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INCREASE IN LIPOPEROXIDES AND PROLYL HYDROXYLASE ACTIVITY IN RAT LIVER FOLLOWING CHRONIC ETHANOL FEEDING

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Abstract—The effect of lipid peroxidation on hepatic collagen synthesis was investigated in male Wistar strain rats after 7 weeks of ethanol feeding. Compared with control rats, the ethanol-fed rats had a significantly higher lipoperoxide content and a significantly lower reduced glutathione content at all times following ethanol treatment. Except for the earliest time (2 days), hepatic prolyl hydroxylase activity was also significantly increased and finally reached up to 214% of the control level. Hepatic hydroxyproline content was slightly increased, but not statistically significant. The lipoperoxides content was significantly correlated with prolyl hydroxylase activity and inversely correlated with reduced glutathione content. These findings were also confirmed in ethanol–pyrazole-treated rats. These results suggest that elevated lipoperoxides mediate an acceleration of collagen synthesis, even at an early stage, in ethanol-induced hepatic injury.

Chronic ethanol consumption is known to produce hepatocellular damage and to alter hepatic collagen metabolism, and finally to produce hepatic fibrosis [1]. Generally, hepatic fibrosis is principally induced by increased synthesis and/or decreased degradation of hepatic collagens. In patients with alcoholic liver disease, as well as animals with experimentally induced alcoholic liver injury, hepatic prolyl hydroxylase activities are increased [2–4]. In addition, serum immunoreactive prolyl hydroxylase increases in alcoholic liver disease [5]. However, the pathogenic mechanism related to the elevation of prolyl hydroxylase in alcoholic liver injury, remains to be elucidated. The induction of collagen synthesis is almost always preceded by inflammation, even in alcoholics [6].

It is generally accepted that active oxygen species such as superoxide ion, hydroxyl radical and hydrogen peroxide are linked to inflammation. These active oxygen species promote lipid peroxidation. In alcoholic liver diseases serum and liver lipoperoxides are increased [7]. Moreover, chemically generated superoxide ion has been shown to stimulate collagen synthesis in cultured lung and liver cells [8, 9].

To elucidate the effect of ethanol on hepatic collagen metabolism, we simultaneously determined prolyl hydroxylase activity, lipoperoxides and reduced glutathione (GSH) levels in rat livers after chronic ethanol feeding.

MATERIALS AND METHODS

Male Wistar strain rats each weighing about 200 g were used. All animals received a nutritionally adequate liquid diet (Kurea Co., Osaka, Japan) as described previously [10, 11]. The control group received 18% of its calories as protein, 35% as fat and 47% as carbohydrate. The ethanol group was fed a similar diet except that ethanol was substituted isocalorically for carbohydrate to provide 36% of the calories. In two additional groups, the pyrazole group was given 2 mmol/kg pyrazole (Nakarai Chemicals Ltd, Kyoto, Japan) daily as in the control diet group, and the ethanol–pyrazole group was prepared similarly to the ethanol diet group [12]. The animals were fed for 7 weeks, and after fasting for 16 hr all rats were killed via aortic exsanguination. The liver was quickly removed, and washed in ice-cold 0.24 M sucrose.

Hepatic prolyl hydroxylase activity was assayed by the method of Hatton *et al.* [13], using ^{3}H-labeled protocollagen as a substrate. Briefly, the protocollagen was prepared from 9-day-old decapitated chick embryos labeled with 3,4-[^{3}H]proline (36.8 Ci/mmol: Amersham Co., Arlington Heights, IL, U.S.A.). The specific activity of the prepared protocollagen was 56×10^4 cpm/mL of protocollagen solution. To 0.2 mL of the substrate solution was added 0.2 mL of 10% liver homogenate supernatant, mixed with 1 mL of 0.1 M Tris–HCl buffer at pH 7.5 containing 1.25 mM L-ascorbic acid, 0.3 mM α-ketoglutarate, 0.08 mM $FeSO_4$, 0.4 mg catalase (Sigma Chemical Co., St Louis, MO, U.S.A.), 2 mg bovine serum albumin and 0.1 mM dithiothreitol and then the mixture was incubated at 37° for 60 min. The enzyme reaction was stopped by the addition of 0.4 mL of 50% TCA. The tritiated water was added to 10 mL of ACS-II (Amersham Co.) and was then counted using a liquid scintillation counter (Packard Tri-Carb 4640). Prolyl hydroxylase activity was expressed as cpm/hr/g protein. Under these conditions, the formation of ^3H_2O rose linearly up to 4 mg of protein in the enzyme solution.

