Effect of Celecoxib, a Selective Cyclooxygenase-2 Inhibitor on Carbon Tetrachloride Intoxication in Rats

Yukiko Washino, Eriko Koga, Yuko Kitamura, Chiaki Kamikawa, Keiko Kobayashi, Tomoka Nakagawa, Chihiro Nakazaki, Ikuyo Ichi, and Shosuke Kojo*

Department of Food Science and Nutrition, Nara Women's University; Nara 630–8506, Japan.
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CCl_4 (0.5 ml/kg as CCl_4) was orally administered to rats. Twelve hours after administration of CCl_4, plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels, indicators of liver necrosis, were significantly higher than those in the control group showing that active liver necrosis took place. At the same time the level of liver vitamin C was decreased significantly compared to that in the control group. Oral administration of 100 mg/kg each of celecoxib 3 and 8 h after CCl_4 treatment did not change plasma ALT and AST and liver vitamin C levels 12 h after CCl_4 treatment, but 24 h after CCl_4 treatment, significantly decreased plasma ALT and AST levels and elevated liver vitamin C level. These finding suggested that celecoxib effectively ameliorated the necrotic action and the oxidative stress induced by CCl_4 in the second phase. Although the plasma levels of all ceramide species were significantly increased 24 h after CCl_4 intoxication, treatment with celecoxib significantly reduced the total ceramide concentration in plasma. These results indicated that celecoxib significantly ameliorated the toxicity of CCl_4 in the second phase.

Key words celecoxib; carbon tetrachloride; ceramide; oxidative stress; ascorbic acid

In the study of radical reactions in biology, one of the most studied models is drug-induced hepatitis. Carbon tetrachloride (CCl_4) is a well-known typical hepatotoxin causing centrizonal necrosis.[1] CCl_4-induced hepatic injury is assumed to involve two phases. The initial phase is generation of radicals and the second phase is activation of Kupffer cells,[2] which release various pro-inflammatory mediators.[3] In the second phase, activities of phospholipase A_2[4] and cyclooxygenase-2 (COX-2)[5—7] are increased. While COX-2-dependent response is assumed to be an important factor to link between oxidative stress and inflammation,[5] COX-2 is also suggested to be hepatoprotective.[8] In this study we evaluated the effect of celecoxib, a specific inhibitor of COX-2 in CCl_4 intoxication.

In addition, CCl_4 intoxication has been used as an animal model of fulminant hepatic failure to develop artificial liver support.[9] In fulminant hepatic failure, toxic substances and cytokines released into the circulation are assumed to cause encephalopathy and renal dysfunction.[10] Recently we reported that plasma ceramides are increased in severe liver failure caused by D-galactosamine[11] or CCl_4.[12,13] It is well established that ceramides cause apoptosis in a variety of cells.[14] In this study we evaluated the effect of celecoxib on the increase of plasma ceramide caused by CCl_4. Since extensive liver damage and the increase in plasma ceramide concentration occurred 24 h after CCl_4 administration,[12,13] we focused our study on the change in ceramides at this time.

MATERIALS AND METHODS

Animals This study was approved by the Animal Care Committee of Nara Women's University. Eight-week-old 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., Osaka, Japan) and water *ad libitum*. To detect the effect of celecoxib definitely, the oral doses of CCl_4 and celecoxib were examined based on preliminary experiments changing the dose of CCl_4 at 0.5, 1, and 2 ml/kg body weight and that of celecoxib at 50 and 100 mg/kg body weight. The dose of CCl_4 was determined to be 0.5 ml/kg as CCl_4. Celecoxib (100 mg/kg body weight 3 h before or after administration of CCl_4) did not give sufficiently high protective effect, then the same dose of celecoxib was added 8 h after administration of CCl_4. Based on these preliminary experiments, after 12 h fasting, CCl_4 groups were orally administered 1 ml/kg of a mixture of CCl_4 and mineral oil (0.5 ml/kg as CCl_4) as previously described.[15] Three and eight hours after administration of CCl_4, the CCl_4+celecoxib group orally received 100 mg/kg each of celecoxib twice. The CCl_4+vehicle group received vehicle (0.5% methylcellulose, 0.025% Tween 20). Analysis of plasma and the liver for the CCl_4+celecoxib group and the CCl_4+vehicle group were made 12 and 24 h after CCl_4 administration. The sham and control groups were administered 1 ml/kg of mineral oil. The sham group received celecoxib 3 and 8 h after administration of mineral oil. Analysis of plasma and the liver for the control and sham were made 24 h after mineral oil administration.

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 liver was 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—5 animals in each group.

