16, 16 DIMETHYL PROSTAGLANDIN E$_2$ DECREASES THE FORMATION OF COLLAGEN IN FIBROTIC RAT LIVER SLICES[1]

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ABSTRACT

The effect of 16,16 dimethyl prostaglandin E$_2$ (DMPG) on fibrogenesis was studied in slices from normal and fibrotic rat liver. Rats received a cirrhogenic diet for seven months; supplemented controls received a diet with the deficient nutrients restored. Slices from fibrotic livers incorporated more ^{14}C-proline and produced more ^{14}C-hydroxyproline in TCA precipitable proteins than slices from control livers. DMPG (10^{-10}M) decreased the incorporation of labeled proline and the synthesis of labeled hydroxyproline in slices from fibrotic livers to the same extent, suggesting that DMPG did not affect the hydroxylation of proline per se. The magnitude of the DMPG induced decrease in labeled proline incorporation correlated with the hydroxyproline content in the liver (i.e. with increasing fibrosis there was a greater effect of DMPG; while in control rat liver slices, DMPG had no effect). DMPG did not change the size of the proline pool, its specific activity, or the activity of proline oxidase. We conclude that under these conditions of enhanced fibrogenesis, DMPG decreases the formation of collagen *in vitro*, possibly by lowering the incorporation of proline into collagen precursors. This may explain, at least in part, the inhibition of fibrogenesis by DMPG *in vivo*.

INTRODUCTION

Prostaglandins have been shown *in vivo* to decrease acute necrosis caused by carbon tetrachloride (1,2), aflatoxins (3), galactosamine (4) and the virus of murine hepatitis (5). In long term studies, rats fed a deficient diet (6) and rabbits fed cheneodeoxycholic acid (7) developed hepatic fibrosis which was partially prevented by concomitant DMPG treatment.

Although the acute studies suggest that DMPG may partially inhibit hepatic fibrosis by prevention of the initial necrotic lesion, *in vitro* data suggests a direct effect on collagen metabolism may also be involved. For example, it has been shown in human skin fibroblasts and in pulmonary fibrosis in hamsters that prostaglandins increase the rate of degradation of intracellular collagen and concomitantly decrease the rate of formation of extracellular collagen (8,9). Furthermore, when isolated rat liver macrophages were stimulated by bacterial lipopolysaccharides, the

addition of prostaglandin E_2 or prostaglandin E_1, increased the activity of extracellular neutral collagenase (10).

In view of this data, we tested whether DMPG directly affects collagen metabolism in a model of dietary induced hepatic fibrosis in vitro.

MATERIALS AND METHODS

Reagents
16,16-dimethyl prostaglandin E_2 was synthesized at the Upjohn Co., Kalamazoo, MI and stored at -80°C in absolute ethanol prior to use. Uniformly labeled L-[^{14}C] proline (268.1 mCi/mmol), was obtained from New England Nuclear, Boston, Mass. Earle's balanced salt solution (EBSS), was obtained from Gibco, Grand Island, N.Y. L-proline, hydroxy-L-proline, penicillin, streptomycin, chloramine T, O-aminobenzaldhyde, cytochrome-C, and cyclohexamide, were obtained from Sigma Chemical Co., St. Louis, MO. HFP-20 liquid scintillation cocktail was obtained from Research Products.International, Mount Prospect, IL. All other reagents were from commercial sources and of the highest purity available.

Animals
Male Sprague Dawley rats bred at the Upjohn Co. and initially weighing approximately 100 gms were used. The rats had unrestricted access for seven months to a nutritionally deficient diet (ICN Nutritional Biochemicals diet # 901387 which was high in fat and deficient in choline, and further modified to exclude vitamin B_{12}, folic acid and methionine), or the same diet with the deficient nutrients added (supplemented control diet).

Preparation and Incubation of Liver Slices
Rats were sacrificed by decapitation and the livers perfused in situ with ice cold physiological saline saturated with oxygen. Slices (about 1 mm thick) were made with a hand-held razor blade and two randomized portions each weighing about 1 gram, were incubated in 5.0 ml of Earle's Balanced Salt Solution containing 0.48mm L-proline (11), 10 µCi ^{14}C-Proline, 50 units/ml penicillin and 50 µg/ml streptomycin. DMPG (200 mg/ml stock solution) was diluted with absolute ethanol and 9.5 µl of the diluted solution was added to the incubation medium so that the final concentration of DMPG was 10^{-10} M. Preliminary experiments over the range of 10^{-6} to 10^{-14}M indicates that the DMPG effects were maximal at 10^{-10}M. Flasks containing vehicle alone were prepared by adding 9.5 µl of absolute ethanol to the incubation mixture. Blanks were made 10 mm in cyclohexamide. The flasks were shaken for four hours in a water bath at 37°C, at 70 cycles/min, under a constant flow of 95% O_2 and 5% CO_2.

