Modification of Carbon Tetrachloride-Induced Hepatotoxicity by Clofibric Acid in Rats

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Effects of clofibric acid (*p*-chlorophenoxyisobutyric acid) on carbon tetrachloride (CCl_4)-induced hepatocellular necrosis and fatty liver were investigated. Male Wistar rats were fed a diet containing 0.5% (w/w) clofibric acid for 7 days before the administration of CCl_4 (1 ml/kg, *p.o.*) and the treatment with clofibric acid continued throughout the time course of the study. In rats given only CCl_4, the serum activity of alanine aminotransferase (ALT) increased rapidly and reached a maximum level at 24 h after the administration of CCl_4. The rats pretreated with clofibric acid exhibited a significantly lower serum level of ALT compared with the rats treated with CCl_4 alone until 24 h following CCl_4 dosing. However, the maximum level that was observed at 48 h after the administration of CCl_4 to the rats pretreated with clofibric acid was similar to the highest level observed in the CCl_4 alone group at 24 h after CCl_4 dosing. The hepatic glycogen level steeply decreased at 3 h after the administration of CCl_4 and reached the lowest level at 12 h preceding the definite appearance of necrosis, with gradual recovery noted by 96 h. An evident decrease in glycogen level was also noted in the group given clofibric acid throughout the time course. In rats treated with both CCl_4 and clofibric acid (clofibric acid + CCl_4), hepatic glycogen was exhausted from 3 h and the depletion lasted until 96 h after dosing of CCl_4. The serum level of glucose was not increased, but rather decreased markedly after the administration of CCl_4 in both the rats receiving CCl_4 alone and in the rats treated with clofibric acid + CCl_4. The hepatic content of triglyceride increased rapidly and reached a level about 5-fold greater than the control at 12 h after the administration, then the elevated level lasted until 96 h of the time course. The increase in the hepatic content of triglyceride induced by CCl_4 was significantly suppressed by pretreatment with clofibric acid and returned to the basal level by 96 h after dosing of CCl_4. The results of histopathological examination of liver sections stained by hematoxylin and eosin, and oil red O, were very consistent with the biochemical changes mentioned above. These results indicate that dietary pretreatment with clofibric acid suppressed the necrosis of hepatocytes in the initial stage, but not in the late stage; rather, the recovery of liver from necrosis was delayed. Also this drug significantly suppressed fatty liver caused by CCl_4.

Key words —— clofibric acid, carbon tetrachloride, hepatocellular necrosis, fatty liver

INTRODUCTION

It has been widely accepted that carbon tetrachloride (CCl_4) causes centrilobular hepatic necrosis and fatty liver. To find compounds which prevent CCl_4-induced hepatocellular toxicity, a number of chemicals have been placed under screening. As a result of great effort, several compounds have been characterized to have preventive effects against CCl_4-induced liver necrosis, such as antioxidants,[1] inhibitors of CYP 2E1,[2,3] inhibitors of phospholipase A_2,[4] and calcium chelators.[5]

Clofibrate (ethyl ester of clofibric acid) is a hypolipidemic agent which stimulates not only fatty acid degradation,[6–8] but also phospholipid synthesis[9] in the liver. It has been reported that pretreatment of mice with clofibrate reduces acetaminophen-caused hepatocellular necrosis[10] and that, moreover, the drug reverses orotic acid-induced fatty liver in rats.[11] These findings imply the possibility that clofibrate becomes one

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of the compounds which prevents CCl_4-induced hepatocellular necrosis and fatty liver. To date, however, only a few studies have been performed to demonstrate this possibility. Namely, Burdino *et al.* reported that clofibrate had no preventive effects against CCl_4-induced hepatocellular necrosis in rats.[12] By contrast, Temcharoen *et al.* showed that dietary pretreatment of rats with clofibrate markedly reduced CCl_4-caused hepatocellular necrosis.[13] However, since these studies were designed with a relatively short time course, information is very limited about whether clofibrate is able to prevent CCl_4-induced hepatocellular injury. In this context, we studied the effect of clofibric acid treatment on hepatocellular necrosis and fatty liver by monitoring several parameters at sufficient selected time points with a longer time course. Interestingly, we obtained the results that clofibric acid suppressed hepatocellular necrosis in the early stage, but not the late stage, of the time course, and that this drug efficiently suppressed triglyceride accumulation in the liver.

MATERIALS AND METHODS

Materials —— Clofibric acid was purchased from Sigma Chemical Co. CCl_4 (ultra pure grade), olive oil, anthrone and D-(+)-glucose were obtained from Wako Pure Chemical Industries (Osaka, Japan). Triheptadecanoin was purchased from Nu-Check-Prep., Inc. All other chemicals were of analytical grade.

Animals and Treatments —— Male Wistar rats aged 6 weeks were obtained from Japan SLC, Inc. (Hamamatsu, Japan) and acclimatized in community stainless-steel cages for 1 week before use. The rats received a standard diet (CE-2, Clea, Tokyo, Japan) and water *ad libitum*. Rats were exposed to a 12-h light-dark cycle and the room was maintained at 23℃ with a relative humidity of 60%.

