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FREE AND SMALL PEPTIDE-BOUND [^{14}C]HYDROXYPROLINE SYNTHESIS *IN VITRO* IN ETHANOL-INDUCED HEPATIC INJURY IN THE RAT LIVER

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Abstract—To clarify the significance of free and small peptide-bound hydroxyproline synthesis in ethanol-induced liver injury, we measured the *in vitro* synthesis of [^{14}C]hydroxyproline in the 67% ethanol-soluble fraction in rat liver slices, together with hepatic protein-bound [^{14}C]hydroxyproline synthesis. The synthesis of free and small peptide-bound [^{14}C]hydroxyproline was 11.1 ± 2.0 dpm × 10^{-4}/g liver/3 hr and the synthesis of protein-bound [^{14}C]hydroxyproline was 10.1 ± 3.3 dpm × 10^{-4}/g liver/3 hr in control rat liver. In the ethanol-fed rat liver, the synthesis of free and small peptide-bound [^{14}C]hydroxyproline significantly increased 1.5-fold and the synthesis of protein-bound [^{14}C]hydroxyproline significantly increased 1.6-fold, while the hepatic collagen content did not change. There was a significant correlation between free and small peptide-bound [^{14}C]hydroxyproline synthesis and protein-bound [^{14}C]hydroxyproline synthesis. These results suggest that free and small peptide-bound hydroxyproline synthesis plays an important role in regulating the content of hepatic collagens.

We previously reported that in CCl_4-induced hepatic fibrosis hepatic collagen synthesis increases, but free and small peptide-bound hydroxyproline (Hyp) synthesis decreases [1,2], indicating that the combination of an increase in hepatic collagen synthesis and a decrease in free and small peptide-bound Hyp synthesis contributes to the rapid accumulation of collagens in chronic CCl_4-induced hepatic fibrosis. In ethanol-induced hepatic injury, hepatic collagen synthesis is also increased. For example, the activity of hepatic prolyl hydroxylase, the incorporation of labeled proline into collagen by liver slices, and the type I procollagen mRNA content are increased after ethanol feeding [3–5], although the hepatic collagen content does not change [4]. However, the significance of free and small peptide-bound Hyp synthesis has been obscure in alcoholic liver injury. In the present study, therefore, we measured the *in vitro* synthesis of free and small peptide-bound [^{14}C]Hyp in rat liver after 7 weeks of ethanol feeding.

MATERIALS AND METHODS

Animals. Fourteen male Wistar strain rats, weighing from 170 to 200 g, were divided into two groups, and for 7 weeks they were pair-fed with a liquid diet in which either ethanol or carbohydrate made up 36% of the calories, as described previously [6]. After fasting overnight, the rats were killed under ether anesthesia by exsanguination from the aorta. The livers were excised and subjected to biochemical analysis.

Synthesis of free and small peptide-bound and protein-bound Hyp. The synthesis of free and small peptide-bound and protein-bound Hyp was measured by the modified method of Diegelmann *et al.* [7] as described previously by us [2]. Liver slices (0.5 g) were incubated with 5 μCi L-[^{14}C]proline (> 250 mCi/mmol; Amersham) in 10 mL of Earle's balanced salt solution containing 0.2 mM L-proline, 25 mM ferrous sulfate and 0.5 mM ascorbic acid. Incubation was done under an atmosphere of 95% O_2 and 5% CO_2 in a shaking bath at 37° for 3 hr and the reaction was terminated by the addition of absolute ethanol to give a final concentration of 67%. The sample was homogenized and centrifuged to separate it into a 67% ethanol-soluble fraction and a 67% ethanol-insoluble fraction. The latter was washed with 20 mL of 67% ethanol, twice, and each supernatant fraction was combined with the first 67% ethanol-soluble fraction and then evaporated. The dried ethanol-soluble fraction and the ethanol-insoluble fraction were hydrolyzed in 10 mL of 6 N HCl for 24 hr at 105°, dried by evaporation to remove the HCl, and dissolved in 20 mL of distilled water. Hydrolysates were neutralized with 1 N NaOH and lyophilized. Ethanol-soluble fractions were lyophilized and then dissolved in 4 mL of 1 M ammonium acetate, pH 6.8, and applied to a column (1 × 30 cm) containing a water-washed Dowex 1-X8 200–400 mesh resin in acetate form. The column was eluted with water, and the initial effluent fractions (50 mL) containing Hyp were pooled and lyophilized.

