Aggravating action of zymosan on acute liver damage in perfused liver of rats treated with D-galactosamine

TAV-XING CUI, MASARU IWAI, TADASHIGE YAMAUCHI, AND TAKASHI SHIMAZU

Department of Medical Biochemistry, Ehime University
School of Medicine, Shigenobu, Ehime 791-0295, Japan

Cui, Tai-Xing, Masaru Iwai, Tadashige Yamauchi, and Takashi Shimazu. Aggravating action of zymosan on acute liver damage in perfused liver of rats treated with D-galactosamine. Am. J. Physiol. 275 (Gastrointest. Liver Physiol. 38): G1361–G1366, 1998.—To study the role of Kupffer cells in the aggravation of liver injury, effects of zymosan on acute liver damage were explored using perfused livers of rats 24 h after intraperitoneal injection of D-galactosamine (800 mg/kg). The leakage of lactate dehydrogenase and aspartate aminotransferase into the effluent was used to indicate acute liver damage. Infusion of zymosan (30 µg/ml) into the portal vein rapidly increased the leakage of lactate dehydrogenase and aspartate aminotransferase from galactosamine-treated liver with decreased perfusion flow. Pretreatment of animals with gadolinium, which diminished an immunostaining of resident macrophages in the injured liver, significantly attenuated the flow reduction induced by zymosan, whereas it did not affect the increases in enzyme leakage. Infusions of PGF2α, PGE2, and leukotriene D4, the eicosanoids mainly produced by Kupffer cells, decreased perfusion flow without rapid augmentation of enzyme leakage from galactosamine-treated liver. These results indicate that zymosan potentiates acute liver damage after galactosamine injection and suggest that certain types of nonparenchymal cells other than Kupffer cells are mainly involved in the action of zymosan.

Kupffer cells; hepatic nonparenchymal cells; gadolinium; eicosanoids

THE HEPATIC SINUSOID consists of different types of nonparenchymal cells, such as the endothelial cells, Kupffer cells, and hepatic stellate cells. These sinusoidal cells have an important role in metabolic and hemodynamic regulations in the liver through interaction between the nonparenchymal cells and parenchymal cells (1, 5, 10). For instance, it has been shown that the effects of hepatic sympathetic nerves and extracellular ATP on carbohydrate metabolism in hepatocytes are mediated by eicosanoids, which are produced by nonparenchymal cells like Kupffer cells and act as intercellular mediators (10, 15, 20, 23). Moreover, Kupffer cells and hepatic stellate cells are known to be involved not only in physiological functions but also in pathological responses of the liver (4, 8, 12, 18).

Recently, we demonstrated that electrical stimulation of the hepatic sympathetic nerves and circulating catecholamines exaggerate acute liver damage induced by D-galactosamine (17). Considering the above findings of interaction between the nonparenchymal cells and hepatocytes for the action of hepatic sympathetic nerves and of the involvement of sinusoidal cells in liver pathology, it might be possible that hepatic sinusoidal cells are involved in the aggravation of acute liver damage induced by the hepatic nerve stimulation.

The present studies were designed to explore this possibility by infusing zymosan into perfused liver from galactosamine-treated rats. Zymosan is known as an activator of sinusoidal cells, especially the Kupffer cells, and it induces acute responses of the liver, such as glucose production (7) and hemodynamic changes (6) by producing eicosanoids. Hence, the mechanism of action of zymosan was also analyzed in terms of Kupffer cell function in both normal and galactosamine-injured livers.

MATERIALS AND METHODS

Materials. Zymosan, D-galactosamine hydrochloride, gadolinium chloride, PGF2α, PGE2, and leukotriene D4 were purchased from Sigma (St. Louis, MO). ED2 was from Serotec (Oxford). Envision polymer reagent was from DAKO J apan (Kyoto, J apan). Other materials were from Wako Pure Chemical Industries (Osaka, J apan).

Animals. Male Sprague-Dawley rats (200–250 g; Clea J apan, Osaka, J apan) were kept on a 12:12-h light-dark cycle (lights on from 6 AM to 6 PM) with free access to water and laboratory chow (MF, Oriental Yeast, Tokyo, J apan). All experiments were started between 11 AM and 12 AM.

