Effect of Carbon Tetrachloride Intoxication on the Type Conversion of Xanthine Dehydrogenase into Xanthine Oxidase in Rats

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Abstract ☐ The conversion of xanthine dehydrogenase(type D) into xanthine oxidase(type O) was significantly increased in serum and liver of all CCl_4 treated rats on the necrosis and early cirrhosis stage of liver tissue. In the pretreatment of prednisolone, the ratio of type O per type O+D showed the decreasing tendency in serum, but the significant decrease in liver. *In vitro*, the conversion of liver xanthine oxidase from type D into type O was markedly increased by following preincubation with lysosomal fraction. The type conversion of xanthine oxidase may be caused by proteolytic enzymes in lysosome.

Keywords ☐ Xanthine oxidase(type O), Xanthine dehydrogenase(type D), CCl_4, Prednisolone, Lysosome.

Xanthine oxidase exists in mammals in two forms ; a dehydrogenase(type D), which used NAD^+ as an electron acceptor, and an oxidase(type O) with O_2 as an electron acceptor. The xanthine oxidase of rat liver supernatant is chiefly an NAD^+-dependent dehydrogenase as its native form[1]. Della Corte *et al.*[1] demonstrated *in vitro* that treatment with proteolytic enzymes caused an irreversible conversion of the dehydrogenase to an oxidase from and they suggested that under certain condition of stress the *in vivo* D→O conversion would be of physiological significance[2]. Furthermore, Mec-Cord *et al.*[3] also postulated that during ischemia or hypoxia xanthine dehydrogenase would be converted to the superoxide producing oxidase.

Toncsev *et al.*[4] found that the damage of lysosomal membrane was caused by an acute CCl_4 intoxication *in vitro* and that a significant increase in the permeability and rigidity of the lysosomal membrane was caused by the injection of CCl_4 to rats. It is postulated that some proteolytic enzymes leaked out of the lysosomes in rat liver cells as a result of damage of lysosomal membrane by CCl_4 intoxication, might convert type D into type O.

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In this study, we observed the type conversion of the enzyme in both liver and serum of rats after administration of CCl_4. Concomitantly, the effects of prednisolone on the conversion of xanthine dehydrogenase into oxidase in CCl_4 intoxicated rats and the activity of lysosomal marker enzyme, acid phosphatase were investigated. In addition, the effect of lysosomal fraction in rat liver on the type conversion of the enzyme was measured *in vitro*.

EXPERIMENTAL METHODS

Animals and CCl_4 treatment

Male Sprague-Dowley rats weighing 210 to 23 g fed a standard diet as described in Table I for on month, and were subcutaneously injected 0.15 m of 50% (v/v) CCl_4 per 100g body weight once i three days. Control rats were given olive oil onl Water was provided *ad libitum*. Rats were sa crificed two days after the last injection. The an mals were divided into six groups, each containin 6 rats.

Treatment of prednisolone

The rats were divided into four groups. Group received 0.15 m*l* of 50% (v/v) CCl_4 subcutaneou ly. The second treatment of CCl_4 was done onc more after 18 hr and then the animals were sa

Table I. Composition of experimental diet(g/kg diet).

Ingredients	Quantities
Casein	200
Corn starch	674.36
Corn oil	64.85
Vitamin A and D mixture(a)	10.2
Vitamin E and K mixture(b)	2
Water soluble vitamin mixture(c)	3
Vitamin B_{12} (d)	1
Salt mixture(e)	40
α-Cellulose	20

*4081 Kcal

a) Vitamin A & D mixture: 51,000 unit of A and 5,100 unit of D dissolved in 100 ml of corn oil.
b) Vitamin E & K mixture: 5g of α-tocopherol and 0.2g of menadion dissolved in 200 ml of corn oil.
c) Water soluble Vitamin mixture: contained(mg); choline chloride 2000, Thiamine hydrochloride 10, Riboflavin 20, Nicotinic acid 120, Pyridoxine 10, Ca-pantothenate 100, Biotin 0.05, Folic acid 4, Inositol 500, p-aminobenzoic acid 100.
d) Vitamin B_{12} : 5 mg of Vitamin B_{12} dissolved in 500 ml of distilled water.
e) Salt mixture: contained(g): $CaCO_3$ 300, Potassium phosphate dibasic 322.5, $MgSO_4$ 102, Ca-phosphate monobasic 75, NaCl 167.5, Ferric citrate 27.5, KI 0.8, $ZnCl_2$ 0.25, $CuSO_4 \cdot 5H_2O$ 0.3, $MnSO_4$ 5, Molybdic acid 0.2.

