$1208 \pm 212^{**}$	$7280 \pm 3190^{**}$	25.2 ± 19.2

D-galactosamine (1 g/kg) was intraperitoneally administered to rats. After 18 and 24 h, plasma GOT and GPT activities were assayed as described in the text. Control rats received saline and the enzyme activity was determined after 24 h. Values are means ± SD for four rats and asterisks indicate significant differences from the corresponding control group (ANOVA Fisher's protected least significant difference test (PLSD), $^{**}P < 0.01$).

respectively, showing that fulminant hepatic failure took place.

2.2. Changes in the liver level of ceramides in rats treated with D-galactosamine

In the control liver, the major ceramides, in decreasing order, were C24:0, C24:1, C16:0, and C22:0 (Table 2). This ceramide composition was similar to that of HepG2[10] and HL-60[8] cells, where C16:0, C24:0, C24:1, and C22:0 were major species. The sum of these major ceramide in the control liver was 302.6 nmol/g tissue (Table 2), which was comparable to that reported previously.[11–13]

Eighteen hours after injection of D-galactosamine, when apoptosis and necrosis of the liver cells started[5] based on the increase in plasma GOT and GPT levels (Table 1), the liver concentration of C18:0 and C22:0 ceramides was increased significantly (Table 2). However, the sum of ceramides did not differ from that in the control group (Table 2). After 24 h, when extensive necrosis had developed, the liver concentration of C18:0, C20:0, C22:1, and C24:2 ceramides was significantly increased compared to that of the control rats (Table 2). Since these are relatively minor components of ceramides, the total concentration of ceramides was not affected by the increase in these ceramides compared to that of the control (Table 2).

Although rat hepatocytes were resistant to the toxicity of even 100 μM C2 ceramide,[14] C8 ceramide caused hepatocyte cell death through disruption of mitochondrial function much more effectively than C2 ceramide.[15] Therefore the increase in ceramides with a long acyl chain induced by D-galactosamine may contribute to the extensive liver cell death.

2.3. Changes in the plasma level of ceramides in rats treated with D-galactosamine

In the control plasma, major ceramides, which exceeded 1 nmol/mL plasma, were C24:0, C24:1, C22:2, C24:2, C22:0, and C16:0 in decreasing order. The first two major ceramides were in common with those in the liver. The ceramide profile of rat plasma was significantly different from that of human plasma,[16] where the total amount of ceramide depended on age and ranged from 27 to 51 nmol/mL,[16] which was much higher than the value for rats (16 nmol/mL) obtained in the present study.

Eighteen hours after the administration of D-galactosamine, no change in the concentration of ceramide in the plasma was observed (Table 3). Twenty-four hours after the D-galactosamine injection, the plasma concentration of major ceramides such as C24:0, C24:1, C22:0, C16:0, C22:1, and C18:0 increased significantly compared to the control (Table 3). The total amount of ceramides also significantly increased to 3.6 times that in the control plasma (Table 3). An elevation in the plasma ceramide concentration by 1.6-fold was reported[17] in rats administered lipopolysaccharide, which is also a well-known toxin appearing in the plasma of rats with fulminant hepatic failure.[18] Since ceramides are a lipid causing death in a variety of cells,[9] the increase in plasma ceramide may cause damages in other tissues such as the brain resulting in encephalopathy and the kidney.

The origin of these increased ceramides in plasma may be ascribed to the necrotized liver, because major ceramide species in the liver increased in plasma. In addition, the extremely high plasma GOT and GPT levels (Table 1) 24 h after the D-galactosamine administration

Table 2. The ceramide concentration (nmol/g tissue) in the liver of D-galactosamine-treated rats after 18 and 24 h and the control group

	C16:0	C18:0	C20:0	C22:0	C22:1
18 h	43.9 ± 5.6	$11.1 \pm 3.9^{*}$	3.9 ± 0.9	$37.8 \pm 7.1^{*}$	4.6 ± 1.2
24 h	36.2 ± 4.3	$11.5 \pm 1.7^{*}$	$5.6 \pm 1.3^{*}$	35.6 ± 5.1	$7.1 \pm 1.4^{*}$
Control	34.1 ± 9.2	7.0 ± 1.9	3.8 ± 0.6	27.2 ± 5.2	5.1 ± 1.0
	C22:2	C24:0	C24:1	C24:2	Total
18 h	4.8 ± 1.6	173.7 ± 41.1	59.6 ± 14.5	12.4 ± 1.7	351.9 ± 60.9
24 h	7.0 ± 5.0	166.3 ± 34.6	67.2 ± 7.5	$17.2 \pm 1.5^{**}$	353.7 ± 32.1
Control	6.7 ± 1.3	137.4 ± 30.5	69.8 ± 22.2	11.6 ± 3.4	302.6 ± 51.0

D-Galactosamine (1 g/kg) was intraperitoneally administered to rats. After 18 and 24 h, concentrations of ceramides in the liver were determined as described in the text. After 24 h of the administration of saline, determinations were made for control rats. Values are means ± SD for four–six rats and asterisks indicate a significant difference from the control group (ANOVA Fisher's protected least significant difference test (PLSD), $^{*}P < 0.05$ and $^{**}P < 0.01$).