Analysis of Liver Slices
After incubation, the liver slices were washed repeatedly in ice cold physiological saline until no counts were detected in the wash. The slices (150 mg/ml) were then homogenized in 53mM Phosphate buffer, pH 7.6 with a Potter Homogenizer in an ice bath using a motor driven teflon pestle. Aliquots of the homogenate were set aside for the determination of total proteins (12) and prompt assay of proline oxidase (13).

To a 4.0 ml aliquot of the homogenate, 400 µl of 50% trichloroacetic acid (TCA) was added and the solution kept on ice for 30 minutes. The solution was then centrifuged at 2500 x g for 30 minutes at 4°C to collect the precipitated proteins. The supernatant was removed and the volume measured. A 2.0 ml aliquot of the supernatant was neutralized with 35 µl of 10 M NaOH. The specific activity of proline in the supernatant was measured in a toluene extract by the method of Rojkind and Gonsalez (14). The radioactive samples were added to 10 ml of HFP-20 scintillation cocktail and counted in a Beckman LS 3801 liquid scintillation counter. The precipitated pellet was washed three times with 5% TCA and the pellet resuspended in 6 M HCl to the original volume. The tubes were sealed with teflon tape, capped, and hydrolyzed at 115°C for 18 hours. The concentration of proline and the radioactivity in proline and hydroxyproline was then measured (14). Total hydroxyproline was measured as previously described (6).

Statistical Analysis
Data from the two one gram portions from each liver were averaged and the mean used for statistical analysis. For analysis of the effect of DMPG the Wilcoxon Signed Rank Test was used. All other differences were evaluated by the Student's Test. The correlations expressed in the text were determined by linear regression.

RESULTS

Rats fed the deficient diet for 7 months developed severe fibrosis (Figure 1), and the concentration of hydroxyproline in the pellet from these rats increased substantially compared with the supplemented controls ($p < 0.01$, **Table 1**). The fibrotic liver slices incorporated approximately 5.5 times the amount of ^{14}C-proline and produced approximately 4.5 times the amount of ^{14}C-hydroxyproline in TCA precipitable proteins compared to normal livers. Incubation of liver slices with 16,16-dimethyl prostaglandin E_2 (10^{-10} M) significantly decreased the appearance of ^{14}C-Proline compared to vehicle ($p < 0.01$) in the TCA precipitate of fibrotic liver slices (Figure 2). No prostaglandin effect was observed in slices from control livers (Figure 2). In the presence of cyclohexamide, very little radioactivity was incorporated into proteins.

The effect of DMPG on the incorporation of label into hydroxyproline is shown in Figure 3. Again there was a significant decrease in the incorporation of the label in fibrotic liver slices ($p < 0.01$) and no effect was observed in livers from the animals receiving the control diet. As shown in Figure 4, the magnitude of the DMPG induced decrease in proline incorporation correlated well with the magnitude of decreased incorporation of the label into hydroxyproline. This figure also shows that the inhibitory effect of DMPG varied over a wide range (30 - 100% of the vehicle).

The extent of the DMPG induced decrease in proline incorporation and the extent of decreased incorporation of label into hydroxyproline correlated with the concentration of hydroxyproline in liver slices and therefore with the degree of hepatic fibrosis {$r = 0.68$ ($p < 0.02$) and $r = 0.59$ ($p < 0.05$) respectively} (Figure 5).

TABLE 1. LIVER HYDROXYPROLINE CONCENTRATION AND INCORPORATION OF RADIOLABELED PROLINE AND HYDROXYPROLINE INTO PROTEINS.

(Mean ± SEM)

DIET	N[a]	HYDROXYPROLINE (μmol/mg protein)	RADIOACTIVITY IN PROLINE (DPM/mg protein)	RADIOACTIVITY IN HYDROXYPROLINE (DPM/mg protein)
Supplemented	4	0.014±0.001	519±135	22.1±4.5
Cirrhogenic	8	0.038±0.005*	2867±794*	99.6±38.6*

[a] Number of animals per group
* p<0.01 compared to the supplemented values.