After acclimatization, the rats were divided into four groups and 4 rats comprised each group. Two groups of the rats were fed the normal diet *ad libitum* for 7 days. On the seventh day, the rats of one of the groups were killed as controls and the other group received a single administration of CCl_4. The latter rats were fed the normal diet after the treatment until termination of the study. The rats of the other two groups received a diet containing 0.5% clofibric acid *ad libitum* for 7 days and, on the seventh day, the rats were injected CCl_4 or vehicle (olive oil); after the treatment with either CCl_4 or vehicle, the rats received dietary treatment with clofibric acid until termination of the time course study. CCl_4 was given orally to rats at a dosage of 1 ml of CCl_4 per kg body weight as a 25% (v/v) solution in olive oil. The rats receiving clofibric acid alone were administered the equivalent amount of olive oil. At 3, 6, 12, 24, 48, 72 or 96 h after CCl_4 or vehicle injection, blood was collected from the inferior vena cava under diethyl ether anesthesia and serum was prepared. The liver was rapidly removed, washed with saline and weighed. Sections of the liver were placed into 10% neutral-buffered formalin for histopathological study. For biochemical analysis, the rest of the liver was perfused with cold saline to wash out traces of blood, then frozen in liquid nitrogen and stored at −80℃ until use.

Serum Levels of Alanine Aminotransferase, Glucose and Triglyceride. —— The activities of alanine aminotransferase (ALT, EC 2.6.1.2) in serum were measured using a commercial reagent kit (Autosera GPT-2, Daiichi Pure Chemical Co., Tokyo, Japan). The activity is expressed in IU/l. Serum levels of glucose and triglyceride were measured enzymatically using the Autosera GLU-2 test kit and Autosera TG-2 test kit (Daiichi Pure Chemical Co., Tokyo, Japan), respectively.

Hepatic Levels of Triglyceride and Glycogen —— The frozen liver was thawed on ice and homogenized in 9 vol of 0.25 M sucrose, 1 mM EDTA and 10 mM Tris–HCl (pH 7.4). After the addition of a known amount of heptadecanoin as an internal standard, total lipid was extracted from liver homogenates according to the method of Bligh and Dyer.[14] Triglyceride was separated by thin-layer chromatography on silica gel G plates (Merck, Darmstadt Germany) using a solvent system comprising hexane : diethyl ether : acetic acid (80 : 30 : 1, v/v/v). After spraying 0.001% (w/v) primuline in acetone, the spot corresponding to triglyceride was extracted from the silica with chloroform/ methanol/ 0.1 M HCl (4 : 4 : 1, v/v/v) as previously described.[15] Fatty acids in triglyceride were transmethylated with sodium methoxide, and the resulting fatty acid methyl esters were analyzed by gas chromatography as described previously.[15] Glycogen was determined by the method of Seifter *et al.* with glucose as a standard.[16]

Histopathology —— The left lateral lobe of the liver was embedded in paraffin, sectioned at 3 μm and stained with hematoxylin and eosin. For the estimation of severity of necrosis, the necrotic area which

contained necrosis with an infiltration of inflammatory cells and granulolation-like tissue in 10 lobules were measured by planimetry under a light microscope using an image analyzer system (Vidas video plan, Carl Zeiss Co., Germany). Frozen sections of the liver fixed in 10% neutral-buffered formalin were stained with oil red O for neutral lipid.

Statistical Analysis —— Homogeneity of variance was established using the one-way ANOVA. When a difference was significant ($p<0.05$), Dunnett's multiple range test was used as a post test. For statistical evaluation between the group of CCl_4 alone and the group treated with clofibric acid + CCl_4, the F test and Student's t test were used between the respective time points after the administration of CCl_4. The acceptable level of significance was $p<0.05$. The logarithm of value was used for the analysis of ALT.

RESULTS

At the end of time course study, 100% survival was noted in all the groups.

Effects of Clofibric acid Pretreatment on CCl_4-Induced Liver Necrosis

An elevation of serum ALT was estimated as a marker of liver necrosis over a time course (0—96 h) after an administration of CCl_4 (Fig. 1). In rats given only CCl_4, the serum activity of ALT increased rapidly after a time lag for 3 h, reached a maximum level at 24 h after the administration, then decreased gradually to the control level by 96 h. When rats were pretreated with clofibric acid for 7 days before dosing of CCl_4 and received continued treatment with clofibric acid throughout the time course of the study (clofibric acid + CCl_4), significant prevention of the elevation of serum activity of ALT occurred with the duration for the early 24 h. Forty eight hour after the administration of CCl_4, however, serum activity of ALT reached the maximal level, which was similar in magnitude to that of the rats at 24 h after the treatment with CCl_4 alone. The ALT level in the rats treated with clofibric acid + CCl_4 was significantly higher than that in the rats receiving only CCl_4 at the time points of 72 and 96 h. Clofibric acid alone did not change the serum level of ALT throughout the time course. Areas under the curves for the groups of clofibric acid + CCl_4 and CCl_4 alone were 441342 and 319500 IU·h/l, respectively.