crificed 24 hr later. Group II was injected with 0.75 mg prednisolone per 100g of body weight intramuscularly an hour before administration of CCl_4[5]. Group III was given the same dose of prednisolone only. Control group received olive oil only.

Preparation of crude hepatic xanthine oxidase

The animals were killed by exsanguination of the abdominal aorta. The liver was exhaustively perfused with cold 0.25 M sucrose through the portal vein. The liver of rat was rapidly removed and homogenized in ice-cold 0.25 M sucrose. Homogenates (20% w/v) were centrifuged at 700g for 10 min. The supernatants obtained were again spun at 15,000*g* for 30 min. The postmitochondrial fractions were used for the assay of both xanthine oxidase and acid phosphatase activities. The process of tissue preparation and the assay of enzyme activities with its preparation were rapidly accomplished.

Separation of lysosomal fraction

Separation of lysosome from rat liver was performed by some modification of the method of Toncsev *et al.*[4]. The liver was homogenized in ice-cold 0.25 M sucrose. Homogenate (10% w/v) was centrifuged at 800*g* for 15 min at 4℃. The supernatant was centrifuged at 16,000*g* for 30 min. The pellet was washed twice with sucrose solution and centrifuged. The precipitate was frozen and thawed. The frozen and thawing procedures were repeated and then the precipitate was dialyzed. Identification of lysosomal fraction was confirmed by determining the activity of lysosomal marker enzyme, acid phosphtase in its preparation using the method of Bessey-Lowry-Brock[6].

Enzyme assay

Xanthine oxidase in both liver and serum was determined at 30℃ by the UV method of Della Corte *et al.*[1] or colorimetric method of Yoon[7].

The xanthine oxidase activity was aerobically determined by measuring the rate of uric acid formation using 100 mM xanthine as substrate, while the combined dehydrogenase-oxidase activity (type D+O) was done using the same assay with buffer supplemented with both 100 μM xanthine and 670 μM NAD^+ or 33 μM methylene blue. Enzyme levels are expressed as n mole of uric acid formed/min/mg of protein. The conversion of xanthine dehydrogenase into oxidase type was indicated as the percent ratio of O/D+O (total type).

For *in vitro* test of conversion of liver xanthine dehydrogenase into oxidase, the supernatant for enzyme assay was promptly prepared with a minimum of manipulation from rat liver.

Lysosomal suspension (16.81 mg of protein/ml) was mixed with freshly prepared supernatant of rat liver at the 1 : 1 or 1 : 2 ratio. After the mixture was preincubated at 37℃ for 10 min, the enzyme assay was performed as described above. The protein content was determined by the method of Lowry *et al*[8].

Preparation of the tissue for light microscopy

Liver tissues were fixed in 10% neutral formalin, made on paraffin embedded tissue and hematoxylin-eosin stain[9]. With these preparations, histological studies were carried out.

RESULTS

Light microscopic finding of rats liver treated with CCl_4

The lobules and portal tracts of control liver were intact. From 3 to 6 days of CCl_4 treatment, fatty hepatocytes randomly distributed in central zone of