TABLE 2. THE EFFECT OF DMPG ON THE FREE PROLINE POOL (TOTAL AND RADIOLABELED), THE SPECIFIC ACTIVITY OF PROLINE AND ON PROLINE OXIDASE ACTIVITY IN FIBROTIC LIVER SLICES.

(MEAN ± SEM)

	Number of Animals	DMPG	VEHICLE
FREE PROLINE (μmoles/mg protein)	8	0.021±0.003	0.020±0.004
FREE ^{14}C-PROLINE (DPM/mg protein)	8	5213±625	5265±741
SPECIFIC ACTIVITY (DPM/μmole) (x10^5)	8	2.72±0.40	2.84±0.52
PROLINE OXIDASE (μmol/min/gm tissue)	4	0.085±0.02	0.083±0.02

There was no effect of DMPG on the concentration or specific activity of free proline, or on the activity of proline oxidase (Table 2).
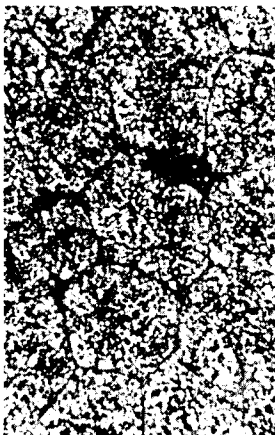
A.
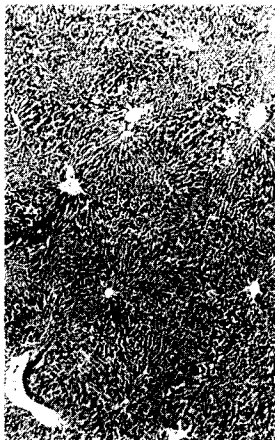
B.

Figure 1.   The effect of the deficient diet on fibrosis. a = nonfibrotic
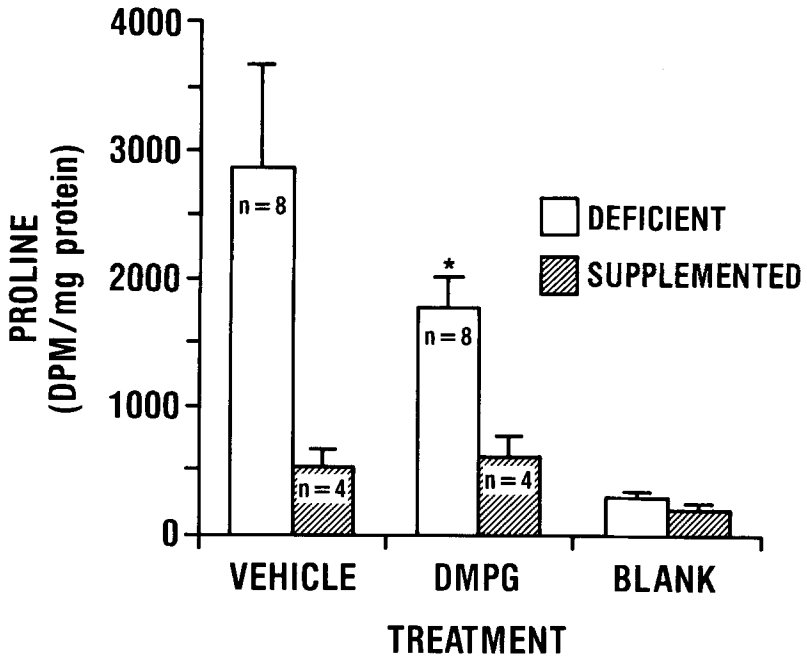liver; b = fibrotic liver.

Figure 2. The effect of DMPG *in vitro* on modifying the incorporation of ^{14}C-proline into proteins in normal and fibrotic liver slices.