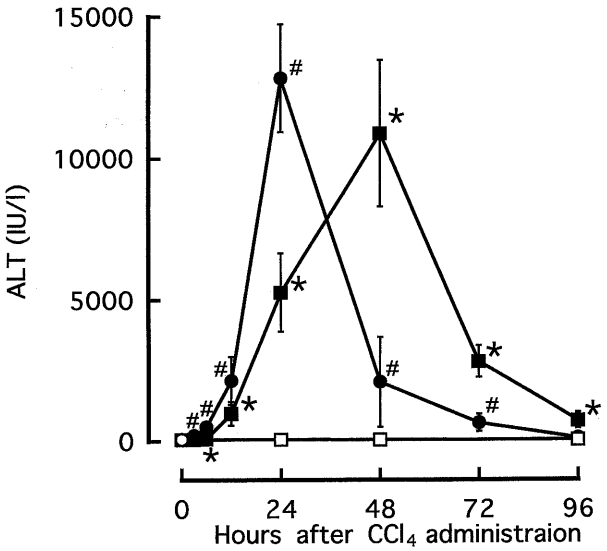

Fig. 1 Effect of Clofibric Acid Pretreatment on CCl_4-Induced Elevation of Serum ALT Activity

Rats were fed a standard diet or a diet containing 0.5 % (w/w) clofibric acid for 7 days before the administration of CCl_4 (1 ml/kg, *p.o.*) and were continually treated with clofibric acid throughout the time course of the study. ○, control; □, clofibric acid alone; ●, CCl_4 alone; ■, clofibric acid + CCl_4. Values represent mean±S.D. of 4 rats. # Significantly different from control ($p<0.05$). *Significantly different from the group of CCl_4 at the respective time points ($p<0.05$).

Liver sections stained by hematoxylin and eosin were examined for necrotic cells (Fig. 2). In the rats receiving only CCl_4, degeneration of hepatocytes was observed between 3 and 12 h (data not shown). Figure 2B depicts injury at 24 h (maximum injury) as observed by biochemical analysis; extensive necrosis was detected predominantly from the centrilobular to the intralobular zone. At 48 h, inflammatory cells infiltrated this necrotic area (Fig. 2C). From 72 h onward, granulation-like tissue was observed in the centrilobular region (Fig. 2D). Vacuoles were evident around the degeneration region between 3 and 12 h (data not shown) and throughout the liver from 24 h onward (Figs. 2C and D). In the rats treated with clofibric acid + CCl_4, necrosis was apparently limited to the centrilobular region at 24 h, but extensive necrosis was detected predominantly at 48 h (Figs. 2F and G). Vacuoles were slightly detected only between 6 and 24 hr (Fig. 2F). In both the groups of clofibric acid alone and clofibric acid + CCl_4, swelling and eosinophilic degeneration in hepatocytes at all time points were observed (Figs. 2E, F, G and H). The lesions were quantified for necrotic cells in the groups of CCl_4 alone and clofibric acid + CCl_4 from 12 h onward (Fig. 3). The results from