Liver injury and treatment with gadolinium. The experimental protocol was approved by the Animal Studies Committee of Ehime University. Acute liver injury was induced in rats by a single administration of D-galactosamine. D-Galactosamine was dissolved in sterilized saline and injected intraperitoneally at a dose of 800 mg/kg after neutralization. In experiments using gadolinium, the animals received further intravenously treatment with either gadolinium chloride (20 mg/kg, dissolved in saline at pH 3) or the same volume of saline as control 1 h after the injection of galactosamine. Gadolinium is taken up exclusively by macrophages, thus selectively destroying Kupffer cells in the liver (3, 19). Animals were used for perfusion experiments 24 h after galactosamine administration.

Liver perfusion. Livers were perfused in situ without recirculation in a 37°C cabinet via the portal vein using Krebs-Henseleit bicarbonate buffer containing 5 mM glucose, 2 mM lactate, and 0.2 mM pyruvate. The medium was equilibrated with 95% O2 and 5% CO2. Perfusion pressure was constant at ~10 cmH2O, with a flow rate of 3.5 to 4.1 ml·min−1·g liver−1 under basal conditions. The flow rate was measured by fractionating the effluent.

Infusion of zymosan and chemical compounds. Zymosan powders were suspended in saline and kept at 95°C for 30 min to eliminate endogenous phospholipase A2 activity as described by Dieter et al. (7). The suspensions were centrifuged at 10,000 g for 5 min. The pellet was washed twice with saline.
and resuspended in a suitable volume of saline. Zymosan pellet was diluted with the perfusion buffer to obtain a final concentration for infusion.

PGF$_2 \alpha$ and PGE$_2$ were dissolved in the perfusion buffer containing 0.1% BSA to obtain a final concentration for infusion. Leukotriene D$_4$ methyl ester was hydrolyzed with a 5% potassium carbonate solution for 3 h and then diluted in the perfusion buffer, which contained 0.1% BSA to obtain a final concentration for infusion.

Measurement of metabolites and enzyme activity. Glucose and lactate were measured with glucose oxidase (Auto-Pak A glucose; Boehringer Mannheim, Mannheim, Germany) and lactate oxidase (Determiner LA; Kyowa Medics, Tokyo, Japan), respectively. The activities of lactate dehydrogenase (LDH) and aspartate aminotransferase (AST) were determined as previously described (17).

Immunohistochemical staining of resident macrophages in the liver. The presence of macrophages in the liver (Kupffer cells) was demonstrated using the monoclonal antibody ED2, which recognizes resident macrophages in rat liver (2, 11). Rat livers were removed after intraperitoneal injection of galactosamine (800 mg/kg), followed by the administration of gadolinium chloride as described in Liver injury and treatment with gadolinium. Control rats were administered the same volume of saline instead of galactosamine and gadolinium. Liver samples were then snap-frozen in liquid nitrogen and stored at $-70^\circ$C. Frozen tissues were cut on a cryostat, and 6 µm-thick sections were picked up on glass slides. Sections were air-dried, fixed in pure acetone for 10 min, and air-dried again. A two-step immunoperoxidase method was carried out using ED2 as primary antibodies and Envision polymer reagent as secondary antibodies to demonstrate the presence of Kupffer cells in the liver. ED2 was diluted 1:600 with 0.1% PBS-BSA. After incubation with ED2 for 1.5 h at room temperature, Envision polymer reagent was applied to the sections for 1 h at room temperature. Peroxidase reaction was visualized with 3,3'-diaminobenzidine tetrahydrochloride.

Statistical analysis. The effects of various compounds were evaluated by ANOVA followed by a Newman-Keuls test for multiple comparisons. Differences were considered significant at $P < 0.05$.

RESULTS

Effects of zymosan on acute liver damage and flow rate in perfused liver after treatment with galactosamine. The leakage of LDH and AST into the effluent was used to indicate acute liver damage. The activities of these enzymes were nearly undetectable in control liver throughout the perfusion period (Fig. 1). The infusion of 30 µg/ml zymosan reduced the perfusion flow but did not alter the enzyme leakage (Fig. 1). In contrast, galactosamine-treated livers exhibited an apparent leakage of LDH and AST under the basal conditions as previously described (17) (Fig. 1). In these livers, the leakage of LDH and AST was increased rapidly and remarkably by infusion of zymosan (Fig. 1). Flow reduction caused by zymosan in galactosamine-treated liver was larger than that in normal liver ($P < 0.05$, Fig. 1).

