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EFFECT OF PREDNISOLONE ON THE ACTIVITIES OF THE INTRACELLULAR ENZYMES OF COLLAGEN BIOSYNTHESIS IN RAT LIVER AND SKIN

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Abstract—The effect of prednisolone on the activities of four intracellular enzymes of collagen biosynthesis was studied in rats. The steroid was given intraperitoneally in doses of 5 or 50 mg/kg/day for 4 days. The growth of the rats was retarded. Prolyl hydroxylase, lysyl hydroxylase, collagen galactosyltransferase, and collagen glucosyltransferase activities were reduced in both the liver and skin, in a manner dependent on the dose of prednisolone. An analysis of variance failed to reveal any significant differences between the changes in activity of the four enzymes.

The intracellular modifications in collagen biosynthesis include the hydroxylation of certain prolyl residues to hydroxyprolyl residues and certain lysyl residues to hydroxylysyl residues, and the glycosylation of some of the hydroxylysyl residues to galactosylhydroxylysyl residues and some of the galactosylhydroxylysyl residues to glucosylgalactosylhydroxylysyl residues. These reactions are catalyzed by four separate intracellular enzymes, prolyl hydroxylase, lysyl hydroxylase, collagen galactosyltransferase and collagen glucosyltransferase (for reviews, see [1, 2]). The relationship between changes in the activity of these four enzymes has been studied by measuring then simultaneously during the development of experimental liver injury induced either by carbon tetrachloride or by dimethylnitrosamine [3, 4]. The activity of hydroxylases was altered considerably more than that of the two collagen hexosyltransferases, pattern of changes similar to that observed during ageing [4]. The data based on the simultaneous measurement of prolyl hydroxylase activity and the amount of immunoreactive enzyme protein indicated that prolyl hydroxylase activity was controlled in part by a mechanism which does not involve changes in the content of total immunoreactive protein [5].

The administration of pharmacological doses of prednisolone produces anti-anabolic effects on the metabolism of collagen [6]. It has been suggested that one of these effects is mediated by a decrease in the activity of prolyl hydroxylase, since this correlated with a decrease in the synthesis of collagen [7, 8]. In that condition, however, the total amount of immunoreactive prolyl hydroxylase decreased to the same extent as the active enzyme, meaning that there was control through the amount of the enzyme protein [7]. Prednisolone (5 mg/kg/day) markedly restricted the increase in the activity of prolyl hydroxylase in thioacetamide-induced liver fibrosis in rats, and also reduced the activity of this enzyme in normal rats [9].

In the present study, the effect of prednisolone in the rat on the activity of four specific intracellular enzymes involved in collagen biosynthesis was studied in liver and skin to find out if specific changes take place in some of their activities.

MATERIALS AND METHODS

Animals and preparation of tissue samples for assay. The animals used were 12-day-old Long–Evans rats. Prednisolone-sodium-succinate (Solu-Dacortin^{R}, Merck, E, Darmstadt, West-Germany) in 0.9% NaCl was injected intraperitoneally in doses of 5 mg/kg/day (first experiment) or 50 mg/kg/day (second experiment) for 4 days (two injections per day). Control animals received equal amounts of 0.9% NaCl. Each experiment comprised five rats in the prednisolone group and five controls.

The rats were killed by decapitation 6 hr after the last injection, the liver rapidly removed, immediately frozen in liquid N_2 and weighed in the frozen state. The skin was immediately cooled to 0° and cleaned free of muscle, fat tissue and hair. Tissues were stored at −70° until assayed. The livers were homogenized in a Teflon/glass homogenizer (Thomas) at about 1500 rpm. for 60 sec in a cold solution consisting of 0.2 M NaCl, 0.1 M glycine, 0.1% (w/v) Triton X-100 and 20 mM Tris–HCl buffer, adjusted to pH 7.5 at 4° [4]. The volume of the solution was 20 ml/g of liver. The skin samples were minced with scissors for 5 min in a cold homogenization solution (8 ml/g of skin) and then homogenized in an Ultra-Turrax homogenizer 2 times for 5 sec each. The homogenates were incubated at 4° for 30 min [3] and then centrifuged at 15,000 *g* for 30 min at 4°. Portions of the supernatant were used for assay of the enzyme activities and of the supernatant protein.