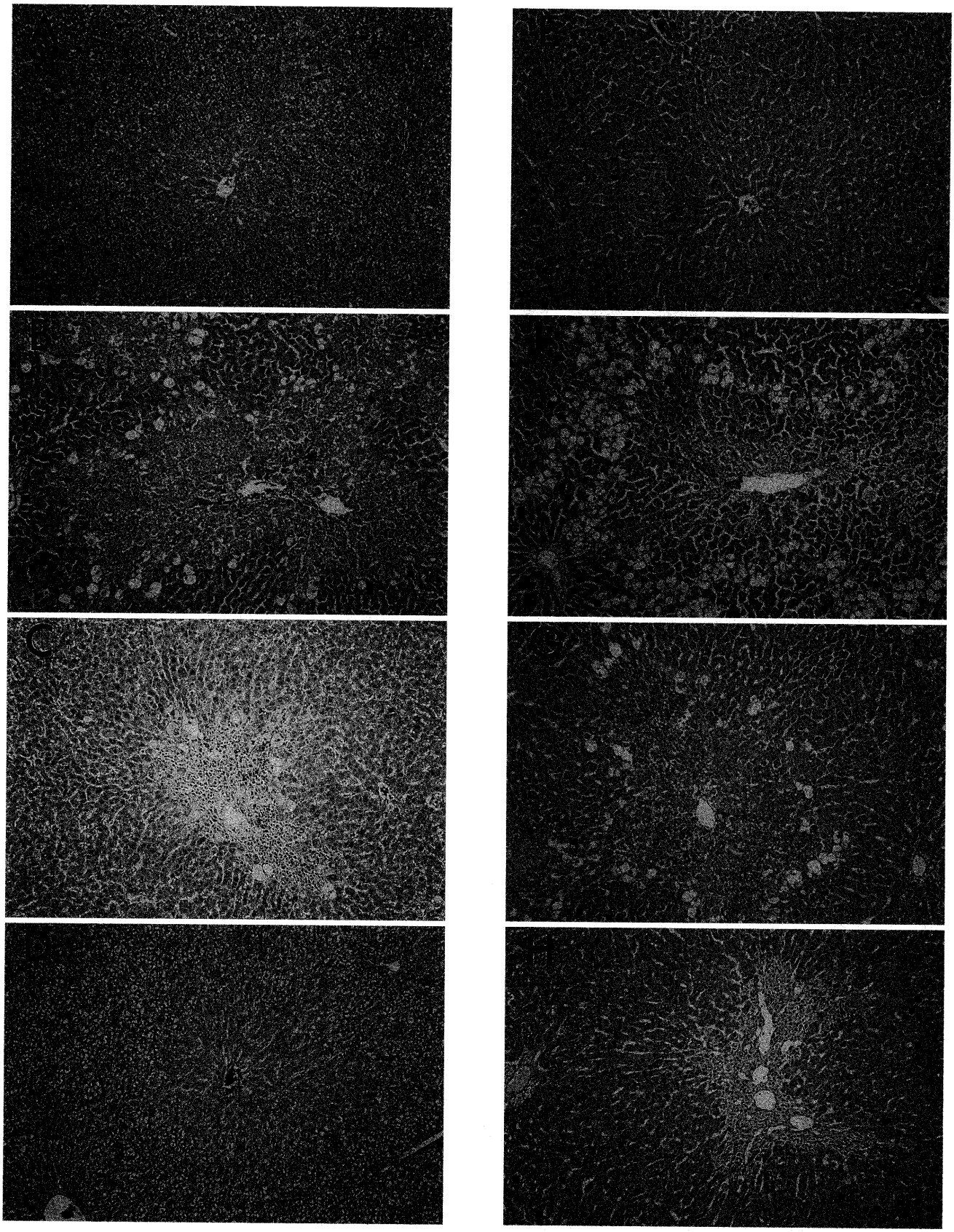

Fig. 2 Representative Photomicrographs of Liver Sections Stained by Hematoxylin and Eosin

The treatment of rats was the same as described in the legend to Fig. 1. A, control ; B, CCl_4 24 h ; C, CCl_4 48 h ; D, CCl_4 96 h ; E, clofibric acid alone ; F, clofibric acid + CCl_4 24 h ; G, clofibric acid + CCl_4 48 h ; H, clofibric acid+CCl_4 96 h. Original magnification 25×. In rats receiving CCl_4 alone, severe necrosis was seen at 24 h after the administration. In rats pretreated with clofibric acid, maximum injury was observed 48 h after the administration of CCl_4, similar to that seen in Fig. 2B.

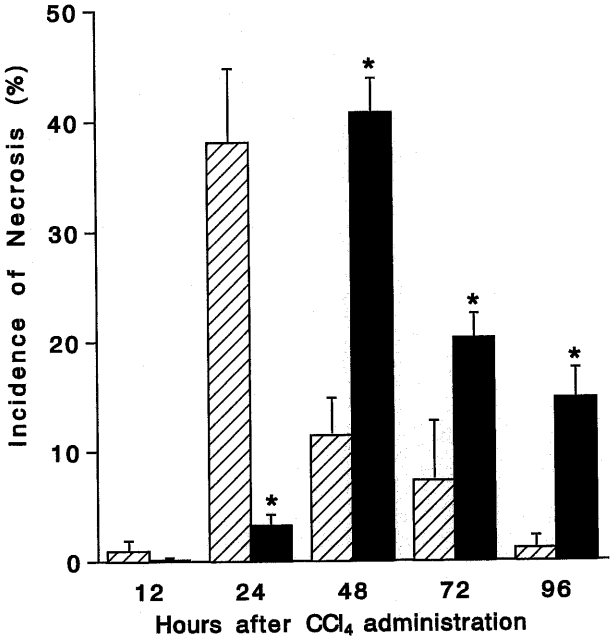

Fig. 3 Effect of Clofibric Acid Pretreatment on CCl_4 -Induced Liver Necrosis

Necrotic indices were estimated by measuring necrotic area / 10 lobules under a light microscope using an image analyzing system. The treatments were the same as described in the legend to Fig. 1. Hatched bars represent CCl_4 alone and closed bars represent clofibric acid + CCl_4. Values represent mean±S.D. of 4 rats. *Significantly different from the group of CCl_4 alone at the respective time points.

histopathological study were concordant with the elevation of serum ALT.

Effects of Clofibric Acid Pretreatment on the Levels of Hepatic Glycogen and Serum Glucose

Figure 4 shows the levels of hepatic glycogen and serum glucose over a time course after the administration of CCl_4. In the rats receiving CCl_4 alone, a drastic decrease in the hepatic level of glycogen was observed from 3 h and the content reached the lowest level at 12 h after dosing of CCl_4. Then, the hepatic content of glycogen gradually returned to the control level by 96 h. An evident decrease in glycogen level was also noted in the group of clofibric acid alone throughout the time course. When rats were treated with clofibric acid + CCl_4, the hepatic content of glycogen was markedly decreased from 3 h and reached the lowest level at 6 h after CCl_4 administration. The reduced level lasted until 96 h after dosing of CCl_4. At 96 h after the administration of CCl_4, the hepatic content of glycogen tended to return to the level of the group of clofibric acid alone (Fig. 4A).

The Serum level of glucose was markedly decreased after the administration of CCl_4 in the rats receiving CCl_4 alone and clofibric acid + CCl_4. In the rats treated with CCl_4 alone, a maximum decrease was observed at 24 h and