Effects of gadolinium on acute liver damage and flow reduction caused by zymosan in galactosamine-treated liver. Gadolinium chloride is known to depress the function of hepatic macrophages, i.e., Kupffer cells (3, 19). Pretreatment of rats with gadolinium did not alter the basal levels of enzyme leakage from galactosamine-treated livers (Fig. 2). The flow reduction caused by zymosan was partially inhibited by gadolinium (cf. Figs. 1 and 2). However, the increases in enzyme leakage during zymosan infusion were not affected even by the treatment with gadolinium (Fig. 2).

---

**Figure 1.** Effects of zymosan (Zym) on the flow rate and enzyme leakage from perfused livers of control and galactosamine-treated rats. Livers were perfused in situ with Krebs-Henseleit bicarbonate buffer containing 5 mM glucose, 2 mM lactate, and 0.2 mM pyruvate. Galactosamine-treated rats were injected intraperitoneally with 800 mg/kg d-galactosamine 24 h before the perfusion. Control rats were injected with the same volume of saline as that in the galactosamine group. Zymosan (30 µg/ml) was infused from 45 to 55 min of perfusion time. Values are means ± SE of 3 rats for control and 4 rats for galactosamine-treated group. Changes in flow rate and enzyme activities during zymosan infusion are significantly different from controls ($P < 0.05$ for flow rate and $P < 0.01$ for enzyme leakage). LDH, lactate dehydrogenase; AST, aspartate aminotransferase.
Effects of gadolinium on metabolic and hemodynamic actions of zymosan in normal liver. To examine whether Kupffer cells are involved in the action of zymosan in normal liver, control livers were perfused with or without gadolinium pretreatment. In the livers without gadolinium pretreatment, infusion of zymosan into the portal vein greatly increased the glucose and lactate output and decreased the perfusion flow (Fig. 3). These metabolic and hemodynamic effects of zymosan were almost completely inhibited by gadolinium (P < 0.01, Fig. 3).

Immunohistochemistry of resident macrophages in the liver. To confirm that Kupffer cells were depleted by treatment with gadolinium, immunohistochemical staining of resident macrophages in the liver, i.e., Kupffer cells, was carried out using monoclonal antibody ED2 (Fig. 4). The staining of resident macrophages with ED2 appeared to be increased slightly by galactosamine treatment (Fig. 4, A and C). Kupffer cells almost totally disappeared with gadolinium both in control and in injured livers (Fig. 4, B and D).

Effects of eicosanoids on acute liver damage in perfused rat liver treated with galactosamine. Because the action of zymosan in the liver is considered to be mediated by eicosanoids (5, 6), the effects of eicosanoids on acutely injured livers were examined (Fig. 5). Infusions of PGF$_{2\alpha}$ and PGE$_2$ at a concentration of 10 µM reduced the perfusion flow, whereas the leakage of LDH and AST was not significantly affected (Fig. 5). On the other hand, leukotriene D$_4$ also decreased the flow rate, and the enzyme leakage was slightly augmented only after cessation of the infusion, coincident with the recovery of the flow (Fig. 5).

DISCUSSION

The present study indicates that zymosan potentiates acute liver damage in isolated perfused liver of rats treated with galactosamine. Acute liver damage induced by zymosan was evaluated by the increases in leakage of LDH and AST into the effluent (Fig. 1). Pretreatment of rats with gadolinium did not attenuate the potentiation of acute liver damage caused by zymosan (Fig. 2), whereas the flow reduction by zymosan was partially inhibited (Fig. 2). Immunohistochemical staining of resident macrophages in the liver was shown to disappear almost completely with gadolinium treatment (Fig. 4). These results suggest that Kupffer cells are involved in the hemodynamic action of zymosan, but the potentiative effects of zymosan on acute liver damage might be mediated by other types of nonparenchymal cells.