All lyophilized Hyp-containing material from the ethanol-soluble or the ethanol-insoluble fractions was dissolved in 2.0 mL of 0.25 N HCl and applied with 0.1 mL of a mixture of 10 mM L-proline and 10 mM L-hydroxyproline to a 1 × 30 cm Dowex 50W-X8 ion-exchange column and eluted with 0.25 N HCl. Effluent fractions of 4.0 mL were collected from fractions 60 to 80. The radioactivity of each aliquot (1.0 mL) was measured in 10 mL of Aqueous Counting Scintillant (ACS II; Amersham) using a liquid scintillation counter (Packard Tricarb 4640).

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Table 1. Effect of chronic ethanol administration on hepatic hydroxyproline content: [^{14}C]Hydroxyproline synthesis in rat liver and hepatic collagenase and collagenolytic cathepsin activity

	Control (N = 7)	Ethanol-fed (N = 7)
Body weight (g)	407 ± 20	285 ± 19*
Liver weight (g)	13.2 ± 1.8	11.7 ± 1.9
(% Body wt)	3.2 ± 0.4	4.1 ± 0.5*
Hepatic triglyceride (mg/g liver)	23 ± 8	47 ± 17†
Hydroxyproline		
Content (mg/liver)	1.80 ± 0.20	1.64 ± 0.45
(μg/g liver)	139 ± 26	140 ± 29
Synthesis (dpm × 10^{-4}/g liver)		
Total [^{14}C]Hyp	21.2 ± 4.5	32.5 ± 7.5*
Protein-bound [^{14}C]Hyp	10.1 ± 3.3	15.9 ± 3.6*
Free and small peptide-bound [^{14}C]Hyp	11.1 ± 2.0	16.7 ± 4.3†
Collagenase (dpm × 10^{-3}/18 hr/g liver)	2.2 ± 0.3	1.8 ± 0.7
Collagenolytic cathepsin (dpm × 10^{-3}/18 hr/g liver)	11.6 ± 0.9	11.8 ± 0.3

Vales are means ± SD.
* $P < 0.01$ compared to control
† $P < 0.05$ compared to control.

Free and small peptide-bound Hyp synthesis was expressed as the amount of [^{14}C]Hyp in the ethanol-soluble fraction, and protein-bound Hyp synthesis as the amount of [^{14}C]Hyp in the 67% ethanol-in soluble fraction. In our previous study [8], the ethanol-soluble fractions before and after hydrolysis with 6 N HCl for 24 hr at 105° were applied on a Dowex 50W-X8 column, and the ratio of free [14C]Hyp and small peptide-bound [^{14}C]Hyp in the control and ethanol rats was determined. We found that free [^{14}C]Hyp was approximately 76% and small peptide-bound [^{14}C]Hyp was 24%, there was no difference in ethanol-treated rats. Therefore, in this study, we did not separate the free and small peptide-bound [^{14}C]Hyp. In addition, the hepatic Hyp content, hepatic triglyceride content, hepatic collagenase activity and hepatic collagenolytic cathepsin activity were determined as described previously [4].

RESULTS

As compared with the controls, ethanol-fed rats had lower mean body weights in spite of pair-feeding, but relatively heavier liver weights, expressed as a percentage of body weight (Table 1). Although the hepatic triglyceride content increased, hepatic Hyp content did not change with ethanol feeding. The protein-bound [^{14}C]Hyp synthesis increased significantly (1.6-fold) in ethanol-fed rats as compared with controls. Free and small peptide-bound [^{14}C]Hyp synthesis in the ethanol-fed rat liver increased to about 1.5-fold of the control liver (Table 1). Free and small peptide-bound [^{14}C]Hyp synthesis correlated significantly with protein-bound [^{14}C]Hyp synthesis in the control and ethanol-fed rats (Fig. 1). On the other hand, hepatic collagenase and collagenolytic cathepsin activities did not change under these conditions.