The determination of total vitamin C was made according to a specific and sensitive method[16] involving chemical derivatization and HPLC. Briefly L-ascorbic acid was oxidized with 2,6-dichloroindophenol to L-dehydroascorbic acid, which was reacted with 2,4-dinitrophenylhydrazine. The resulting osazone was extracted with ethyl acetate and applied to HPLC using μ-Bondasphere 5-μm C_{18}-100A column

* To whom correspondence should be addressed. e-mail: kojo@cc.nara-wu.ac.jp

(3.9×150 mm, Waters), eluted with a 1 : 1 mixture of acetonitrile and water adjusted at pH 3.5 with triethylamine (10 g/l) and phosphoric acid.[16] The absorption at 505 nm was recorded. The vitamin C level was expressed by nmol/g wet liver.

Blood was centrifuged at 8400 ***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 Chemicals Co., Osaka) and expressed as Karmen Units. The levels of plasma ceramides were determined as described previously.[13] Briefly, ceramides were extracted with a mixture of $CHCl_3/CH_3OH$, followed by sequential TLC separations.[17] The ceramide spot was extracted with a mixture of $H_2O/CH_3OH/CHCl_3$ (0.8 : 2 : 1, v/v/v) and subjected to LC-MS/MS analysis.[17]

Statistical analysis was carried out with Statcel (Excel 2000). Differences between the group means were considered significant at $p<0.05$ using Bonferroni/Dunn procedure.

RESULTS

Effect of Celecoxib on the Necrosis and Oxidative Stress Caused by CCl_4 To evaluate the extent of liver necrosis, plasma ALT and AST levels were determined. Twelve hours after administration of CCl_4, plasma ALT level of the CCl_4+vehicle and the CCl_4+celecoxib groups were 600±101 and 706±35.4 Karmen units, respectively ($n=5$). No significant difference was observed between these values, while these values were significantly higher than those in control group shown in Table 1. The data of the control group were determined 24 h after administration of mineral oil and the ALT and AST values were identical with normal rats without any treatment (data not shown). Plasma AST level of the CCl_4+vehicle and the CCl_4+celecoxib groups were 1278±207.5 and 1351±68.8 Karmen units, respectively ($n=5$). No significant difference was also observed between these values, while these values were significantly higher than those in control group shown in Table 1. At the same time the levels of liver vitamin C, a sensitive indicator of oxidative stress,[18] in the CCl_4+vehicle and the CCl_4+celecoxib were decreased to 636.4±71.0 and 583.7±43.4 nmol/g liver, respectively ($n=5$). These values were significantly low compared to those in the control group (Table 1), the vitamin C level of which was identical with normal rats without any treatment (data not shown). The effect of celecoxib on plasma ALT and AST and liver vitamin C were not observed 12 h after CCl_4 treatment, *i.e.*, during the initial phase of intoxication.

Plasma ALT and AST levels were further increased and the level of liver vitamin C remained at a low level 24 h after CCl_4 intoxication (Table 1). Treatment with celecoxib significantly decreased plasma ALT and AST levels compared to the CCl_4+vehicle group, showing that celecoxib effectively ameliorated the necrotic action of CCl_4 at the second phase. Consistent with this observation, the liver vitamin C level in the CCl_4+celecoxib group was higher than that in the CCl_4+vehicle group (Table 1).

No difference was observed between the control and the sham groups, showing that celecoxib did not affect these parameters (Table 1).

Effect of Celecoxib on Plasma Ceramide after CCl_4 Treatment The plasma levels of all ceramide species were significantly increased 24 h after the treatment with CCl_4 (0.5 ml/kg) (Table 2). Treatment with celecoxib significantly reduced the concentrations of total ceramide and all ceramide species except C 24:2 in plasma (Table 2), although all ceramide levels were significantly higher than those in the control and sham groups. The decrease in plasma ceramides corresponded with the decreased liver damage as evidenced by decreased ALT and AST by celecoxib.

Table 1. Plasma ALT and AST and Vitamin C Level in the Liver 24 h after CCl_4 Intoxication

	CCl_4+vehicle	CCl_4+celecoxib	Sham (celecoxib+oil)	Control (oil only)
ALT (Karmen units)	3514.7±431.1*,#	1910.5±243.3*,#	29.5±2.6	33.4±1.4
AST (Karmen units)	7675.7±664.4*	3525.4±412.6*,#	67.5±1.7	83.2±5.9
Vitamin C (nmol/g liver)	437.4±68.6*	862.2±49.4*,#	1679.9±46.6	1878.1±162

CCl_4 (1 ml/kg; as a mixture of CCl_4 : mineral oil=1 : 1) was orally administered to rats. After 24 h, plasma ALT and AST and concentration of vitamin C in the liver were determined as described in the text. After 12 h of fasting, the control rats received mineral oil alone (1 ml/kg body weight). The sham rats received celecoxib at 3 and 8 h after administration of mineral oil. Determinations for the control and sham were made 24 h after mineral oil administration. Values are means±S.E. for 4 rats. Asterisk (*) indicates a significant difference from both the sham and control group and a signal (#) indicates a significant difference from the CCl_4+vehicle group (ANOVA Bonferroni/Dunn, $p<0.05$).