Assay of prolyl hydroxylase activity. The assay was carried out using [^{14}C]proline-labelled protocollagen as substrate [5]. Portions of the 15,000 *g* supernatants were incubated under agitation for 30 min at 37° in a final vol. of 2 ml containing 50,000 dpm. of [^{14}C]proline-labelled protocollagen substrate, 0.08 mM $FeSO_4$, 2 mM ascorbic acid, 0.5 mM α-oxoglutarate, 0.2 mg/ml catalase (Calbiochem Ltd., London, U.K.), 2 mg/ml bovine serum albumin (Sigma

Chemical Co., Kingston-upon-Thames, U.K.), and 50 mM Tris–HCl buffer, adjusted to pH 7.8 at 25° [4]. The reaction was stopped by adding an equal vol. of concentrated HCl, and after hydrolysis at 120° overnight the amount of hydroxy[^{14}C]proline formed was assayed [10].

Assay of lysyl hydroxylase activity. The assay was carried out using [^{14}C]lysine-labelled protocollagen as substrate [4]. Portions of the 15,000 *g* supernatants were incubated under agitation for 45 min at 37° in a final vol. of 1 ml containing 120,000 dpm. of [^{14}C]lysine-labelled protocollagen substrate, 0.05 mM $FeSO_4$, 0.5 mM ascorbic acid, 0.5 mM α-oxoglutarate, 0.1 mg catalase, 1.5 mg bovine serum albumin, and 50 mM Tris–HCl buffer, adjusted to pH 7.8 at 25° [11]. The reaction was stopped by adding 10 ml of cold acetone, and hydroxy[^{14}C]lysine was measured [11, 12].

Assay of collagen galactosyltransferase and collagen glucosyltransferase activities. For the assay of galactosyltransferase activity, portions of the 15,000 *g* supernatants were incubated under agitation for 45 min at 37° in a final vol. of 100 μl containing 40 mg/ml of gelatinized calf skin collagen as substrate, 60 μM UDP-[^{14}C]galactose (55 Ci/mol), 10 mM $MnCl_2$, and 50 mM Tris–HCl buffer, adjusted to pH 7.4 at 20° [13]. The assay of the glucosyltransferase activity was carried out in 100 μl of a similar mixture, except that 60 μM UDP-[^{14}C]glucose (12 Ci/mol) was used [13]. Both reactions were stopped by adding 2 ml of cold 1% (w/v) phosphotungstic acid in 0.5 M HCl. The samples were centrifuged at 4000 *g* for 5 min and the pellets washed twice with 10% trichloroacetic acid and once with ethanol–ether (1:1). The dried pellets were hydrolyzed with 2 M NaOH at 105° for 18 hr. After hydrolysis the [^{14}C]galactosylhydroxylysine and [^{14}C]glucosylgalactosylhydroxylysine formed were assayed by a specific procedure [13] involving purification of the samples in small columns of Dowex 50-X8. The paper-electrophoresis step of the original assay procedure was omitted (see [4]).

Other assays. The protein content of the supernatant of the liver and skin was assayed by the method of Lowry *et al.* [14]. All [^{14}C]radioactivity counting was performed in a Wallac liquid-scintillation spectrometer with an efficiency of 85% and a background of 25 cpm.

Statistical methods. The statistical significance of difference between means was calculated using the Student's *t*-test. An analysis of variance was used to detect significant differences in changes of the four enzyme activities in the liver or skin at a given dose of prednisolone. The calculations were performed on a Compucorp^{R} 344 using a program for one factor analysis of variance. The F ratio obtained was then compared with the F distribution with 16 degrees of freedom (within the sample) and 3 degrees (between samples).

