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SHORT COMMUNICATION

Increase of Caspase-3 Activity in Rat Liver and Plasma by Thioacetamide

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ABSTRACT. Twelve and twenty-four hours after intraperitoneal administration of thioacetamide (200 mg/kg body weight) to rats, caspase-3 activity in the liver and plasma of the treated rats increased significantly compared with that of the control group. These results demonstrated that thioacetamide caused apoptosis, which involved activation of caspase-3, although there may be a number of pathways to apoptosis in the liver that may or may not involve the activation of this protein. The results also indicated that the activated caspase-3 was released in plasma along with glutamate-oxaloacetate transaminase (GOT) by successive necrosis. This is the first study that shows an increase of caspase-3 activity in plasma. BIOCHEM PHARMACOL **58**;12:1941–1943, 1999.

KEY WORDS. apoptosis; thioacetamide; caspase-3; necrosis; liver

Two different modes of cell death, apoptosis and necrosis, have received much attention recently in the biological sciences. Although apoptosis was originally characterized morphologically in animal tissue [1], biochemical mechanisms leading to apoptosis have been studied extensively using cultured cell systems. Therefore, only limited studies are available concerning the mechanism of apoptosis and its relationship to necrosis in animal tissues [2].

Although thioacetamide has long been known to be a typical hepatotoxin causing centrilobular necrosis [3–5], Ledda-Columbano *et al.* [6] reported that thioacetamide induces apoptosis in rat liver, based on histochemical observations. To shed more light on the toxic mechanism of this chemical, we investigated the possible involvement of caspase-3 [7] in chemically induced apoptosis. In this paper, we report that caspase-3 was indeed activated in the liver by thioacetamide and released in the plasma.

MATERIALS AND METHODS

Materials

Ac-DEVD-CHO† and Ac-DEVD-MCA were purchased from Peptide Institute Inc. All other reagents were of analytical grade and were purchased from the Nacalai Tesque Co.

Animals

Guidelines from the Prime Minister's Office of Japan (No. 6; 27 March 1980) for the care and use of laboratory animals were followed. Eight-week-old male rats (SLC: Wistar strain) were obtained from the Japan SLC Co. The animals were housed in a room with a temperature of 24 ± 2° and a 12 hr/12 hr light–dark cycle. Animals were fed commercial laboratory chow (MF, Oriental Yeast Co.) and water *ad lib*. Thioacetamide (200 mg/kg body weight) dissolved in saline was injected intraperitoneally into the treated rats. Control rats received saline.

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 vol. of PBS under ice cooling. All determinations were made in duplicate with 3–7 animals in each group. Blood was centrifuged at 10,000 *g* for 5 min at 4° to separate the plasma. The activity of plasma GOT (EC 2.6.1.1) was determined using a diagnostic kit (GOT-UV Test Wako, Wako Pure Chemicals Co.) and expressed as Karmen Units.

Protein concentrations were determined according to the method of Lowry *et al.* [8] using bovine serum albumin as the standard.

Assay for Caspase-3

Caspase-3 activity was measured using a modified procedure of Hasegawa *et al.* [9]. Liver homogenate was centrifuged at

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† *Abbreviations*: Ac-DEVD-CHO, acetyl-Asp-Glu-Val-Asp-CHO; Ac-DEVD-MCA, acetyl-Asp-Glu-Val-Asp-α-(4-methylcoumaryl-7-amide); AMC, aminomethylcoumarin; and GOT, glutamate-oxaloacetate transaminase.

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TABLE 1. Caspase-3 activity of the liver and plasma in thioacetamide-treated rats 6, 12, and 24 hr after drug administration

	Hours after thioacetamide treatment	Caspase 3 activity (pmol/mg protein/min)	
		Liver	Plasma
Control		15.4 ± 1.2 (7)	0.16 ± 0.29 (7)
Treated	6	22.3 ± 1.1 (3)	0.52 ± 0.13 (3)
	12	31.2 ± 7.2* (6)	4.96 ± 1.23* (6)
	24	24.9 ± 6.3† (7)	5.56 ± 3.16* (7)

Thioacetamide (200 mg/kg) was administered i.p. to rats. After 6, 12, and 24 hr, caspase-3 activities in the liver and plasma were determined as described in the text. Control rats received saline, and the enzyme activity was determined after 24 hr. The number of rats is shown in parentheses. Values are means ± SD and are expressed as pmol AMC liberated/mg protein/min.