It was previously reported that zymosan induces glycogenolysis and vasoconstriction in perfused rat liver (6, 7). These effects of zymosan are considered to be mediated by eicosanoids produced mainly by Kupffer cells.
cells, since zymosan stimulated Kupffer cell eicosanoid production (5, 7) and the effects of zymosan were blocked by inhibitors of eicosanoid synthesis (7). This was also supported by the results of the present study, which showed that the metabolic and hemodynamic actions of zymosan in normal liver were almost totally abolished by the depletion of resident macrophages in the liver with gadolinium treatment (Fig. 3).

In galactosamine-treated livers, the vascular response induced by zymosan appears to be increased (Fig. 1) and mediated at least partly by Kupffer cells (Fig. 2). However, the effect of zymosan on acute liver injury in galactosamine-treated livers appears not to be mediated by Kupffer cells for the following reasons. First, the increases in enzyme leakage caused by zymosan in injured liver were not inhibited even after depletion of Kupffer cells by gadolinium (Figs. 2 and 4). Second, eicosanoids, which act as mediators after production by Kupffer cells (5, 7), could not induce the rapid increases in enzyme leakage (Fig. 5). Only leukotriene D4 could increase slightly the enzyme leakage, but its kinetics was different from that caused by zymosan (Fig. 5). The dose of eicosanoids used in the present study seems high enough to cause metabolic and hemodynamic changes, judging from the data reported previously (14, 16) and from the results on flow reduction in galactosamine-treated liver (cf. Figs. 1 and 5). Previous studies with cultured cells showed that zymosan strongly stimulated Kupffer cells but not endothelial cells for PG production (9). Thus these
results suggest that nonparenchymal cells other than Kupffer cells and endothelial cells may probably be involved in the effect of zymosan on acute liver damage, although the possibility is not totally excluded that zymosan directly acts on parenchymal cells or on inflammatory cells like neutrophils still remaining in the liver after perfusion (preliminary histological observations).

It is unlikely that effects of zymosan are due to the mechanical action of particulate zymosan on liver vessels, e.g., closing the vessels by microemboli with the particles. Histological examination of perfused liver after infusion of zymosan revealed that there was no apparent accumulation or embolus with zymosan in the vessels and/or sinusoids, although phagocytosis of zymosan particles by Kupffer cell-like sinusoidal cells could be observed (data not shown).

Concerning the mediators for the action of zymosan, the involvement of eicosanoids seems unlikely as discussed above. In a preliminary study, we also noted that superoxide dismutase, an enzyme responsible for the degradation of superoxide, or N-nitro-L-arginine, an inhibitor of nitrogen-oxide synthase, could not inhibit the acute liver injury caused by zymosan (data not shown). These results suggest that eicosanoids, superoxide, and nitrogen oxide are all not involved in the action of zymosan on acute liver damage after galactosamine treatment.

Furthermore, zymosan-induced potentiation of acute liver damage does not seem to be secondary to the hemodynamic change but is due to the direct action of zymosan on liver cells because infusion of eicosanoids into the portal vein reduced the perfusion flow without rapid increases in enzyme leakage from the liver (Fig. 5) and because artificial reduction of the perfusion flow could not induce the enzyme leakage as previously demonstrated (17).

The results of the present study suggest the possibilities that factors produced by the nonparenchymal cells other than Kupffer cells are capable of potentiating acute liver damage and that eicosanoids are not the candidate factors. Recently, we found that endothelin-1 potentiates the acute liver damage with perfused liver of rats treated with galactosamine (18). Endothelin-1 is reported to be produced by the hepatic nonparenchymal cells, such as the hepatic stellate cells (13) and endothelial cells (22). Thus it might be possible that endothelin-1 could act as a mediator in the action of zymosan to induce acute liver damage, which deserves further investigation.

We thank K. Tanimoto (Ehime University School of Medicine) for helpful advice during histological examination.

This work was supported in part by a grant-in-aid for scientific research from the Ministry of Education, Science, Sports, and Culture of Japan.

Address for reprint requests: M. Iwai, Dept. of Medical Biochemistry, Ehime Univ. School of Medicine, Shigenobu, Ehime 791-0295, J Japan.

Received 9 March 1998; accepted in final form 13 August 1998.