Table 3. The ceramide concentration (nmol/mL plasma) in the plasma of D-galactosamine-treated rats after 18 and 24 h and the control group

	C16:0	C18:0	C20:0	C22:0	C22:1
18 h	1.06 ± 1.35	0.18 ± 0.29	0.42 ± 0.34	1.16 ± 2.18	0.69 ± 0.40
24 h	3.13 ± 0.66**	0.90 ± 0.15**	0.65 ± 0.03	5.45 ± 0.55**	0.91 ± 0.04**
Control	1.07 ± 0.41	0.19 ± 0.07	0.10 ± 0.17	1.07 ± 0.32	0.21 ± 0.35
	C22:2	C24:0	C24:1	C24:2	Total
18 h	2.57 ± 1.12	6.31 ± 6.59	5.78 ± 7.31	0.98 ± 0.98	18.83 ± 5.70
24 h	2.33 ± 0.38	26.31 ± 3.44**	13.83 ± 1.22**	1.94 ± 0.39	58.18 ± 4.94**
Control	1.70 ± 1.45	7.20 ± 0.99	3.13 ± 2.72	1.31 ± 0.71	15.96 ± 6.52

D-Galactosamine (1 g/kg) was intraperitoneally administered to rats. After 18 and 24 h, concentrations of ceramides in plasma were determined as described in the text. After 24 h of the administration of saline, determinations were made for control rats. Values are means ± SD for four–six rats and asterisks indicate a significant difference from the control group (ANOVA Fisher's protected least significant difference test (PLSD), $^{*}P < 0.05$ and $^{**}P < 0.01$).

indicated that massive liver components were released from the necrotized liver into the circulation.

3. 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 determined by LC–MS/MS in the liver and plasma of D-galactosamine-intoxicated rats. The total concentration of ceramide in the liver was not affected by the administration of D-galactosamine, while that in the plasma was increased to 3.6 times that in the control 24 h after administration of D-galactosamine. Based on these observations we propose that the increased concentrations of ceramides in plasma during fulminant hepatic failure are one of causes of damages in other organs including the brain and kidney.

4. Experimental

4.1. 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. They were fed commercial laboratory chow (MF, Oriental Yeast Co., Osaka, Japan) and water ad libitum. Six-week-old rats were intraperitoneally administered D-galactosamine (1 g/kg body weight). The control rats received saline.

4.2. Materials

All solvents were purchased from Wako Pure Chemicals Co. (Osaka, Japan). Silica gel 60 TLC plates were purchased from Merck (Germany). All other reagents were obtained from Nacalai Tesque Co. (Kyoto, Japan). C8:0 and C16:0 ceramides were purchased from Funakoshi Co. (Tokyo, Japan).

4.3. 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, the organs were removed. The excised tissue was homogenized in five volumes of phosphate-buffered saline (PBS: 10 mM, pH 7.4) under ice cooling. Blood was centrifuged at 13,000*g* for 5 min at 4 °C to separate the plasma. The activities of plasma glutamate-oxaloacetate transaminase (GOT: EC 2.6.1.1) and glutamate-pyruvate transaminase (GPT: EC 2.6.1.2) were determined using diagnostic kits (Transaminase CII Test Wako, Wako Pure Chemicals Co., Osaka) and expressed as Karmen Units.

4.4. Liver lipid extraction

To liver homogenate (4 mL), 12 mL of a mixture of chloroform and methanol (2:1, v/v) was added. After vigorous shaking and centrifugation for 5 min at 1000*g*, the chloroform layer was collected. To the residue, 8 mL of chloroform was added and the extraction was performed again. The combined chloroform layer was evaporated with an evaporator and the resulting lipid was dissolved in 1 mL of chloroform to perform TLC. The plasma (1 mL) was diluted with 1 mL of PBS and extractions were made as for the liver homogenates. The resulting lipid was dissolved in 1 mL of a mixture of chloroform/methanol (1:1, v/v) and subjected to TLC.

4.5. TLC separation and extraction

TLC separating was performed as previously described.[8] In brief, the first elution was made with a mixture of *n*-butanol/acetic acid/water (3:1:1, v/v/v) to the one third mark of the plate and the second elution was made to the top of the plate with a mixture of diethyl ether/*n*-hexane/acetic acid (90:10:1, v/v/v). Ceramide spot was visualized under UV by staining with primulin spray. The ceramide spot was scratched from TLC plates and collected into a glass tube. Extraction was conducted with 2 mL of a mixture of $H_2O/CH_3OH/CHCl_3$ (0.8:2:1,

v/v/v) under shaking for 30 min. After centrifugation, the lower layer was collected. Then, 1 mL of H_2O and 2 mL of $CHCl_3$ were added to the residue and the extraction was repeated. The $CHCl_3$ layer was collected in a glass tube. To the upper phase was added 1.5 mL of $CHCl_3$. The extraction was performed additional 2 times. The collected $CHCl_3$ solution was evaporated and re-suspended in 200 μL of a mixture of chloroform and methanol (1:1, v/v). To calibrate the loss during the extraction and TLC procedures, the recovery using C16:0-ceramide added to the tissue homogenate was measured. The recovery of C16:0-ceramide from rat liver homogenate was 79–88%. Therefore recoveries were not taken into account in each determination.

4.6. 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] HPLC was conducted using a Waters 600S system equipped with a μ-Bondasphere column (5 μC_{18} 100A Waters). Elution 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 ionspray interface of an ESI-MS/MS system. Standards and cellular ceramide extracts were stored at −20 °C. Mass analysis was performed in the positive mode in a heated capillary tube at 250 °C with an electrospray potential of 4.5 kV, a seath gas pressure of 70 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[8,9,13] of the sphingoid base. Standard and sample were injected with 5 μL of 5 pmol/μL 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 the mean ± SD and analyzed using STATVIEW software (Abacus Concepts, Berkeley, CA, USA). Differences between group means were considered significant at $P < 0.05$ using the Fisher's protected least significant difference test (PLSD) generated by this program.

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