The concentration of DMPG in the medium was 10^{-10}M. The blanks were incubated in 10mM cyclohexamide. Each point represents the mean $\pm$ SEM.
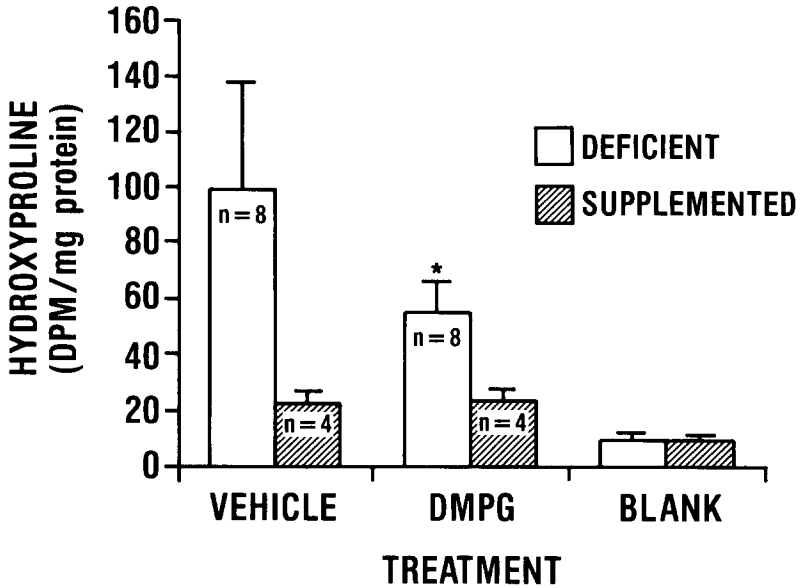
Figure 3.   The effect of DMPG *in vitro* on modifying the appearance of
^{14}C-hydroxyproline in proteins in normal and fibrotic liver
slices.

The concentration of DMPG in the medium was 10^{-10}M. The
blanks were incubated in 10 mM cyclohexamide. Each point
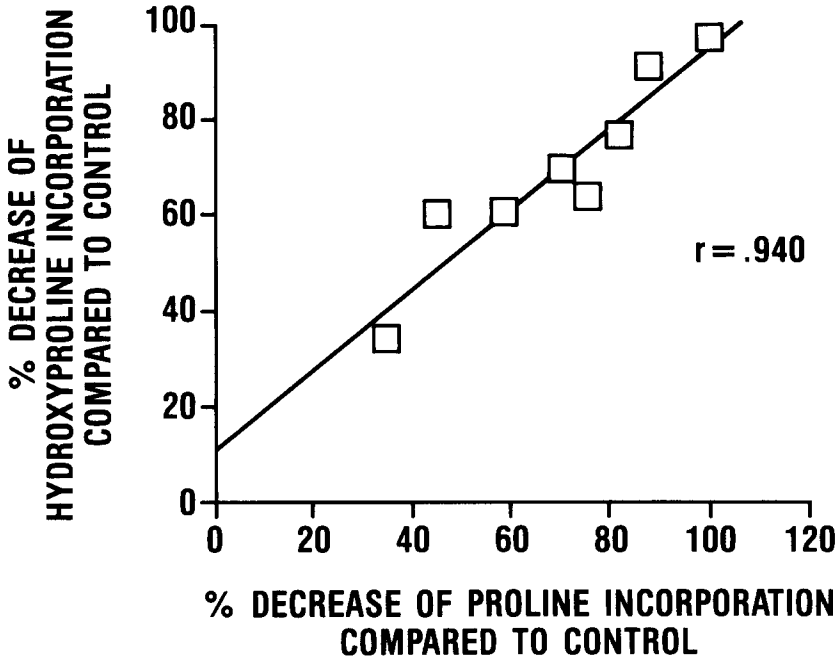represents the mean $\pm$SEM.

Figure 4. The relationship between the percent decrease of ^{14}C-proline and ^{14}C-hydroxyproline incorporation due to DMPG treatment expressed as a percent of the vehicle treated liver slices.

The graph illustrates data from eight fibrotic livers. The intercept of the line on the ordinate is statistically indistinguishable from zero.
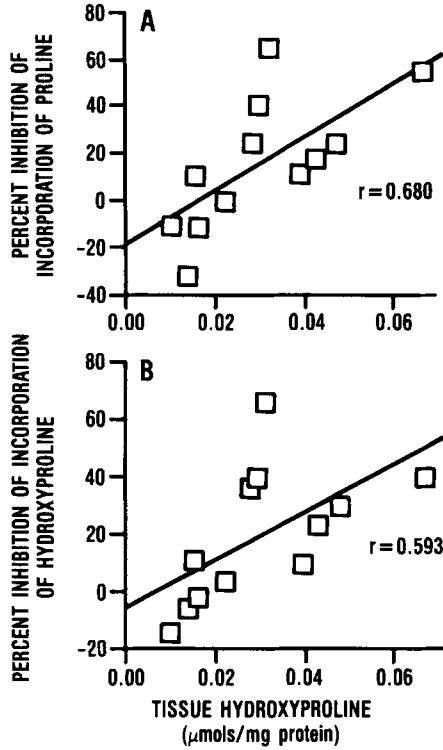
Figure 5. The effect of DMPG on inhibition of incorporation of ^{14}C-proline (A) and ^{14}C-hydroxyproline (B) into TCA precipitable proteins. Correlation with the concentration of hydroxyproline in the liver.

