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EFFECTS OF GLUCOCORTICOSTEROIDS ON CULTURED HUMAN SKIN FIBROBLASTS—IV

SPECIFIC DECREASE IN THE SYNTHESIS OF COLLAGEN BUT NO EFFECT ON ITS HYDROXYLATION

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Abstract—Confluent cultures of normal baby foreskin fibroblasts were exposed for 6 days to hydrocortisone-17-butyrate (5 μg/ml) or to clobetasol-17-propionate (1 or 5 μg/ml). On day 5 [^{3}H]proline was added to the cultures and on day 6 both medium and cell layer were analyzed for [^{3}H]protein and protein-bound [^{3}H]hydroxyproline. The synthesis of labeled protein was little affected, while that of labeled collagenous protein was greatly depressed, as compared to that of the control cells. This depression occurred for the collagenous protein present in the cell layer as well as for that released into the growth medium. This effect was not accompanied by a decrease in cellular prolyl hydroxylase activity or in collagen proline hydroxylation.

In previous work [1, 2] we have shown that the addition of a variety of glucocorticosteroids will cause a transient inhibition of the proliferation of cultured human skin fibroblasts in their early growth stages. With increasing cell density the addition of the steroids resulted in a decrease in this inhibition and, eventually, in no inhibition. This disappearance of an inhibitory effect on growth occurred already before confluence was reached. In further studies [3] it was found that the addition of the steroids to confluent cultures resulted in a depression in the formation of protein-bound hydroxyproline. We concluded that this latter effect could be due to (1) a depression of prolyl hydroxylase activity, (2) an increase in collagen degradation, (3) a depression of procollagen peptidase activity, (4) a selective decrease in the synthesis of either collagen Type I or III, (5) a decrease in the translation or transcription of collagen mRNA. In this paper we report further experiments to obtain information on these points.

While this work was in progress a number of other reports have appeared on the effects of glucocorticosteroids on protein and collagen synthesis in fibroblast cultures. In one case only [4] an increase was reported in both collagen and non-collagenous protein synthesis. In this study confluent fetal human skin fibroblasts were employed in short-term exposure (up to 11 hr) to hydrocortisone. In all the other studies confluent cultures or dense cell suspensions were either exposed *in vitro* usually for a number of days to a variety of glucocorticosteroids or cells were obtained from animals treated for extended periods with the steroids and then studied *in vitro*. In all these studies a decrease was found in the synthesis of protein-bound hydroxyproline and, except in one study [5], this decrease was more extensive than that of protein-bound proline. The cell types used in these studies were suspensions of chicken embryo fibroblasts [6] or from steroid treated animals [7, 8], confluent mouse dermal sponge granuloma fibroblasts from steroid treated mice [5], adult human fibroblasts from normal and keloid skin [9], fetal human skin fibroblasts [10], and isolated polysomes from rat dermal fibroblasts [11–13]. In five of these reports [5, 7, 8, 11, 12] a concomitant decrease in cellular prolyl hydroxylase activity was noted with no decrease, however, in proline hydroxylation. In addition, a decrease in the activity of lysyl hydroxylase and also of galactosyl and glucosyl transferase activity for the synthesis of hydroxylysyl-bound mono- and disaccharides was found in one of these studies [7].

Since most of these studies [9, 10–13], except for two [4, 5], showed a specific decrease in the collagen synthesis by normal dermal fibroblasts under the influence of a variety of glucocorticosteroids, we decided to continue our studies on the mechanism of this effect with our experimental system of cultured normal baby foreskin fibroblasts.

Our results show that in this system there was a specific depression of collagen synthesis, with no concomitant effect either on cellular prolyl hydroxylase activity or on collagen hydroxylation.