RESULTS

Effect of prednisolone treatment on the weights of the rats and their livers, and on the protein content of the supernatant of the liver and skin homogenates. Prednisolone treatment had a marked effect on the growth of the rats (Table 1), the weight gain being even reversed with larger doses. The wet weight of liver nevertheless increased in all animals, but this was largely due in the prednisolone group to an increase in the water content, as the 15,000 *g* supernatant protein content of the liver homogenates was significantly lower (Table 1). The soluble protein content of skin homogenates was only about one third of that in the liver. Macroscopically the skin of the treated animals was much thinner than that of the controls. The 15,000 *g* supernatant protein content of the skin homogenates was not changed significantly by the treatment (Table 1). The slight increase in the protein of the supernatant in the prednisolone group may be due to a reduction in water content [6].

Effect of prednisolone treatment on the four enzyme activities in the liver. Conditions for the extraction and assay of the four enzyme activities in the liver have been studied previously [3, 4]. All the assays were carried out in the linear part of the standard curves. The changes in the four enzyme activities in the liver compared with values in the control rats, are shown in Fig. 1A and B. In both experiments the decreases in the activity of the four enzymes were statistically significant ($P < 0.01$; Student's *t*-test). The reductions in activities were dependent on the dose of prednisolone. With a dose of 5 mg/kg/day the mean value in the prednisolone treated rats was 85.0 per cent of that in the control, and with a dose of 50 mg/kg/day it was of 71.3 per cent. The analysis of variance showed that the differences between changes among the four enzymes were not significant. Thus prednisolone produced a similar effect on all four enzymes in the liver.

Effect of prednisolone treatment on the activity of

Table 1. Effect of prednisolone on the weight of rats, their livers and the protein content of the supernatant of liver and skin homogenates

Dose and group	Initial weight	Terminal weight	Weight of liver	Supernatant protein /g of liver	/g of skin
	g	g	mg	mg	mg
5 mg/kg/day					
Controls	20.4 ± 1.6	24.3 ± 1.4	825 ± 47	163 ± 3	47 ± 8
Prednisolone	20.5 ± 1.2	21.2 ± 1.1†	884 ± 42*	155 ± 9*	54 ± 12
50 mg/kg/day					
Controls	22.1 ± 2.0	25.3 ± 1.8	921 ± 103	189 ± 4	44 ± 7
Prednisolone	22.1 ± 2.1	20.0 ± 1.2‡	1159 ± 110†	182 ± 7*	50 ± 11

Prednisolone was given intraperitoneally in the doses indicated above for four days. Results are expressed as mean ± S.D. of five rats. Significance levels: *$P < 0.05$; †$P < 0.01$; ‡$P < 0.001$.

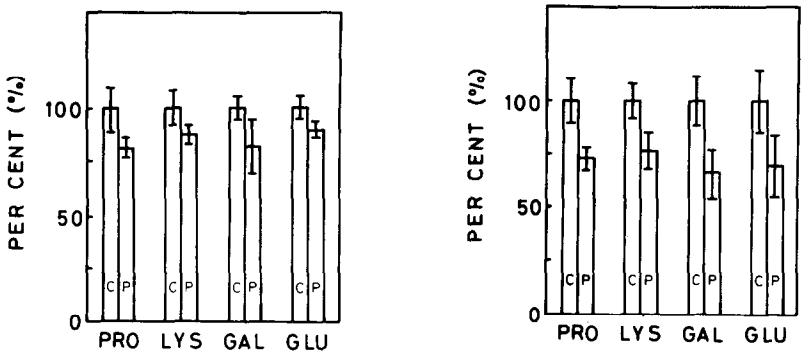

Fig. 1. Effect of prednisolone treatment on the four enzyme activities in the liver. The values are expressed as percentages (mean ± S.D.) of those in the control rats. Pro = prolyl hydroxylase; Lys = lysyl hydroxylase, Gal = collagen galactosyltransferase, and Glu = collagen glucosyltransferase activity. C = controls, P = prednisolone treated. (A) Dose of prednisolone 5 mg/kg/day, (B) dose of prednisolone 50 mg/kg/day.

the four enzymes in the skin. Standard curves were prepared for all enzymes to ensure that the assays would be carried out under conditions in which the relationship between enzyme activity and product formation is linear. The changes in the four enzymes in the skin, as compared with values in the controls, are shown in Fig. 2A and B. In both experiments all reductions in activities were statistically significant ($P < 0.01$; Student's *t*-test). As in the liver, the decrease in the enzyme activities was dependent on the dose of prednisolone. With a dose of 5 mg/kg/day the mean value in the prednisolone rats was 77.8 per cent of that in the control rats, and with a dose of 50 mg/kg/day it was of 71.3 per cent. The analysis of variance showed no significant differences between the changes among the four enzymes. Thus the effect of prednisolone was also similar on all four enzyme activities in the skin.