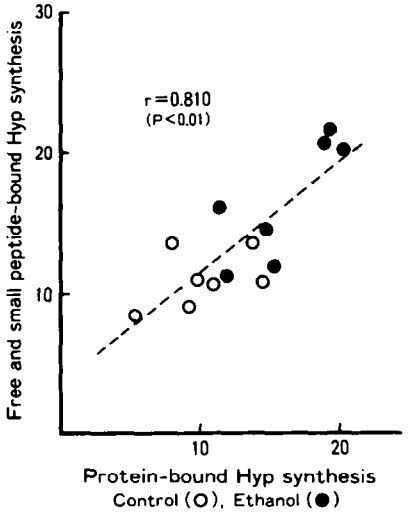

Fig. 1. Correlation between free and small peptide-bound [^{14}C]Hyp synthesis and protein-bound [^{14}C]Hyp synthesis. Units on both ordinate and abscissa are dpm × 10^{-4}/g liver.

DISCUSSION

It is well known that collagen-producing cells, including hepatocytes, produce free and small peptide-bound Hyp under culture conditions [7–11]. Free and small peptide-bound Hyp is not made from degradation products of the collagen secreted into the extracellular space and is considered to be produced by the intracellular degradation of newly synthesized collagen before its secretion. Recent work suggests that the intracellular degradation of newly synthesized collagen serves two distinct roles in modulating collagen production [12]. First, intracellular collagen degradation provides a mechanism

for the destruction of defective molecules before their secretion, thus preventing the incorporation of defective molecules into the extracellular matrix. For example, incubation of cells with analogues of proline such as *cis*-hydroxyproline [13, 14] or azetidine [10] results in an increase in the intracellular degradation of newly synthesized collagen. Second, intracellular degradation provides one means to regulate the amount of collagen and the relative amounts of collagen types produced by the cell. For example, elevated levels of cyclic-AMP result in a decrease in collagen secretion and in an increase in the intracellular degradation in firboblast cultures [15].

With regard to pathogenic roles in the degradation of newly syntehsized collagen in hepatic fibrosis, we previously reported that free and small peptide-bound Hyp synthesis decreases inversely with hepatic collagen content in CCL_4-induced fibrotic liver [2]. In the present study concerning ethanol-induced hepatic injury, we confirmed that the hepatic Hyp content does not change, and found that free and small peptide-bound Hyp synthesis increased significantly in response to hepatic collagen synthesis. It has been reported that chronic ethanol administration causes no hepatic fibrosis, but hepatic collagens are increased [3]. These results are thought to be due to the difference in the extent and the stage of hepatic fibrosis. In fact, in bleomycin-induced pulmonary fibrosis of hamsters, free and small peptide-bound Hyp synthesis has been shown to increase significantly 8 days after bleomycin administration, but it was not significantly different from control values at subsequent examination [16].

The reason for the lack of hepatic fibrosis development in rats resulting from the chronic ethanol feeding could be explained by an exaggerated collagen degradation system. For example, chronic ethanol feeding has resulted in increases in the urinary excretion of Hyp [17], hepatic collagenase activity [18] and hepatic collagenolytic cathepsin activity [3] in rats. In the present study, however, hepatic collagenase activity and hepatic collagenolytic cathepsin activity were not changed, but free and small peptide-bound Hyp synthesis was increased. Therefore, it is conceivable that in ethanol-induced hepatic injury newly synthesized collagen is efficiently removed by an intracellular collagen degrading system which is different from collagenase and collagenolytic cathepsin. In contrast to advanced hepatic fibrosis as seen in CCl_4 injury, the intracellular degradation of collagen increases in the early stages of hepatic fibrosis, supporting the conclusions that the degradation system of newly synthesized collagen plays an important role in the development of hepatic fibrosis.