MATERIALS AND METHODS

Fibroblasts, growth conditions, analysis of radioactivity and cell counting

The fibroblasts used in these studies were 11th–16th passage cells obtained from explanted biopsies of normal baby foreskin. Confluent cell cultures were exposed for 6 days to hydrocortisone-17-butyrate (5 μg/ml) or to clobetasol-17-propionate (1 or 5 μg/ml) by adding the appropriate amount of steroid in 1.0 μl of ethanol per ml of medium.* On day 5 [^{3}H]proline was added to

* Hydrocortisone-17-butyrate (Locoid®, H-17-B) and clobetasol-17-propionate (Dermovate®, C-17-P) were kindly provided by Gist-Brocades and Glaxo, respectively.

Table 1. Effects of H-17-B and C-17-P on total protein synthesis, hydroxyproline formation and proline hydroxylation*

Experiment no.		[^{3}H]Protein (d.p.m. × 10^{-6} and %)†									[^{3}H]Hydroxyproline (d.p.m. × 10^{-6} and %)‡									Proline		
		1			2			3			1			2			3			1	2	3
Conditions¶		d.p.m.	Δ(%)‖	Distr. (%)**	d.p.m.	Δ(%)	Distr. (%)	d.p.m.	Δ(%)	Distr. (%)	d.p.m.	Δ(%)	Distr. (%)	d.p.m.	Δ(%)	Distr. (%)	d.p.m.	Δ(%)	Distr. (%)			
Control	c	11.21		70.5	9.44		62.7	9.95		69.4	1.03		56.6	0.82		50.9	0.56		55.4	9.2	8.7	5.6
	m	4.70		29.5	5.57		37.3	4.39		30.6	0.79		43.4	0.79		49.1	0.44		44.6	16.8	14.2	10.0
	t	15.91	0	100	15.01	0	100	14.34	0	100	1.82	0	100	1.61	0	100	1.00	0	100	11.4	10.7	7.0
Ethanol	c	9.53		69.7	9.33		65.3	7.63		64.7	0.83		57.2	0.82		57.3	0.61		53.5	8.7	8.8	8.0
	m	4.14		30.3	4.95		34.7	4.17		35.3	0.62		42.8	0.61		42.7	0.53		46.5	15.0	12.3	12.7
	t	13.67	−14.1	100	14.28	−4.9	100	11.80	−17.7	100	1.45	−20.4	100	1.43	−11.0	100	1.14	+14.0	100	10.6	10.0	9.6
H-17-B, 5 μg/ml	c	—		—	10.55		71.8	10.98		73.5	—		—	0.22		36.0	0.23		38.3	—	2.1	2.1
	m	—		—	4.24		28.2	3.96		26.5	—		—	0.39		64.0	0.37		61.7	—	9.2	9.3
	t	—		—	14.69	−2.1	100	14.93	+4.1	100	—	—	—	0.61	−63.2	100	0.60	−40.0	100	—	4.2	4.0
C-17-P, 1 μg/ml	c	9.24		73.4	9.87		73.2	12.37		72.9	0.26		43.3	0.20		40.0	0.29		38.2	2.8	2.0	2.3
	m	3.35		26.6	3.61		26.8	4.61		27.1	0.34		56.7	0.30		60.0	0.47		61.8	10.1	8.3	10.2
	t	12.59	−20.9	100	13.48	−10.2	100	16.98	+18.4	100	0.60	−67.0	100	0.50	−69.0	100	0.76	−24.0	100	4.8	3.7	4.5
C 17-P, 5 μg/ml	c	7.38		72.4	—		—	—		—	0.22		38.6	—		—	—		—	3.0	—	—
	m	2.81		27.6	—		—	—		—	0.35		61.4	—		—	—		—	12.4	—	—
	t	10.91	−31.4	100	—	—	—	—	—	—	0.57	−69.7	100	—	—	—	—	—	—	5.6	—	—

* Given are the total amounts of [^{3}H]protein and of [^{3}H]hydroxyproline incorporated per flask during the 24 hr labeling period (3 experiments) in the cell layer (c), in the non-dialyzable polypeptides of the growth medium (m), and the sum of these two (t). For each condition triplicate flasks were used in all experiments. Thus each value is the average of three individual flasks which varied within 10 per cent.

† Total amount of ^{3}H-protein and

‡ of ^{3}H-hydroxyproline incorporated.