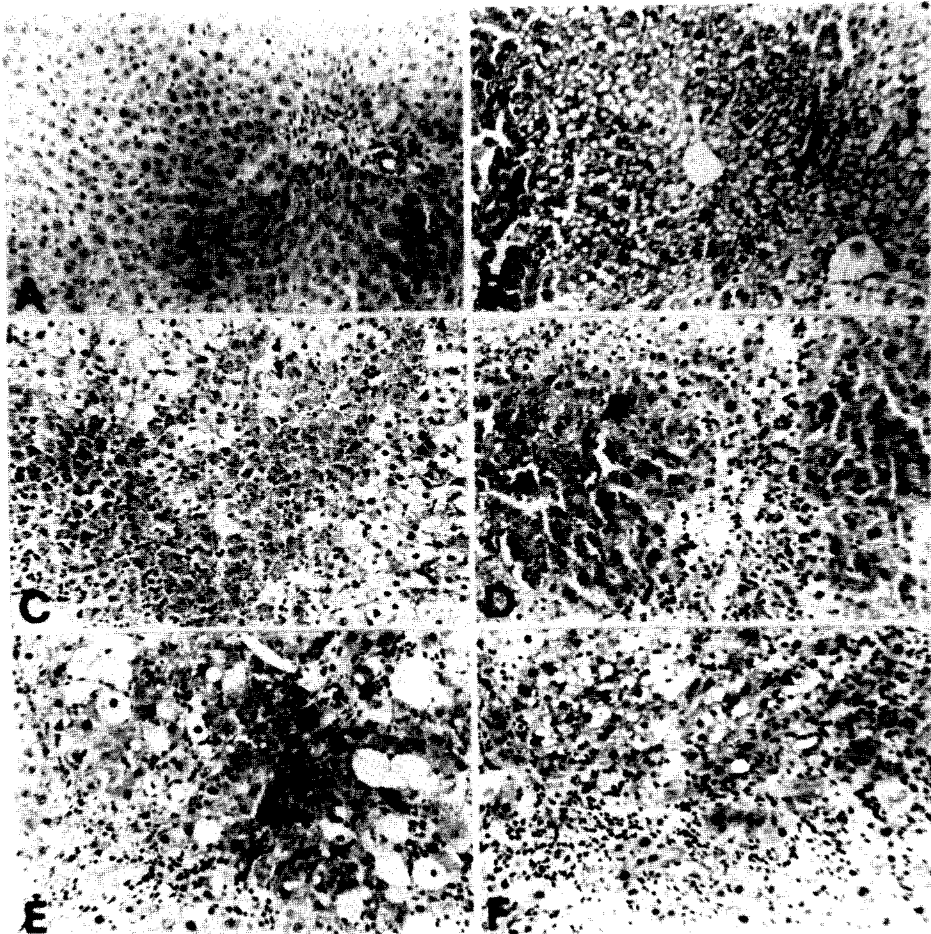


Fig.1. Light microscopic finding of rat liver treated with CCl_4 and normal control group.

A; Control group, the lobules and portal tracts are intact. B to F; CCl_4 treated group. B; Four doses of CCl_4 in 12days, severe fatty change is seen in entire lobules and fibrosis of portal tracts is characteristic with widening of portal tracts(D). C; Six doses in 21 days, zonal necrosis is demonstrated in large areas. E; Fifteen doses in 48 days, Early cirrhosis with fatty change of hepatocytes occurs. F; Thirty doses in 92 days, marked cirrhosis characterized by broad fibrosis and microlobulation with fatty change is well demonstrated.

the hepatic lobules. On 12 days, severe fatty changes appeared in entire lobules. After 21 days of treatment, zonal necrosis was demonstrated in large area. On 48 days, early cirrhosis with fatty change of hepatocytes occurred and inflammatory cells were infiltrated in areas of fibrosis. After 92 days, marked cirrhosis characterized by broad fibrosis and microlobulation with fatty change were well demonstrated (Fig.1).

Effect of CCl_4 on the ratio O/D+O of both serum and liver xanthine oxidase

In both liver and serum xanthine oxidase, the percent ratio of O/D+O was generally increased in all CCl_4 treated group. The O/D+O ratio of serum xanthine oxidase that showed a gradual increase from the first group (3 days) reached the plateau on early cirrhosis stage (48 days). On necrosis stage (21 days), markedly increased about 50% as compared with the control group. The ratio of O/D+O in liver xanthine oxidase significantly increased to about 1.7 fold on necrosis stage (21 days) and 1.5 fold on early cirrhosis stage, while in severe cirrhosis (92 days), the ratio corresponded with the control value(Fig.2).