REFERENCES

1. Hirayama C, Morotomi I and Hiroshige K, Quantitative and metabolic changes of hepatic collagens in rats after carbon tetrachloride poisoning. *Biochem J* **118**: 229–232, 1970.
2. Koda M, Murawaki Y and Hirayama C, Free and small peptide-bound [^{14}C]hydroxyproline synthesis in rat liver *in vitro* in CCl_4-induced hepatic fibrosis. *Biochem Biophys Res Commun* **151**: 1128–1135, 1988.
3. Feinman L and Lieber CS, Hepatic collagen metabolism. Effect of alcohol consumption in rats and baboons. *Science* **176**: 795, 1972.
4. Kato S, Murawaki Y and Hirayama C, Effects of ethanol feeding on hepatic collagen synthesis and degradation in rats. *Res Commun Chem Pathol Pharmacol* **47**: 163–180, 1985.
5. Zern MA, Leo MA, Giambrone M-A and Lieber CS, Increased type I procollagen mRNA levels and *in vitro* protein synthesis in the baboon model of chronic alcoholic liver disease. *Gastroenterology* **89**: 1123–1131, 1985.
6. Shiota G. Murawaki Y and Hirayama C, Hepatic collagen content and lysyl oxidase activity in rats fed a low protein–ethanol diet. *Res Commun Chem Pathol Pharmacol* **58**: 115–127, 1987.
7. Diegelmann RF, Cohen IK and Guzelian PS, Rapid degradation of newly synthesized collagen by primary cultures of adult rat hepatocytes. *Biochem Biophys Res Commun* **97**: 819–823, 1980.
8. Murawaki Y, Koda M, Shiota G and Hirayama C, Hepatic injury and hepatic collagen metabolism: Hepatic hydroxyproline synthesis in low molecular weight fraction in ethanol- and CCL_4-induced hepatic injury (Japanese). In: *Progress in Study of Liver Disease III*. pp. 130–140. Medical Review, Tokyo, 1987.
9. Bienkowski RS, Cowan MJ, McDonald JA and Crystal RG, Degradation of newly synthesized collagen. *J Biol Chem* **253**: 4356–4363, 1978.
10. Bienkowski RS, Baum BJ and Crystal RG, Fibroblasts degrade newly synthesized collagen within the cell before secretion. *Nature* **276**: 413–416, 1978.
11. Bienkowski RS, Intracellular degradation of newly synthesized collagen. *Coll Relat Res* **4**: 399–412, 1984.
12. Rennard SI, Stier LE and Crystal RG, Intracellular degradation of newly synthesized collagen. *J Invest Dermatol* **79**: 77–82, 1982.
13. Berg RA, Schartz ML and Crystal RG, Regulation of the production of secretary proteins: Intracellular degradation of newly synthesized "defective" collagen. *Proc Natl Acad Sci USA* **77**: 4746–4750, 1980.
14. Neblock DS and Berg RA, The effect of *cis*-4-hydroxy-L-proline on intracellular degradation of newly synthesized collagen by freshly isolated chick tendon fibroblasts. *Connect Tissue Res* **10**: 297–301, 1982.
15. Baum BJ, Moss J, Breul SD, Berg RA and Crystal RG, Effect of cyclic AMP on the intracellular degradation of newly synthesized collagen. *J Biol Chem* **255**: 2843–2847, 1980.
16. Clark JG, Overton JE, Marino BA, Uitto J and Starcher BC, Collagen biosynthesis in bleomycino-induced pulmonary fibrosis in hamsters. *J Lab Clin Med* **96**: 945–953, 1980.
17. Mezey E, Potter JJ, Slusser RJ and Abdi W, Changes in hepatic collagen metabolism in rats produced by chronic ethanol feeding. *Lab Invest* **36**: 206–214, 1977.
18. Maruyama K, Feinman L, Fainsilber Z, Nakano M, Okazaki I and Liber CS, Mammalian collagenase increases in early alcoholic liver disease and decreases with cirrhosis. *Life Sci* **30**: 1379–1384, 1982.