§ Ratio of the ^{3}H-hydroxyproline over the ^{3}H-protein in per cent.

¶ H-17-B: Hydrocortisone-17-butyrate. C-17-P: Clobetasol-17-propionate.

‖ Change for the experimental relative to the control cells expressed as per cent.

** Amounts of the ^{3}H-protein and ^{3}H-hydroxyproline incorporated in the cell layer (c) and growth medium (m) as the percentage of the sum of these two (t).

For further details see Materials and Methods.

the cultures and on day 6 both medium and cell layer were analyzed for [^{3}H]protein and protein-bound [^{3}H]hydroxyproline. For details concerning growth conditions, labeling with [^{3}H]proline, analysis of radioactivity and cell counting see our previous paper [3]. At the end of the 3 experiments there were $10–15 \times 10^{-6}$ cells per flask. In each experiment cell counts differed less than 10 per cent for the different growth conditions.

Cellular prolyl hydroxylase activity

Cell extracts. The cells (3×10^7 for each growth condition) were washed in their culture flasks with ice-cold PBS and then collected by means of a Teflon policeman. After pelleting at 500 *g* and resuspending in 0.5 ml of ice-cold 40 mM-Tris–HCl (pH 7.4) containing 250 mM of sucrose [14], they were lysed by sonification for 1 min with a Branson sonifier®B-12. The supernatant obtained after centrifugation in the cold for 75 min at 50,000 *g* was assayed for protein content [15] and then stored at −70° until used as the cell extract. The protein content of the cell extracts from steroid treated cells and from control cells differed less than 10 per cent.

Labeled substrate. This was prepared from the frontal bones of 15 day-old chicken embryos by labeling with [^{3}H]proline according to Peterkofsky and Di-Blasio [16] and stored at −70°. In each enzyme assay 150,000–220,000 d.p.m. of the [^{3}H]proline-labeled substrate was used that had been preincubated for 15 min at 37°.

Assay of the enzyme. The total volume of each assay mixture was 2 ml. It contained 50mM-Tris–HCl (pH 7.6), 1mM-ascorbate, 1mM-α-ketoglutarate, 0.2mM-$FeSO_4$, 0.5mM-dithioerytritol, 2 mg/ml of bovine serum albumine, 0.4 mg/ml of catalase [14], 150,000–220,000 d.p.m. of preincubated [^{3}H]proline-labeled substrate and 20 to 100 μg of protein as the cell extract. Blank tubes contained all components except the cell extract. Incubation was for 10 or 20 min at 37°. The reaction was stopped by the addition of 2 ml of 12M-HCl and the contents of the tubes were hydrolyzed and analyzed for radioactivity by means of an oxidation method, as previously described [3].

Treatment of growth medium proteins with prolyl hydroxylase or purified bacterial collagenase

Concentrating the medium proteins. These proteins were precipitated in the cold with 45% or 65% ammonium sulfate and then dissolved in small volumes of NAT-buffer (0.2M-NaCl, 0.05M-Tris–HCl, pH 7.6). The dissolved proteins were then dialyzed exhaustively against the same buffer.

Treatment with prolyl hydroxylase. For each growth condition the precipitated medium proteins in NAT-buffer were first denatured for 15 min at 60° and then treated for 60 min with 200 μg of protein present in the extract of cells grown under control conditions. The assay conditions were as described for the assay of the enzyme in the cell extracts. In each experiment tubes containing labeled chicken embryo substrate were used as a control on enzyme activity. At the end of the incubation an equal volume of 12M-HCl was added and the mixture was hydrolyzed and analyzed for radioactivity [3].

Treatment with collagenase. Aliquots of 0.3–0.8 ml of medium proteins (dissolved in NAT-buffer) and either 30 or 60 μg of purified bacterial collagenase (Calbiochem, A Grade, dissolved at 300 μg/ml in collagenase-buffer: 0.33M-calcium acetate + 25mM-Tris–HCl, pH 7.6) were adjusted to a total volume of 0.5 or 1.0 ml with NAT-buffer and incubated for 30–120 min at 37°. Blank tubes in which collagenase was omitted were used in all experiments. The proteins were then precipitated by adding TCA to 10% and washed once with 10% TCA. The combined TCA supernatants and wash were hydrolyzed and analyzed for radioactivity [3]. In separate experiments the collagenase was tested for general proteolytic activity. Growth medium proteins labeled with tritiated tryptophan rather than with proline were not digested to a significant extent when incubated with the enzyme for 120 min under these conditions.