The hepatic lipoperoxides content was measured by the method of Uchiyama *et al.* [14] using the thiobarbituric acid (TBA) test. Briefly, to 0.5 mL of 10% liver homogenate was added 3 mL of 1% H_3PO_4 and 1 mL of 0.6% TBA (Merck, Darmstadt, F.R.G.) aqueous solution, stirred and then the mixture was heated on a boiling-water bath for 45 min. After cooling, 4 mL of *n*-butanol was added, shaken and then

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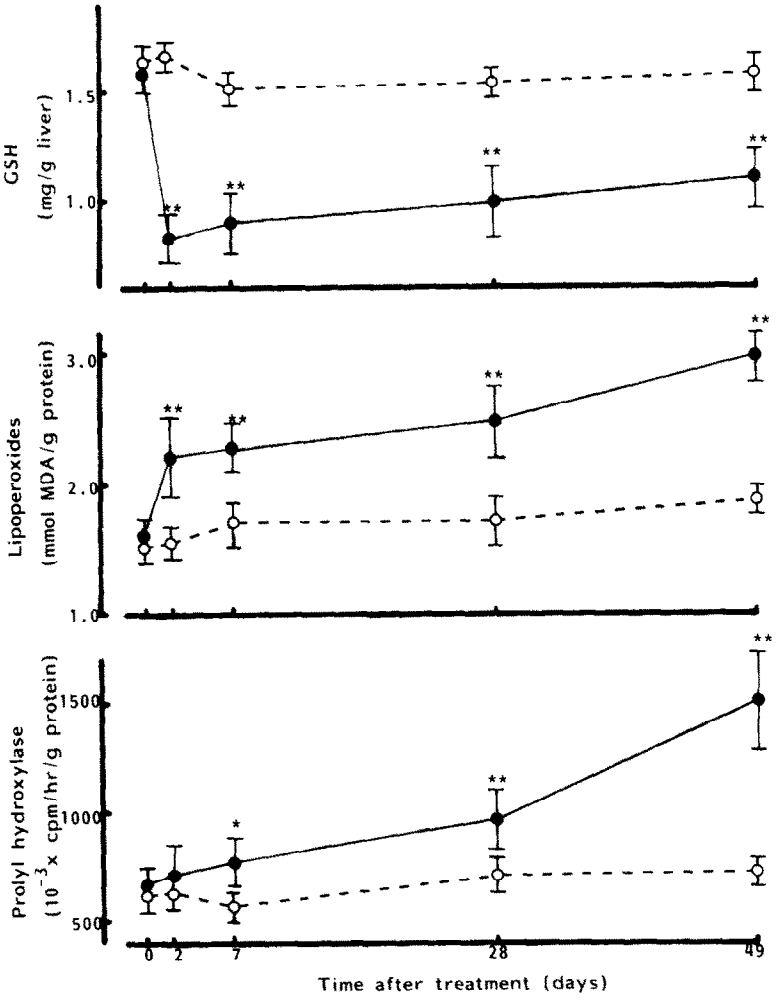

Fig. 1. Time course of the effect of ethanol treatment on GSH, lipoperoxides and prolyl hydroxylase activity in rat liver. Each point is the mean ± SD of determination from three rats. (○) control; (●) ethanol.

the butanol layer was separated and determined at 520 and 535 nm. The difference in optical densities between the two determinations was calculated as the TBA value and expressed as the malondialdehyde (MDA) value.

The hepatic GSH content was determined by the method of Kaplowitz *et al.* [15]. The hepatic hydroxyproline content was assayed by the method of Bergmann *et al.* [16]. The protein content was assayed by the method of Lowry *et al.* [17] using bovine serum albumin as the standard.

RESULTS

As shown in Fig. 1, in a time-course experiment, hepatic GSH content was significantly decreased to 49% of the initial level 2 days after ethanol treatment, but slightly recovered to 75% of the initial level at 7 weeks. The lipoperoxide content was significantly increased to 147% of the initial level in 2 days, and gradually increased during the time of ethanol treatment, finally reaching up to 169% of the initial level at 7 weeks. On the other hand, hepatic prolyl hydroxylase activity was not stimulated 2 days after ethanol treatment, and thereafter it was significantly increased up to 214% of the initial level at 7 weeks.