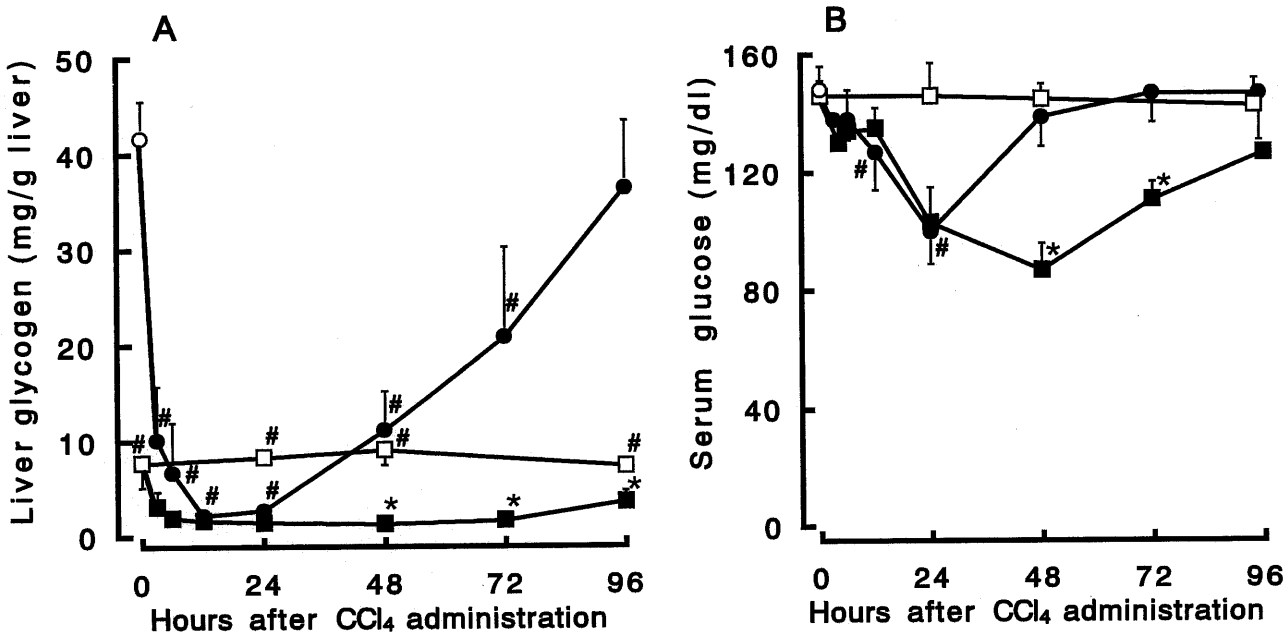

Fig. 4 Effect of Clofibric Acid Pretreatment on the Levels of Hepatic Glycogen and Serum Glucose Levels

The treatment of rats was the same as described in the legend to Fig. 1. A, hepatic glycogen; B, serum glucose. ○, control; □, clofibric acid alone; ●, CCl_4 alone; ■, clofibric acid+CCl_4. Values represent mean±S.D. of 4 rats. # Significantly different from control ($p<0.05$). *Significantly different from the group of CCl_4 alone at the respective time points ($p<0.05$).

recovered at 48 h after the administration of CCl_4. In the group of clofibric acid + CCl_4, the decrease in serum glucose lasted until 48 h after the administration of CCl_4 and recovered slowly thereafter. Clofibric acid alone caused no change in the serum level of glucose throughout the time course (Fig. 4B).

Effects of Clofibric Acid Pretreatment on CCl_4-Induced Fatty Liver

As shown in Fig. 5, the hepatic content of triglyceride increased rapidly following the administration of CCl_4 alone, and the value reached about 5-fold greater than the control at 12 h after the administration. The elevated level lasted until 96 h of the time course. The treatment with clofibric acid alone did not significantly change the hepatic content of triglyceride. Although an increase in triglyceride level was brought about in the rats treated with clofibric acid + CCl_4 as well, the extent of the elevation was markedly suppressed by the treatment with clofibric acid.

Liver sections stained by oil red O were examined for the accumulation of triglyceride (Fig. 6). In the rats receiving CCl_4 alone, oil red O positive droplets had already appeared in the centrilobular zone of the liver at 3 h after dosing of CCl_4 (data not shown). At 12 h, lipid droplets were detected widely, from the central to the peripheral zone, and the injury as characterized by lipid deposits lasted until 96 h (Figs. 6B, C and D). Pretreatment with clofibric acid evidently reduced the CCl_4-induced accumulation of lipid droplets with all the durations tested (Figs. 6F, G and H). No neutral fat droplets were observed in the control rats or the rats treated with clofibric acid alone (Figs. 6A and E).

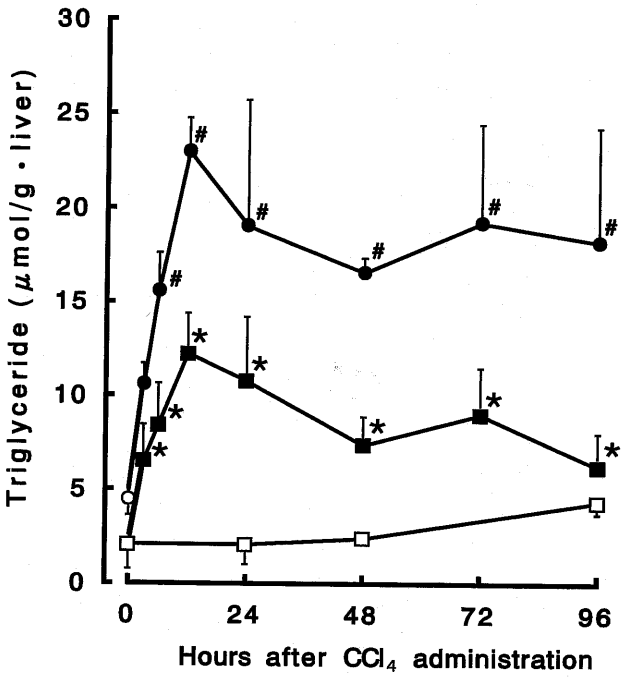

Fig. 5 Effect of Clofibric Acid Pretreatment on CCl_4-Induced Accumulation of Hepatic Triglyceride

The treatments were the same as described in the legend to Fig. 1. ○, control; □, clofibric acid alone; ●, CCl_4 alone; ■, clofibric acid + CCl_4. Values represent mean±S.D. of 4 rats. # Significantly different from control ($p<0.05$). *Significantly different from the group of CCl_4 alone at the respective time points ($p<0.05$).