RESULTS

Decrease in hydroxyproline formation. As shown previously for human adult arm skin fibroblasts [3] the addition to the growth medium of 5 μg of H-17-B/ml or of 1 μg of C-17-P/ml resulted in a small but variable change in the overall incorporation of total radioactivity with the baby foreskin fibroblasts (Table 1). Again, 5 μg of C-17-P/ml depressed this incorporation. The addition of glucocorticosteroids caused a large decrease in the overall incorporation of hydroxyproline radioactivity. This decrease was somewhat larger than that previously found with the arm skin fibroblasts [3]. Overall proline hydroxylation was significantly lowered by the glucocorticosteroids. As shown previously [3] ethanol alone (which served as the solvent for the steroids) caused a small decrease in the amounts of both total and hydroxyproline radioactivity.

We showed previously [3] that under these conditions 40–50 per cent of total radioactive protein and 15–20 per cent of protein-bound [^{3}H]hydroxyproline were present in the cell layer after 24 hr of exposure to [^{3}H]proline of the cultures of arm skin fibroblasts. For the control baby fibroblasts these values were 65–75 per cent and 35–55 per cent respectively.

Table 1 also shows that for the baby cells the relative distribution of both total and hydroxyproline radioac-

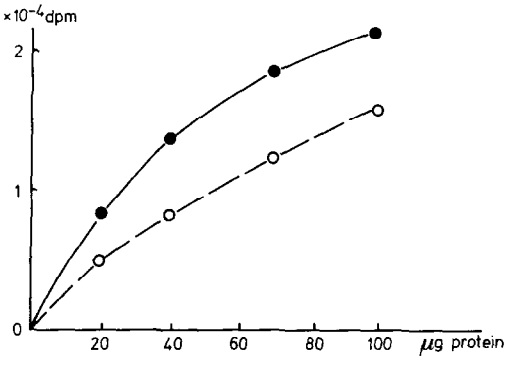

Fig. 1. Enzyme concentration curve for proline hydroxylation. All incubation mixtures contained 220,000 d.p.m. of ^{3}H-proline labeled substrate and varying amounts of control cell extract. Incubation was for 10 (○) and 20 (●) min. Analysis was by the chemical oxidation method. The hydroxyproline formed (d.p.m. × 10^{-4}) is plotted against the amount of cell extract protein.

Table 2. Prolyl hydroxylase activity in control and treated cells*

		Protein (μg)†	^{3}H-hyp. produced (d.p.m.)‡	s.a.§
Exp. 2	Control	22.5	4545	2 02
		45.0	8775	1 95
	Ethanol	20.3	4445	2 19
		40.5	8580	2 12
	H-17-B, 5 μg/ml	16.2	3460	2 13
		33.3	6690	2 01
	C-17-P, 1 μg/ml	18.0	3920	2 18
		36.0	7520	2 09
Exp. 3	Control	19.6	4330	2 21
		39.2	7880	2 01
	Ethanol	16.0	3696	2 31
		32.0	6400	2 00
	H-17-B, 5 μg/ml	18.1	4250	2 35
		36.2	7420	2 05
	C-17-P, 1 μg/ml	20.2	4650	2 30
		40.4	8000	1 98

* Cell extracts were prepared from the cells in experiments 2 and 3 (see Table 1) as described in Materials and Methods and prolyl hydroxylase activity in these extracts was assayed as discussed in Results and in Materials and Methods. For each condition two different concentrations of cell extract were used, one of which was twice the amount of the other. Each incubation mixture contained 145,000 d.p.m. of ^{3}H-proline labeled substrate. Incubation was for 20 min.