Table 2. Plasma Level of Ceramide (nmol/ml) 24 h after CCl_4 Intoxication

	C 16:0	C 18:0	C 22:0	C 24:0	C 24:1	C 24:2	Total
Control (oil only)	0.50±0.04	0.05±0.01	0.37±0.03	3.62±0.27	2.63±0.20	0.75±0.05	7.92±0.60
CCl_4+vehicle	1.33±0.19*	0.40±0.07*	1.13±0.10*	8.52±0.21*	6.18±0.24*	1.86±0.16*	19.4±0.82*
CCl_4+celecoxib	0.93±0.09	0.24±0.03*	0.85±0.06*	6.68±0.36*,#	4.90±0.26*,#	1.55±0.12*	15.2±0.83*,#
Sham (celecoxib+oil)	0.55±0.04	0.06±0.002	0.39±0.04	3.55±0.41	2.61±0.28	0.81±0.08	7.97±0.84

CCl_4 (1 ml/kg; as a mixture of CCl_4 : mineral oil=1 : 1) was orally administered to rats. After 24 h, plasma ceramide concentrations were determined as described in the text. After 12 h of fasting, the control rats received mineral oil alone (1 ml/kg body weight). The sham rats received celecoxib at 3 and 8 h after administration of mineral oil. Determinations for the control and sham were made 24 h after mineral oil administration. Values are means±S.E. for 4 rats. Asterisk (*) indicates a significant difference from both the sham and control group and a signal (#) indicates a significant difference from the CCl_4+vehicle group (ANOVA Bonferroni/Dunn, $p<0.05$).

DISCUSSION

This study demonstrated that treatment with celecoxib significantly ameliorated liver cell necrosis based on plasma ALT and AST levels 24 h after CCl_4 intoxication. At the same time, celecoxib significantly reduced the oxidative stress in the liver during the second phase of CCl_4 intoxication based on hepatic vitamin C level, which was the most sensitive indicator of oxidative stress during hepatitis caused by chemicals such as CCl_4,[15] thioacetamide,[19] or D-galatosamine.[20] Although an antioxidant such as α-tocopherol inhibited liver necrosis caused by CCl_4 *via* direct reduction of oxidative stress,[21] a different mechanism should operate in the inhibition of oxidative stress by celecoxib. Because it was unlikely that celecoxib functioned as a radical scavenger.

CCl_4 activated Kupffer cells,[2] causing secretion of chemical mediators such as TNF-α (tumor necrosis factor-α), IL (interleukin)-1, and IL-6[3,22] and induction of COX-2.[23] Soluble TNF-α receptor prevented the increase in serum ALT 24 h after CCl_4 intoxication and thereafter, showing an important role of TNF-α in the second phase of liver cell injury.[24] Upregulation of TNF-α associated with the induction of COX-2,[25,26] products of which might have a conceivable link between inflammatory response and oxidative injury.[5] Overexpression of COX-2 in the mouse liver resulted in a marked induction of the proinflammatory cytokines such as TNF-α, IL-1β, and IL-6, inducing hepatitis, which was recovered by celecoxib administration.[27] These results indicated the close link among COX-2, proinflammatory cytokines, and oxidative stress.

On the other hand, inhibition of COX-2 with NS-398, another selective COX-2 inhibitor, aggravated the liver injury caused by a higher dose of CCl_4 at 2 ml/kg.[8] The reason of this difference is not easily explained but the effect of CCl_4 on COX-2 and inflammation may vary with an applied dose. Indeed, a moderately hepatotoxic dose of CCl_4 (2 ml/kg) increased hepatic COX-2, while a highly hepatotoxic dose of CCl_4 (3 ml/kg) was accompanied by minimal COX-2 activity.[28]

The effect of celecoxib on the liver is still controversial. In chronic applications of CCl_4 to rodents, COX-2 inhibitor reduced,[29—31] or potentiated[32] liver fibrosis. The critical role of COX-2 and the effect of celecoxib on liver inflammation remained to be explored.

Treatment with celecoxib significantly reduced the plasma level of ceramide, which caused cell death in a variety of cells[14] and we reported that it might be a cause of multi-organ failure in fulminant hepatic failure.[13] Therefore it is possible that celecoxib is beneficial to prevent multi-organ failure in fulminant hepatic failure, although the role of COX-2 in ceramide metabolism is not clear at present.

In contrast to our study, celecoxib induced *de novo* synthesis of sphingolipids including ceramide in human cancer cell lines.[33] The difference may be explained thus: ceramide is produced by neutral sphingomyelinase, *via* a salvage pathway in CCl_4 intoxication.[13]

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