Figure 7 represents changes in the acyl composition of hepatic triglyceride. The amounts of palmitic acid (16 : 0), stearic acid (18 : 0), oleic acid (18 : 1) and linoleic acid (18 : 2) in hepatic triglyceride increased rapidly following the administration of CCl_4, as was observed with the hepatic content of triglyceride. Nevertheless, no considerable change in the proportions of fatty acids occurred. Although the administration of clofibric acid alone did not significantly change the content of individual fatty acid in hepatic triglyceride, the treatment caused an increase of 18 : 1 and a decrease of 18 : 2 in their proportions. Treatment with clofibric acid + CCl_4 also caused a significant increase in the content, of 16 : 0, 18 : 0, 18 : 1 and 18 : 2 in triglyceride, whereas the increased levels of each fatty acid were evidently lower than those of the rats treated with CCl_4 alone. Moreover, the content of each fatty acid returned to basal levels by 96 h. Compared with the administration of CCl_4 alone, treatment with clofibric acid + CCl_4 decreased the proportion of 18 : 2 and increased the proportions of 16 : 0 and 18 : 1.

Changes in the serum level of triglyceride are shown in Fig. 8. In the rats receiving CCl_4 alone, serum levels of triglyceride decreased markedly up to 6 h after the administration. Thereafter, the triglyceride level turned toward an increase, then reached a level of about 2.5-fold greater than the control at 72 h after the CCl_4 dosing. In the rats treated with clofibric acid + CCl_4, the serum triglyceride level changed in a similar way to the group treated with CCl_4 alone. However, the elevated level in the rats receiving clofibric acid + CCl_4 was significantly lower than that in the groups receiving CCl_4 alone. The serum level of triglyceride of the rats treated with clofibric acid alone was significantly lower than that of the controls.