† Amount of protein added as cell extract.

‡ Amount of ^{3}H-hydroxyproline formed in the assay (averages of duplicates). The blanks of 400–500 d.p.m. have been substracted.

§ Amount of ^{3}H-hydroxyproline formed per 1 μg of protein.

tivity between cell layer and growth medium was affected by the glucocorticosteroids. There was a small relative shift to the cell layer for the former and a large one to the growth medium for the latter radioactivity.

No effect on prolyl hydroxylase activity. The decrease in the formation of polypeptide-bound [^{3}H]hydroxyproline under the influence of glucocorticosteroids suggested that this could be due to a decrease in the activity of intracellular prolyl hydroxylase. We decided therefore to test the activity of the enzyme in extracts obtained from the steroid treated cells of experiments 2 and 3 (Table 1).

In order to arrive at appropriate assay conditions we varied independently both the added amount of extract protein from control cells and the incubation time at a constant substrate concentration. The results shown in Fig. 1 enabled us to maximize possible differences in enzyme activity between control and experimental flasks by utilizing quantities of the extract amounting to 16–45 μg of protein and an incubation time of 20 min for each incubation mixture.

Table 2 shows that the enzyme activity in extracts from the steroid treated cells was not significantly different from that of control cells under conditions where product formation was roughly proportional to the amount of enzyme added. We concluded from these results that the steroids did not have a significant effect on the amount of active enzyme present in the cells.

The addition of the glucocorticosteroids to the incubation mixture containing control extract, at the same concentrations as were used in the growth medium for the steroid-treated cells, did not affect the measured enzyme activity (results not shown).

No underhydroxylation of medium collagen. We next turned our attention to the degree of hydroxylation of the collagenous polypeptides present in the growth medium. The medium proteins of cells grown in the absence or presence of steroids were precipitated with 45% ammonium sulfate, dissolved in a small volume of the appropriate buffer and treated with an excess of prolyl hydroxylase extract from control cells, as described under Materials and Methods. In this way more than 79 and 87 per cent of total and hydroxyproline radioactivity, respectively, present in the medium protein was obtained in a solution at a concentration sufficient for carrying out the assays in a small volume.

Separate experiments showed that the standard amount of non-hydroxylated prolyl hydroxylase substrate was hydroxylated essentially to completion when incubated for 60 min with 200 μg of protein from control cell extracts (Fig. 2). For this reason this amount of the extract and 60 min incubation were used to obtain further hydroxylation of the precipitated medium proteins by added enzyme.

The treatment of medium proteins with prolyl hydroxylase gave rise to varying degrees of further hydroxylation (Table 3). These variations, however, fell within experimental error. A significant underhydroxylation of the collagenous proteins made (i.e. an increase in hydroxylatable protein) in the presence of glucocorticosteroids was not apparent. The large decrease in hydroxyproline formation in the presence of these compounds did not seem to be due therefore to underhydroxylation of the collagen made.

Decrease in the amount of collagen. We then decided to determine whether the amount of collagen synthesized was affected. To arrive at suitable assay conditions the medium proteins of control cells were precipitated with ammonium sulfate and dissolved in a small volume of collagenase buffer. Aliquots of this protein

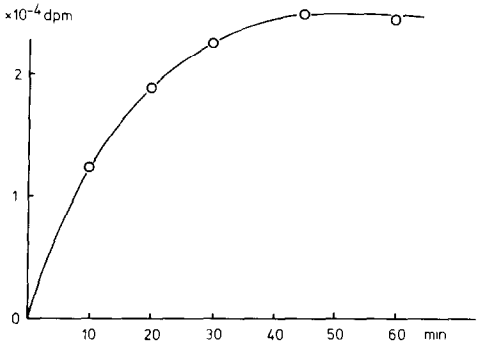

Fig. 2. Requirements for complete hydroxylation. An amount of 180,000 d.p.m. of ^{3}H-proline labeled substrate was incubated with 200 μg of control cell extract for 10–60 min and the hydroxylation (d.p.m. × 10^{-4} of hydroxyproline formed) was measured by the chemical oxidation method.