Effects of prednisolone on the conversion of xanthine dehydrogenase into oxidase and acid phosphatase activities

In the liver homogenate of CCl_4 treated rats

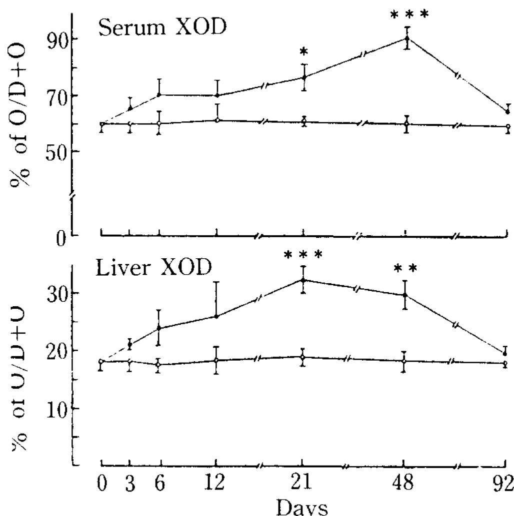
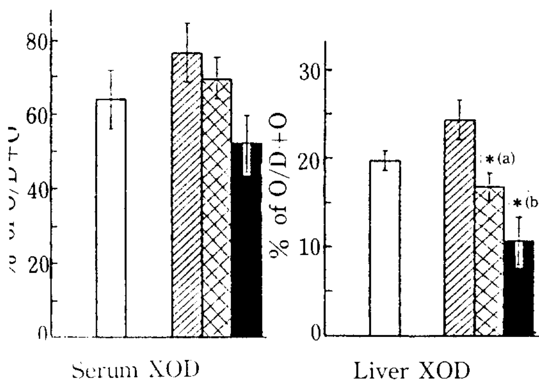


Fig.2. Changes in O/D+O ratio of serum and liver xanthine oxidase from CCl_4 treated rats.

Each point represents the mean±SE of 6 rats. ○－○ ; Control, ●－● ; CCl_4 treatment. * ; $p<0.05$, ** ; $p<0.01$, *** ; $p<0.001$



ig.3. Effect of prednisolone on the conversion of xanthine dehydrogenase(type D) into oxidase(type O) in CCl_4intoxicated rats.

Pretrement of prednisolone, 0.75 mg/100g of body weight was done intramuscularly an hour before administration of CCl_4. The vertical bar expressed as mean±SE with 5 animals in each group. *(a); significantly different from the CCl_4 treated group. ($p<0.05$) ** (b); significantly different from the control group. ($p<0.05$)▭; Control, ▨; CCl_4, ▩; CCl_4+Prednisolone, ■; Prednisolone

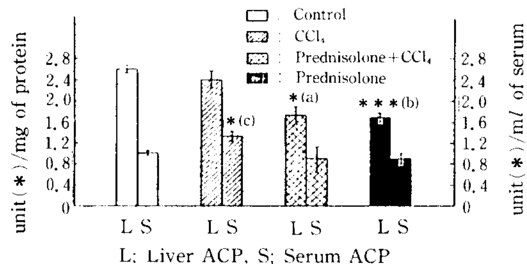


Fig.4. Effect of prednisolone on a lysosomal marker, acid phosphatase activity in the liver and serum of CCl_4 treated rats.

Each point represents mean±SE of 5 rats. (b), (c); Significantly different from the control group, (a); significantly different from the CCl_4 treated group. uint(*) ; Bessey-Lowry unit. * ; $p<0.05$, *** ; $p<0.01$

the ratio of O/D+O in xanthine oxidase and the acid phosphatase activities were significantly decreased by the prior injection of prednisolone. Injection of prednisolone alone to the rats led to the markable decreased ratio of O/D+O in hepatic xanthine oxidase and the significant decreased acid phosphatase activities as compared with control group or CCl_4 treated rats. In the sera of CCl_4 treated rats, the ratio of O/D+O in xanthine oxidase and the acid phosphatase activities also showed decreased tendencies in rats treated with prednisolone alone(Fig.3, 4).