As shown in Table 1, the mean levels (±SE) of hydroxyproline, lipoperoxides and GSH in normal livers were 79 ± 9 μg/g liver, 1.76 ± 0.16 mmol MDA/g protein and 1.62 ± 0.10 mg/g liver, respectively. Prolyl hydroxylase activity was 666 ± 60 × 10^{-3} cpm/hr/g protein. After 7 weeks of ethanol feeding there was a significant decrease in bodyweight up to 70% of the control level ($P < 0.01$) and a slight increase in liver weight, expressed as a percentage of body weight. In the ethanol group, hepatic prolyl hydroxylase activity was significantly increased to 190% of the control value ($P < 0.01$), but the hepatic hydroxyproline content was not altered. The hepatic lipoperoxide content significantly increased to 173% of the control value, but the hepatic GSH content significantly decreased to 73% of the control value ($P < 0.01$ for both).

Ethanol given with pyrazole also resulted in a

Table 1. Effect of ethanol feeding with and without pyrazole on body weight, hydroxyproline content, GSH, lipoperoxides and prolyl hydroxylase activity

Group	Control	Ethanol	Pyrazole	Ethanol–pyrazole
No. of rats	12	12	5	5
Body weight (g)	320 ± 13	233 ± 15*	264 ± 8	209 ± 12†
Liver weight (g/100 g body wt)	3.0 ± 0.1	3.4 ± 0.1	2.8 ± 0.1	3.0 ± 0.2
Protein (mg/g liver)	163 ± 5	174 ± 3	185 ± 4	169 ± 7
Hydroxyproline (μg/g liver)	79 ± 9	109 ± 11	87 ± 13	105 ± 15
GSH (mg/g liver)	1.62 ± 0.10	1.19 ± 0.08*	1.70 ± 0.07	1.30 ± 0.15
Lipoperoxides (mmol MDA/g protein)	1.76 ± 0.16	3.04 ± 0.14*	1.65 ± 0.09	2.22 ± 0.13‡
Prolyl hydroxylase (10^{-3} × cpm/hr/g protein)	666 ± 60	1264 ± 94*	905 ± 53	1182 ± 35‡

Mean ± SE, * $P < 0.01$ compared to control
† $P < 0.01$, ‡ $P < 0.05$ compared to pyrazole.

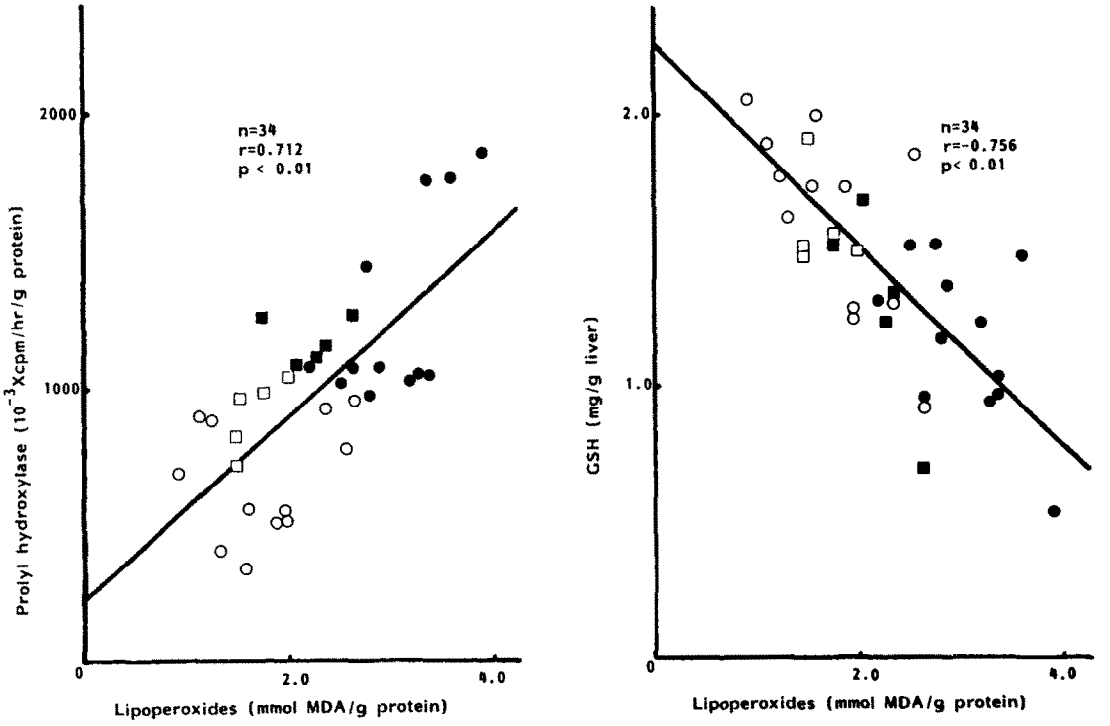

Fig. 2. Correlation of hepatic lipoperoxides content to prolyl hydroxylase activity and GSH content. (○) Control; (●) ethanol; (□) pyrazole; (■) ethanol–pyrazole.