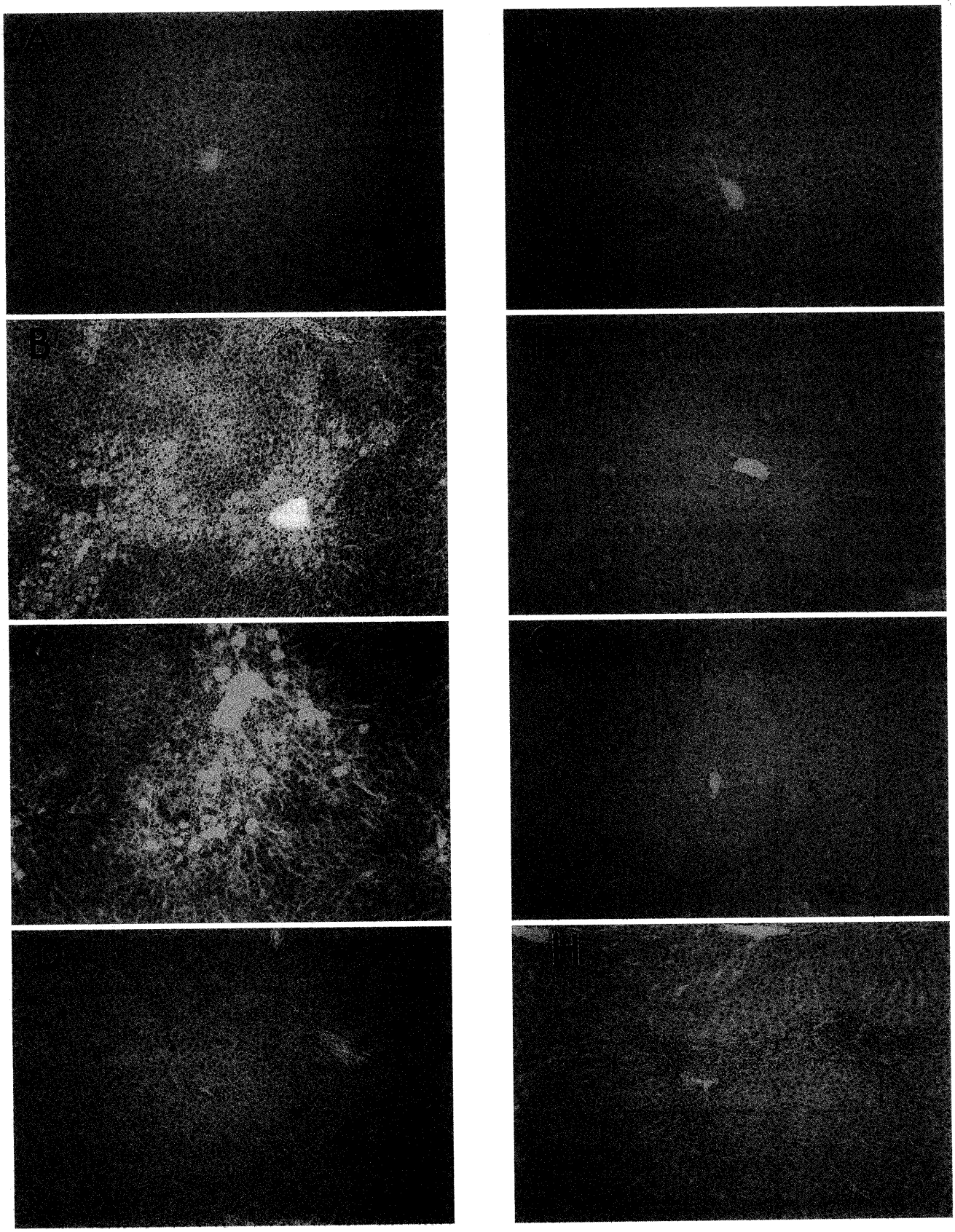

Fig. 6 Representative Photomicrographs of Liver Sections Stained by Oil Red O

The treatment of rats was the same as described in the legend to Fig. 1. A, control ; B, CCl_4 24 h ; C, CCl_4 48 h ; D, CCl_4 96 h ; E, clofibric acid alone ; F, clofibric acid + CCl_4 24 h ; G, clofibric acid + CCl_4 48 h ; H, clofibric acid+CCl_4 96 h. Original magnification 62.5×. In rats receiving CCl_4 alone, neutral fat droplets were detected in the central and peripheral zones at the respective time points. In rats pretreated with clofibric acid, the area of fatty degeneration induced by the administration of CCl_4 was decreased. No neutral fat droplets were observed in either the control group or the group of clofibric acid alone.

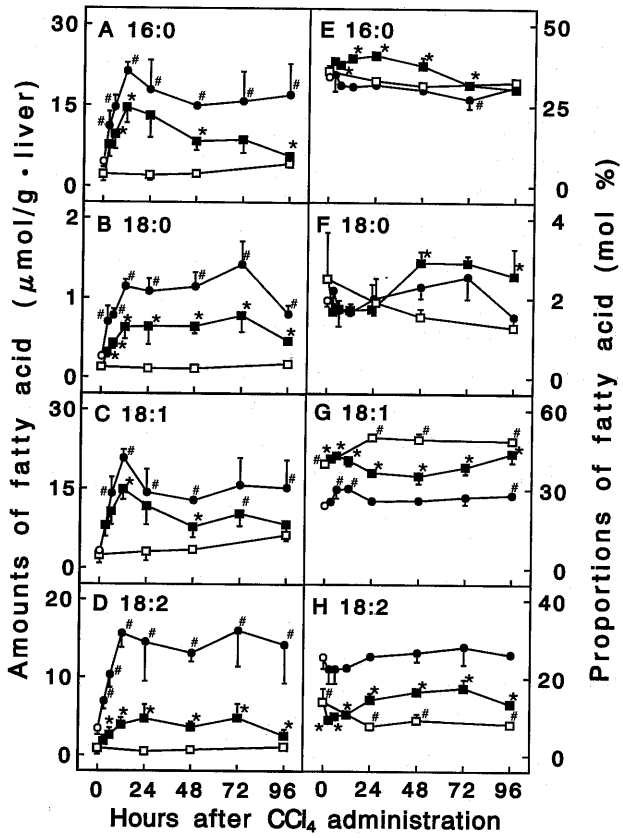

Fig. 7 Effects of Clofibric Acid Pretreatment on Fatty Acid Composition of Triglyceride in the Liver

The treatment of rats was the same as described in the legend to Fig. 1. A, B, C and D, contents of fatty acid; E, F, G and H, proportions of fatty acid. A and E, palmitic acid (16 : 0); B and F, stearic acid (18 : 0); C and G, oleic acid (18 : 1); D and H, linoleic acid (18 : 2). ○, control; □, clofibric acid alone; ●, CCl_4 alone; ■, clofibric acid + CCl_4. Values represent mean±S.D. of 4 rats. # Significantly different from control ($p<0.05$). *Significantly different from the group of CCl_4 alone at the respective time points ($p<0.05$).

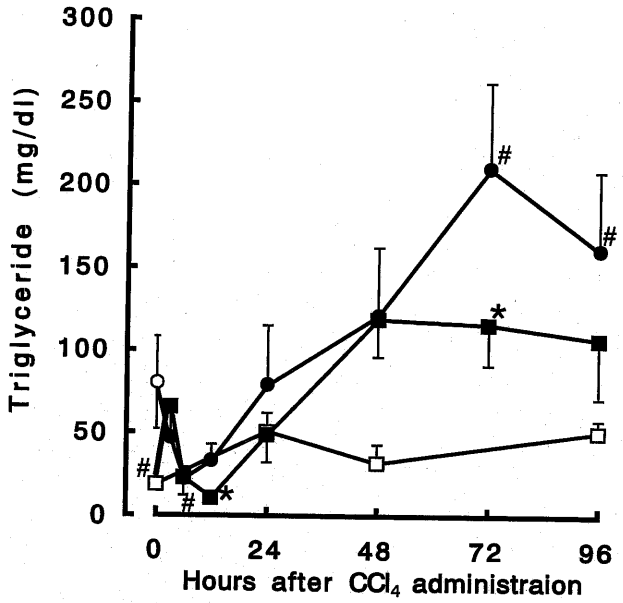

Fig. 8 Effect of Clofibric Acid Pretreatment on Serum Level of Triglyceride

The treatment of rats was the same as described in the legend to Fig. 1. ○, control; □, clofibric acid alone; ●, CCl_4 alone; ■, clofibric acid + CCl_4. Values represent the mean±S.D. of 4 rats. # Significantly different from control ($p<0.05$). *Significantly different from the group of CCl_4 alone at the respective time points ($p<0.05$).