Conversion of rat liver xanthine dehydrogenase into an oxidase by preincubation with lysosomal preparations in vitro

As shown in Fig.5, the ratio of O/D+O in xanthine oxidase increased to 1.6 fold as using mixture of liver homogenate and lysosomal suspension (2 : 1) and to 2.5 fold as doing 1 : 1 mixture. Especially, it was observed that the conversion rate of liver xanthine dehydrogenase into an oxidase was calculated to 45% when using the enzyme material with the mixture of liver homogenate and lysosomal suspension (1 : 1). The lysosome -dependency was paralled with the acid phosphatase activity.

DISCUSSION

It has been reported that most of the activity of xanthine oxidase in rat liver supernatant appears as NAD^+-dependent dehydrogenase if the supernatant is prepared with a minimum of manipulation and is assayed promptly[1]. The enzyme is converted into an oxidase by the treatment of some proteolytic enzymes *in vitro*[1]. MecCord *et al.*[3] postulated that during ischemia or hypoxia xanth-

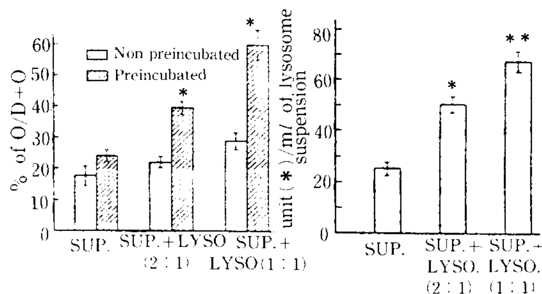


Fig.5. Conversion of rat liver xanthine dehydrogenase(type D) into a xanthine oxidase(type O) by preincubation with lysosomal preparations and the activities of lysosomal marker enzyme, acid phosphatase containing in lysosomal preparation.

The values are mean±SE of 7 experiments. Sup.; rat liver supernatant, Sup.+Lyso.(2 : 1) ; a mixture of supernatant(2 parts) with lysosomal preparation(1 part), Sup.+Lyso. (1 : 1) ; a mixture of supernatant with the same amount of lysosomal preparations. Samples of liver supernatant were previously incubated with the indicated amounts of lysosomal suspensions at 37℃ for 10 min. unit(*) ; Acid phosphatase, Bessey-Lowry unit. * ; $p<0.01$, ** $p<0.001$

ine dehydrogenase is very rapidly converted to its oxidase. Moreover, Tubaro *et al.*[10] could not ruled out the possibility that dehydrogenase is converted into oxidase form of the enzyme in response to infection. In this study, the dehydrogenase of the liver or serum enzyme, *in vivo*, generally brought about by the injection of CCl_4 to rats, with a significant increase on the necrosis and early cirrhosis stage of liver tissue. These experiments demonstrated that the conversion would be obtained by proteolytic enzymes released from lysosome in liver cell of rat. Toncsev *et al.*[4] reported that a significant increase in the permeability and rigidity of the lysosomal membrane in liver of rat was caused by the injection of CCl_4 to an animal.

In order to clarify the effect of a lysosomal enzyme on the conversion of xanthine dehydrogenase into oxidase, it was tested that prednisolone which was known as lysosomal membrane stabilizing agent[11,12] prevent liver from the acute lysosomal membrane damage caused by CCl_4.

After treatment of prednisolone, injection of CCl_4 to rats showed a significant decrease in the ratio of type O per type D of the liver and decreasing tendency of the serum *in vivo*.

Concomitantly the activities of lysosomal enzyme, acid phosphatase showed a significant decrease in liver and decreasing tendency in serum of rats, by injection of prednisolone prior to CCl_4 administration *in vitro*, the ratio of O/D+O was gradually increased by following preincubation with the lysosome suspension prepared from the liver of rats, in proportion to its additional amount.

According to our observations, both *in vitro* and *in vivo*, it is indicated that the conversion of the type D into type O in the CCl_4 intoxicated rat was caused by some proteolytic enzymes in lysosome.

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