Table 3. Hydroxylation of medium protein before and after treatment with prolyl hydroxylase*

Conditions	Exp. No.	− Prolyl hydroxylase†			+ Prolyl hydroxylase†		
		^{3}H-protein	^{3}H-hyp	% hyp‡	^{3}H-protein	^{3}H-hyp.	% hyp‡
Control	1	165.0	23.2	14.1	165.0	20.8	12.6
	2	142.0	16.5	11.6	142.0	15.5	10.9
Ethanol	1	162.0	18.5	11.4	162.0	19.8	12.2
	2	175.0	16.8	9.6	175.0	23.3	13.3
H-17-B, 5 μg/ml	1	—	—	—	—	—	—
	2	248.0	27.6	11.1	248.0	22.2	9.0
C-17-P, 1 μg/ml	1	192.1	14.3	7.4	192.0	18.5	9.6
	2	300.0	10.3	3.4	300.0	15.3	5.1
C-17-P, 5 μg/ml	1	105.0	14.9	14.2	105.0	16.0	15.2
	2	—	—	—	—	—	—
Labeled substrate§	1	114.0	0.3	0.3	114.0	20.0	17.5
	2	110.0	0.2	0.2	110.0	18.3	16.6

* Aliquots of the growth medium proteins of experiments 1 and 2 (see Table 1) were precipitated with 45 per cent ammonium sulfate and redissolved as described in Materials and Methods. Duplicate samples of the solubilized medium proteins were incubated either as such (−Prolyl hydroxylase) or with 200 μg of control cell extract (+Prolyl hydroxylase) for 60 min and then analyzed according to the oxidation method (see Text and Materials and Methods).

† Shown are the values for d.p.m. × 10^{-3} ^{3}H-protein and ^{3}H-hydroxyproline and proline hydroxylation (averages of duplicate samples) both in the absence of the enzyme and in its presence.

‡ The small increases or decreases in the proline hydroxylation values after treatment with the enzyme can be explained by the experimental variation between the duplicates.

§ Labeled substrate prepared from chicken embryo frontal bone (see Materials and Methods) serving as a control on enzyme activity.

solution were treated for 30–120 min with 30 μg of purified bacterial collagenase, as described in Materials and Methods. Figure 3 shows that after 30 min digestion was essentially complete. For this reason incubation for 90 min appeared adequate for further experiments.

Next, aliquots of the medium proteins from experiments 1 and 2 (Table 1) were precipitated with 65% ammonium sulfate and dissolved in small amounts of NAT-buffer. This yielded protein solutions in collagenase buffer containing more than 98 per cent of the protein-bound [^{3}H]hydroxyproline present originally in the aliquots of the growth media. These solutions were treated with collagenase for 90 min together with blank incubation mixtures in which the collagenase was omitted.

Table 4 summarizes the results. In the blank tubes (bl) more than 95 per cent of the radioactive protein and more than 91 per cent of the protein-bound [^{3}H]hydroxyproline was TCA-precipitable (values for *n*). In the presence of collagenase these values were between 43.8 and 70.0 per cent and between 9.6 and 16.5 per cent, respectively. The remainder stayed in solution in the TCA-supernatants (TCA).

These results show that more than 90 per cent of the collagenous proteins were TCA-precipitable and that 85–90 per cent of these became non-precipitable with TCA after collagenase treatment. More than 90 per cent of the [^{3}H]hydroxyproline solubilized by the collagenase was present as dialysable peptides.