DISCUSSION

In the present study, we investigated whether clofibric acid, a well known hypolipidemic drug and peroxisome proliferator, is preventive against liver necrosis and fatty liver which are induced by CCl_4. Liver necrosis was initiated as early as 12 h after dosing of CCl_4, progressed with time and reached a maximum level at 24 h, in accordance with the findings of Kim *et al.*[17] The liver injury, which is characterized by necrosis and neutrophil infiltration, almost regressed by 48 h after dosing of CCl_4, suggesting that compensatory cell proliferation and hepatocellular regeneration occurred following CCl_4-caused damage to the liver, as suggested previously.[18-19] The dietary pretreatment of rats with clofibric acid significantly suppressed hepatocellular necrosis in the initial stage of the time course until 24 h after the administration of CCl_4. These results are consistent with the previous findings that dietary pretreatment of rats with clofibrate for 3 weeks reduced CCl_4-caused liver necrosis up to 12 h after the treatment with CCl_4,[13] and that repeated intraperitoneal pretreatment of mice with clofibrate prevented hepatocellular necrosis at 24 h after a challenge with CCl_4.[20] Our results illustrate the critical importance of conducting time course studies rather than examining liver injury at just one or two time points and making conclusions. Despite the beneficial effects of this drugs in the initial stage of the toxicity, our results showed that the suppression of CCl_4-induced liver necrosis was no longer observable at 48 h. The injury, which occurred at 48 h after the administration of CCl_4 in combination with clofibric acid, was as severe as that observed at 24 h after dosing of CCl_4 alone. Since the area under the ALT *vs.* time course for the group of clofibric acid + CCl_4 was slightly greater than that for the group of CCl_4 alone, clofibric acid may have delayed the injury but the total damage appears to be the same or even more pronounced. Moreover, our results showed that the restoration from necrosis was retarded in the rats treated with clofibric acid +

CCl_4. These results suggest the possibility that there exist two separate operating mechanisms for clofibric acid in modifying the time course of toxicity for CCl_4. One is the suppression of necrosis induced by CCl_4 in the initial stage; the other is the retardation of repair from necrosis induced by CCl_4 in the late stage. The mechanism by which clofibric acid suppresses necrosis in the initial stage of toxicity is currently being investigated. This mechanism must be separated from that for the retardation of repair from necrosis. The final outcome of acute toxicity is considered to be governed by a balance between the progression of injury and the extent of repair. In the process of tissue repair following exposure to CCl_4, hepatic glycogen is utilized as an energy source, but is not used to increase serum glucose level.[19] In accordance with previous findings, our results showed that hepatic glycogen levels in the initial stage of the time course of toxicity for CCl_4 decreased without increasing the serum level of glucose. It should be noted here that the depletion of hepatic glycogen preceded the definite appearance of necrosis. This is consistent with the concept that intracellular Ca^{2+} rises, which activates phosphorylase a, thereby explaining the glycogen depletion even before the appearance of necrosis.[21] On the other hand, pretreatment of rats with clofibric acid extensively lowered the hepatic level of glycogen and the glycogen which remained in the liver was exhausted rapidly following the administration of CCl_4, much faster than after dosing with CCl_4 alone. The depletion of glycogen preceded the definite outcome of necrosis. Due to this low storage of glycogen, therefore, the liver could not obtain enough energy to repair itself, leading to the retardation of compensatory cell proliferation or hepatocellular regeneration for tissue repair.

Although it has been reported that a number of chemicals, such as quinacrine, calcion and thioridazine, suppress induced liver necrosis, these compounds do not always diminish the revelation of fatty liver induced by CCl_4.[4,22,23] The present study showed that the time course of fat accumulation, as evidenced by hepatic levels of triglyceride and histopathology, was completely different from that of liver necrosis. Namely, no recovery from fatty liver was observed throughout the time course. The pretreatment of rats with clofibric acid significantly suppressed the accumulation of triglyceride in the liver. Moreover, clofibric acid did not suppress fatty liver by stimulating the secretion of lipoprotein into the blood circulation, because pretreatment of rats with clofibric acid did not increase the serum triglyceride level after dosing with CCl_4. These results are consistent with previous findings that clofibric acid suppressed lipoprotein secretion.[15,24] Clofibric acid is known to stimulate the degradation of fatty acid by inducing activities of peroxisomal and mitochondrial β-oxidation,[6-8,24] which may prevent triglyceride accumulation in the liver following the administration of CCl_4. Clofibric acid considerably changed the acyl composition of triglyceride. However, the relationship between the acyl composition and accumulation of triglycerides in the liver remains to be investigated.

We conclude that repeated pretreatments with clofibric acid are preventive in the initial stage, but not in the late stage, of the time course for CCl_4-induced necrosis, and that this drug significantly prevents liver from fat accumulation throughout the time course.

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