*,† Significantly different from the corresponding control group (ANOVA Bonferroni/Dunn procedure): *$P < 0.01$ and †$P < 0.05$.

15,000 *g* for 5 min, and the supernatant, diluted 3- to 4-fold with PBS, was assayed for caspase-3 activity. Plasma diluted 5-fold with PBS was used as an enzyme source. An enzyme solution (1.0 mL) was mixed with 790 μL of a mixture containing 100 mM Tris–HCl, 2 mM EDTA, and 20 mM EGTA. To the resulting mixture, 200 μL of dithiothreitol (final concentration 1 mM) solution and 10 μL of Ac-DEVD-MCA (final concentration 50 μM) solution were added. The reaction was performed at 37°, pH 7.5. After 0, 10, and 20 min, an aliquot (190 μL) was taken from the reaction mixture, and 10 μL of $HClO_4$ was added to terminate the reaction. After centrifugation at 15,000 *g* for 5 min, the fluorescence of the supernatant containing released AMC was determined using a fluorescence spectrophotometer (Shimadzu, RF-1500) with excitation at 380 nm and emission at 460 nm. All assays were essentially linear during this time interval. A standard curve was prepared using solutions of AMC at various concentrations in the assay solution containing 5% $HClO_4$. The activity of the enzyme was expressed as picomoles of AMC liberated per milligram of protein per minute.

Data were expressed as means ± SD and analyzed by ANOVA using StatView software (Abacus Concepts). Differences between group means were considered significant at $P < 0.05$, using the Bonferroni/Dunn procedure generated by this program.

RESULTS AND DISCUSSION

Activation of Caspase-3 by Thioacetamide in Rat Liver

In the control rat liver, caspase-3 activity was 15.4 ± 1.2 pmol/mg protein/min. At 6 hr after the administration of thioacetamide at a dose of 200 mg/kg body weight, the activity of caspase-3 was not changed (Table 1). At 12 and 24 hr after the injection, the activity of caspase-3 increased significantly compared with that of the control group (Table 1). These caspase-3 activities in the liver were inhibited completely by 50 μM Ac-DEVD-CHO, a specific inhibitor of the enzyme [10]. Therefore, the proteolytic activity observed in the liver was due to caspase-3 itself and not to other proteases. Combined with the finding by Ledda-Columbano *et al.* [6] that thioacetamide causes apoptosis in rat liver (based on histochemical studies), these results indicate that thioacetamide caused apoptosis involving activation of caspase-3, although there may be a number of pathways to apoptosis that may or may not involve the activation of the protein. Caspase-3 activity in the liver may be a useful biochemical indicator to investigate whether apoptosis is involved in the toxic action of chemicals, because apoptosis has been investigated based on histochemical studies [6, 11–14] in animal experiments. Besides, thioacetamide does not cause activation of liver transglutaminase [6], a presumptive marker of apoptosis [15].

Jaeschke *et al.* [16] reported that endotoxin, a hepatotoxin leading to necrosis, causes a 17-fold activation of caspase-3 in mouse liver 7 hr after administration. The activation caused by endotoxin is much higher than that observed in the present study. It remains to be clarified whether the difference is brought about by acute induction of apoptosis by endotoxin or if thioacetamide causes only partial apoptosis.

Increase of Caspase-3 Activity in Plasma by Thioacetamide

The activity of caspase-3 in the plasma of control rats that had received saline 24 hr previously was barely detectable (Table 1). Six hours after the administration of thioacetamide, plasma caspase-3 activity was not different from that of the control group (Table 1). At 12 hr after the injection, the caspase-3 activity in plasma increased dramatically (Table 1). At 24 hr, the caspase-3 activity in plasma still remained at a higher level than that of the control group (Table 1). Caspase-3 activities in plasma were also inhibited completely by 50 μM Ac-DEVD-CHO. Therefore, it is concluded that the proteolytic activity determined in the plasma was due to caspase-3 itself. At 12 hr after the injection of thioacetamide, the activity (664 ± 156 units) of plasma GOT was significantly higher than that of the control group (72.2 ± 3.7 units), and it increased further to 3325 ± 550 units at 24 hr. These results indicate that the necrotic process proceeded for approximately 12–24 hr, consistent with the literature [4, 5], and that caspase-3 was released from the liver to the plasma. This is the first study showing an elevation of caspase-3 (a protease) activity in plasma, which contains a strong anti-protease activity. It may be worthwhile noting that plasma caspase activity is a useful and convenient index to evaluate the occurrence of apoptosis in pathological conditions.

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