significant decrease in bodyweight to 79% of the control–pyrazole group ($P < 0.01$). In the ethanol-pyrazole group, the hepatic hydroxyproline content did not change, but prolyl hydroxylase activity increased to 131% of the control–pyrazole group ($P < 0.01$). The hepatic lipoperoxide content was significantly increased to 135% of the control–pyrazole group ($P < 0.05$), but the hepatic GSH content decreased slightly to 76% of the control–pyrazole group. There were no differences in these parameters between the ethanol and ethanol–pyrazole groups.

As shown in Fig. 2, the hepatic lipoperoxide level correlated well with hepatic prolyl hydroxylase activity and was inversely related to hepatic GSH content, both of which were statistically significant differences ($P < 0.01$). However, hepatic prolyl hydroxylase activity was not correlated with hepatic hydroxyproline content.

DISCUSSION

It has been reported that in the early stage of ethanol feeding collagen metabolism is accelerated in rat liver, but biochemical collagen content does not change [11, 18, 19]. We previously reported that at 4 and 7 weeks after ethanol feeding, hepatic collagen synthesis and lysyl oxidase activity were significantly increased, however hepatic collagenolytic cathepsin activity was also significantly increased [11, 19, 20]. During these times, hepatic collagenase activity was not altered, so our findings showed that ethanol feeding caused increased collagen synthesis,

which could be efficiently eliminated intracellularly by collagen peptidase and/or collagenolytic cathepsin, without leading to fibrosis [11, 19]. In the present study, the hepatic hydroxyproline content was not changed, but hepatic prolyl hydroxylase activity was significantly increased in ethanol and ethanol–pyrazole groups. These results suggest that ethanol-induced liver damage stimulates collagen synthesis and may also promote the degradation of collagen, which have been described previously [19, 20].

It has been reported that chronic ethanol feeding causes hepatic GSH depletion and initiates lipid peroxidation [7, 21]. In this experiment, compared to the control group, the hepatic GSH content was significantly decreased and the hepatic lipoperoxide content was significantly increased in the ethanol group, and they were inversely correlated with each other. Because GSH seems to act as a scavenger for toxic chemical agents, including acetaldehyde, these results also support the extent of peroxide generation in ethanol-induced liver injury. Also, thiol-compounds such as GSH prevent ethanol-induced liver injury through the elimination of acetaldehyde [22]. Therefore, increased peroxides cause liver damage via lipid peroxidation of the cell membrane and may initiate collagen synthesis. Indeed, *in vivo* studies show that prolyl hydroxylase activity and collagen synthesis are greatly stimulated in hepatocytes exposed to superoxide [9]. In the present study, the hepatic lipoperoxide content was significantly correlated with hepatic prolyl hydroxylase activity.

In a time-course experiment hepatic GSH content was significantly decreased in the early days of ethanol feeding (2 days), but slightly recovered to the initial level at 7 weeks. By contrast, hepatic lipoperoxides content was significantly higher than the initial level at 2 days and gradually increased during the time of ethanol feeding. These results also show that hepatic GSH depletion initiates the increase of hepatic lipoperoxides and the recovery of GSH content takes a little effect to scavenge the peroxides. The increase of prolyl hydroxylase activity was paralleled with that of hepatic lipoperoxide content except in the early days. This time lag in the appearance of prolyl hydroxylase activity suggests that macromoleculars such as enzyme protein synthesis may be involved.

The mechanisms of GSH depletion and/or lipoperoxide generation which mediate collagen synthesis in the liver are not yet known. One explanation is as follows: free radicals are involved in the hydroxylation of proline, probably via the reduction of ferri-enzyme to ferro-enzyme [23]. Therefore, these reactive oxygen species, such as superoxide and lipoperoxides, may play important roles in lung and liver fibrosis [24, 25].

In ethanol–pyrazole-treated rats, compared to the pyrazole group, hepatic lipoperoxide content and prolyl hydroxylase activity were also increased. Though pyrazole is an inhibitor of alcohol dehydrogenase (ADH), increased lipoperoxides may be generated through a non-ADH pathway. Indeed, non-ADH pathways in ethanol metabolism show greater acetaldehyde and lipoperoxide generation than the ADH-pathway [26, 27]. Thus, these results that, unlike the mechanism, increased lipoperoxides in chronic ethanol-fed livers may be the key to collagen synthesis stimulation.

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