The degree of hydroxylation of the collagenous proteins present in the growth media which became non-precipitable with TCA after collagenase treatment varied between 17.3 and 27.2 per cent and was not consistently lower after steroid treatment. This again showed that the presence of glucocorticosteroids did not result

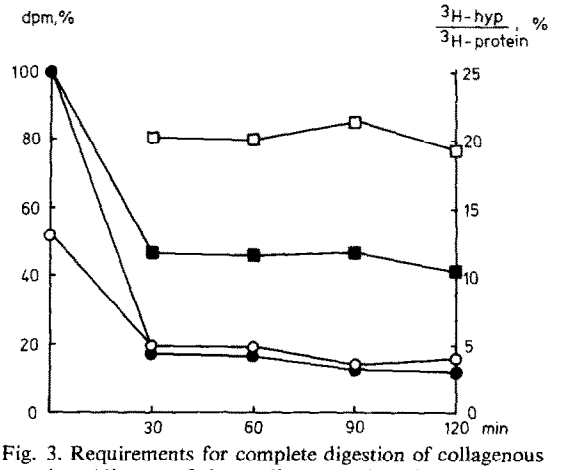

Fig. 3. Requirements for complete digestion of collagenous proteins. Aliquots of the medium proteins of control cells prepared according to the method described in Table 3 (1.8×10^{-5} d.p.m. per assay) were incubated with 30 μg of purified bacterial collagenase for 30–120 min at 37°. In blank tubes, incubated for 120 min, collagenase was omitted. They showed no change in the amounts of precipitable ^{3}H-protein and ^{3}H-hydroxyproline during incubation. The proteins were reprecipitated with 65 per cent ammonium sulfate and analyzed for radioactivity. ^{3}H-protein: ■; ^{3}H-hydroxyproline: ●; and proline hydroxylation: ○ of precipitated protein. Proline hydroxylation of digested protein: □.

Table 4. Collagenase treatment of medium protein*

Exp. No.		^{3}H-protein				^{3}H-hydroxyproline				Proline hydroxylation			
		1		2		1		2		1		2	
Enzyme		−	+	−	+	−	+	−	+	−	+	−	+
Cond.	fr.												
Control	n	95.7	51.5	95.0	43.8	93.6	13.0	92.3	11.4	13.1	3.7	13.4	3.9
	TCA	4.3	48.5	5.0	56.2	6.4	87.0	7.7	88.6	19.8	26.2	20.9	23.6
Ethanol	n	94.9	54.5	95.5	46.1	91.2	10.9	93.9	14.1	13.1	2.7	12.5	3.9
	TCA	5.1	45.5	4.5	53.9	8.8	89.1	6.1	85.9	23.4	26.8	17.2	20.4
H-17-B, 5 μg/ml	n	—	—	96.5	64.6	—	—	93.2	9.6	—	—	8.7	1.4
	TCA	—	—	3.5	35.4	—	—	6.8	90.4	—	—	17.7	24.1
C-17-P, 1 μg/ml	n	96.6	70.0	97.4	64.8	92.6	15.1	93.9	16.5	8.1	5.5	7.5	1.9
	TCA	3.4	30.0	2.6	35.2	7.4	84.9	6.1	83.5	18.5	27.2	18.1	17.3
C-17-P, 5 μg/ml	n	96.4	62.6	—	—	93.5	13.5	—	—	10.5	2.5	—	—
	TCA	3.6	37.4	—	—	6.5	86.5	—	—	19.5	26.3	—	—

* Aliquots (1.9–5.1 × 10^5 d.p.m.) of the growth media of experiments 1 and 2 (see Table 1) were precipitated with 65 per cent ammonium sulfate as described in Materials and Methods. The resulting precipitate was dissolved and dialysed exhaustively against collagenase buffer. The dissolved precipitates were used as such.

For each growth condition a blank solution (−) without added collagenase was incubated for 90 min together with samples containing 30 μg of purified bacterial collagenase (+) and incubated for 90 min. At the end of the incubation TCA was added to precipitate the proteins as described under Materials and Methods.

Shown are ^{3}H-protein and ^{3}H-Hydroxyproline radioactivity. The values listed are percentages of each radioactivity precipitated with TCA: n, and of that remaining in the TCA-supernatants (TCA).

Proline hydroxylation is listed as the percentage.

in increased underhydroxylation, and furthermore that the decrease in hydroxyproline formation in the presence of the steroids was due to a decrease in collagen synthesis.