DISCUSSION

The biosynthesis of collagen depends upon a number of unique post-translational modifications which are catalyzed by a number of specific enzymes. Knowledge of these modifications has increased rapidly in recent years (see [1, 2]), and preliminary information is available on the regulation and possible specific pharmacological control of collagen biosynthesis at these stages.

Glucocorticoids and several synthetic anti-inflammatory steroids reduce the rate of collagen biosynthesis when administered in pharmacological doses (see [8, 15, 16]). This effect is partly mediated by a reduction in the activity of prolyl hydroxylase *in vivo* [7, 8, 17], but *in vitro* no decrease in the activity of this enzyme was observed, even though collagen synthesis was reduced [17]. The present work demonstrates that prednisolone administration decreases the activity of four specific enzymes involved in collagen biosynthesis, and thus the changes are not selectively directed against prolyl hydroxylase [7], but to all four intracellular enzymes.

After liver injury the levels of the two hydroxylase activities alter much more than those of the two collagen hexosyltransferases [3, 4]. After prednisolone treatment, however, the activity of all these four enzymes is reduced similarly, possibly due to a general reduction in the synthesis of the enzyme proteins. In the case of prolyl hydroxylase the amount of enzyme protein has been shown to decrease to the same extent as the activity of the enzyme [7, A. Oikarinen, personal communication], but it has not

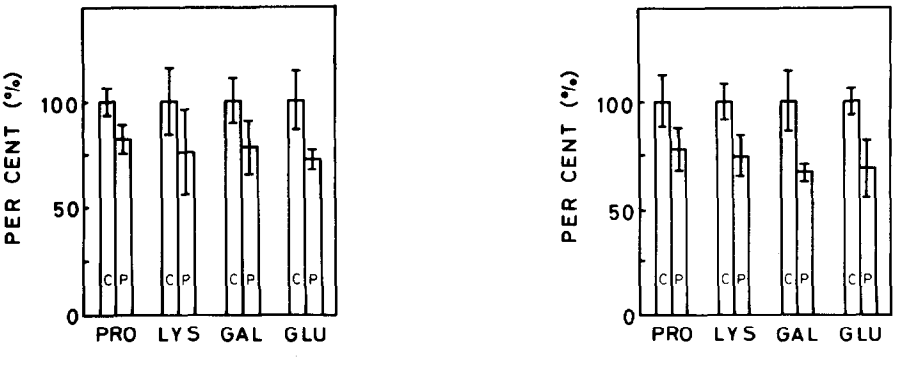

Fig. 2. Effect of prednisolone treatment on the four enzyme activities in the skin. The values are expressed as percentages (mean ± S.D.) of those in the control rats. Pro = prolyl hydroxylase, Lys = lysyl hydroxylase, Gal = collagen galactosyltransferase, and Glu = collagen glucosyltransferase activity. C = controls, P = prednisolone treated. (A) Dose of prednisolone 5 mg/kg/day, (B) dose of prednisolone 50 mg/kg/day.

been possible so far to measure the enzyme proteins of the other three enzymes to verify this finding.

Glucocorticoids display inhibitory effects on fibroblast multiplication [18]. This explains the retarded growth of the prednisolone rats here, but it need not mean that the reduction in activity of the four enzymes is due to a decrease in the number of fibroblasts, as it has been shown that the activity of prolyl hydroxylase decreases even when expressed per cell [17]. In addition it has recently been found that the activities of the other three enzymes also decrease in matrix-free tendon cells when hydrocortisone is administered to chick embryos before the isolation of the cells [A. Oikarinen, personal communication].

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