DISCUSSION

Collagen is synthesized by the incorporation of proline into the procollagen polypeptide chain and subsequent hydroxylation of some of the proline to form hydroxyproline, a virtually specific marker of collagen. The objective of this study was to determine whether 16,16-dimethyl prostaglandin E_2 modifies collagen synthesis in vitro in fibrotic or normal liver slices. In these experiments, livers were made fibrotic by feeding rats a cirrhogenic diet for 7 months and the extent of fibrosis assessed by measuring the concentration of hydroxyproline. As expected, livers from rats fed the cirrhogenic diet had a significantly increased concentration of hydroxyproline (Table 1). The incorporation of proline into TCA precipitable proteins and the synthesis of hydroxyproline in these proteins was assessed by incubating liver slices for four hours with ^{14}C-proline and measuring the amount of labeled proline and hydroxyproline in TCA precipitates from liver homogenates.

Fibrotic liver slices incorporated more labeled proline and generated more labeled hydroxyproline than did control livers (Table 1). Incubation of liver slices with DMPG in vitro significantly decreased the amount of ^{14}C-proline in the TCA precipitate in fibrotic, but not in control livers (Figure 2). This suggests that DMPG is not a general inhibitor of protein synthesis in normal rat liver slices, but specifically inhibits the incorporation of proline into proteins from liver slices undergoing fibrogenesis. This is in agreement with our in vivo observations that in the normal rat, DMPG has no effect on the appearance of stainable collagen, the concentration of hydroxyproline in the liver, or the concentration of N-Terminal procollagen peptide in serum (15). Figure 3 shows that DMPG produces a significant decrease in the amount of label in hydroxyproline in the TCA precipitates of fibrotic liver slices, while again no such decrease was seen in liver slices from normal rats. The magnitude of the inhibitory effect, expressed as a percentage of the incorporation seen in the vehicle treated liver slices was virtually the same for proline and hydroxyproline (Figure 4). These data suggest that at least some of the proline not incorporated because of the action of DMPG, is destined for conversion to collagen and that the rate of hydroxylation of proline is not affected. Furthermore, Figure 4 shows that the inhibitory effect of DMPG varies over a wide range. The extent of the inhibitory effect of DMPG on incorporation of label into proline and hydroxyproline correlated with the extent of hepatic fibrosis in the liver ($r = 0.68$, $r = 0.59$, respectively) (Figure 5). This correlation is, however, not very striking and suggests that factors other than fibrosis may also contribute to this effect.

It has been proposed that the concentration of free proline may regulate the biosynthesis of collagen in the liver of both rat (16) and man (17). In the experiments reported here, DMPG modified neither the size of the free proline pool, its specific activity nor the activity of proline oxidase, the enzyme capable of degrading proline to form glutamate (Table 2). Thus DMPG inhibits hepatic fibrosis by a mechanism apparently not involving the amount of free proline available for collagen synthesis.

Our observations demonstrate that in this model of enhanced fibrogenesis, DMPG decreases the net amount of labeled proline and hydroxyproline in TCA precipitable proteins in vitro, possibly by decreasing the incorporation of proline into collagen precursors, increasing the rate of degradation of these precursors or both. The effect of DMPG on lowering collagen formation in vitro, may explain at least in part, the protective effect by

DMPG on liver fibrosis *in vivo*. Whether this effect is specific for one or more of the genetically determined types of collagen, and whether it is initiated by action of DMPG on the corresponding procollagen mRNAs remains to be tested. Enhanced activity of procollagen mRNA for collagen Types I, III, and IV has recently been observed in dimethylnitrosamine induced liver injury (18).

We have tested the effect of one representative of a family of naturally occurring substances (prostaglandins) on modifying collagen synthesis in rat liver slices. DMPG has been shown *in vivo* to modify hepatic fibrosis in two species of animals (rat and rabbit).using different means of inducing hepatic damage (6,7). The data presented here demonstrates that DMPG also affects collagen formation *in vitro*. Our findings suggest that prostaglandins may have a place in the treatment of liver fibrosis in man.

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