DISCUSSION

Our results show that in our experimental system with cultured baby foreskin fibroblasts hydrocortisone-17-butyrate (5 μg/ml) and clobetasol-17-propionate (1 or 5 μg/ml) cause a specific depression of the formation of protein-bound hydroxyproline (Table 1). This depression appears to be due to an overall decrease in the collagenous protein deposited in the cell layer and released into the growth medium. This effect is not accompanied by a decrease in cellular prolyl hydroxylase activity (Table 2), as measured in cell extracts, nor in a decrease in collagen proline hydroxylation (Tables 3 and 4). Whether the decrease in collagenous protein occurs for both Type I and III collagen to a similar degree remains to be established. Neither do we know, at the moment, whether the decrease in collagenous protein is due to a decrease either in the translation or in the transcription of collagen mRNA. The studies of Cutroneo *et al.* [11–13], however, suggest that there may well be less translatable mRNA in the presence of glucocorticosteroids.

A decrease in prolyl hydroxylase activity may not affect the degree of proline hydroxylation [17–19]. The degree of proline hydroxylation may be cell-specific rather than dependent on growth conditions. This is suggested by the results of Russell *et al.* [9] who found that hydrocortisone specifically depressed the synthesis of collagenous protein by normal human fibroblasts, while it had no effect on collagen synthesis by fibroblasts from keloid skin. Also, the co-ordinate decrease of collagenous and non-collagenous protein synthesis and of prolyl hydroxylase activity of mouse dermal sponge fibroblasts grown in the presence of hydrocortisone, as found by Kruse *et al.* [5], may be an effect specific for this type of cell. In view of this evidence the overall degree of hydroxylation of the collagen synthesized in the presence of glucocorticosteroids may not be influenced if the prolyl hydroxylase activity in the experimental system is either lowered [5, 7, 8, 11, 12] or unaffected (this study).

The absence of an effect on prolyl hydroxylase activity in our system, as measured in an *in vitro* assay with an excess of cofactors and with cell extracts does not imply, however, that enzyme activity *in vivo* was not influenced by the steroids. Such an *in vivo* effect could arise secondarily by an effect on the cofactors required for enzyme activity, such as ketoglutarate and Fe^{2+} [16]. In this study we did not investigate this point. In any case, the steroids did not cause significant underhydroxylation of the collagenous protein present in the medium.

Several reports have appeared showing that in a variety of systems glucocorticosteroids will inhibit the cellular uptake of amino acids [20–24] and increase that of hexoses [25, 26]. Although this conceivably may influence protein synthesis in our system, it seems unlikely that this would have resulted specifically in diminished collagen synthesis.

Glucocorticosteroids have been shown to have an effect on collagenase production. Hook *et al.* [27] have found that dexamethasone will stimulate such activity in corneal fibroblasts. On the other hand, Koob *et al.*

[28] reported that in various *in vitro* systems the activity of this enzyme is reduced in the presence of hydrocortisone or dexamethasone. Studies on collagenase production have to be performed in serum-free medium, because serum is known to contain potent inhibitors of this enzyme and of other proteolytic activities [29, 30]. Since we have performed all our experiments with media containing 12 per cent of fetal calf serum, it seems therefore unlikely that the steroids could have stimulated collagen degradation in our system.

The values for the degree of hydroxylation of the collagenous protein solubilized from the medium protein by the purified bacterial collagenase (which was free from other proteolytic activities, see Materials and Methods) varied between 17.3 and 27.2 per cent at the various conditions (see Table 4). These values are lower than those of 40–45 per cent reported for embryonic chick cranial bone [31–33], embryonic chick tendon fibroblasts [34], and skin fibroblasts [35]. The reason for this is unclear, at the present time, since tryptophan labeled non-collagenous proteins were not digested by collagenase (see Materials and Methods).

Besides decreasing collagen synthesis by cultured dermal fibroblasts glucocorticosteroids also appear to lower the synthesis of various kinds of proteoglycans [10, 23, 36–39]. Since these steroids have a variety of biological effects on fibroblasts (see ref. 40 for a review), it is to be expected that the synthesis of still other products specific for these cells will also be affected by these drugs.

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