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EFFECT OF A SINGLE INJECTION OF BETAMETHASONE DISODIUM PHOSPHATE ON THE SYNTHESIS OF COLLAGEN AND NONCOLLAGEN PROTEIN OF CARRAGEENIN GRANULOMA IN RATS

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Abstract—The effect of a single injection of betamethasone disodium phosphate on the incorporation of [^{3}H]proline into collagen and noncollagen protein of rat carrageenin granuloma was studied. In all series of experiments, both [^{3}H]proline and the steroid were given intravenously and the animals were killed at 30 min after the labeled proline injection in order to investigate precisely the time course of the drug action. The incorporation was not influenced at all by the steroid when it was given 30 min before the injection of [^{3}H]proline. The inhibitory effect of the steroid, for both the collagen and the noncollagen protein, became significant at 1 hr after its intravenous administration, increasing progressively with the passage of time. Inhibition of the labeled proline incorporation into collagen hydroxyproline was significantly greater than that into noncollagen protein. Neither accumulation of protocollagen nor any change of protocollagen proline hydroxylase activity was demonstrated in the granuloma obtained from the rats treated with the steroid. The results show that the steroid does not inhibit the process of hydroxylation of protocollagen, suggesting indirectly the inhibition of synthesis of protocollagen or of [^{3}H]proline transport through cell membrane, or of both.

THE MODE of action of anti-inflammatory glucocorticoids is not yet well understood, although a number of studies have been reported on this subject.[1–4] Fukuhara and Tsurufuji[5] reported that, among steroid and some nonsteroid anti-inflammatory drugs, only steroids were effective for inducing the involution of pre-existing carrageenin granuloma in rats. Concerning the mechanism of this involution, Ohno and Tsurufuji[6] demonstrated that the action of glucocorticoids on the protein metabolism of the granuloma was anti-anabolic rather than catabolic. Fukuhara and Tsurufuji[7] reported also that betamethasone disodium phosphate injected daily into the pouch of pre-existing carrageenin granuloma for 4 days markedly inhibited collagen synthesis as well as synthesis of noncollagen protein, although some nonsteroid anti-inflammatory drugs were ineffective.

Further biochemical study on the action of the steroid would be important to elucidate the mode of action of glucocorticoids. In the present experiments, therefore, the acute effect of a single injection of betamethasone disodium phosphate on the protein synthesis of preformed carrageenin granuloma has been investigated by measuring the incorporation of labeled proline into collagen hydroxyproline and noncollagen protein. The degree of hydroxylation of protocollagen and the level of

protocollagen proline hydroxylase activity in the granulomatous tissue from rats treated with the steroid were also measured in order to analyze the mechanism of the inhibitory effect of the steroid on collagen synthesis.

EXPERIMENTAL

Treatment of animals

A granuloma pouch was induced in male rats of the Donryu strain weighing from 100 to 120 g (42 days old) by injecting a 2% solution of carrageenin s.c. on day 0. Details of the procedure described previously[5] were followed with slight modification using Seakem 202 carrageenin (Marine Colloid Inc., Springfield, N.J., U.S.A.) instead of TS-36 carrageenin. On day 8, betamethasone disodium phosphate (2 mg/rat; Δ^1, 9α-fluoro-16 β-methylcortisol-17-disodium phosphate) was administered i.v., while control animals were given 0·9% NaCl i.v. A dose of 30 μc per rat of [^{3}H]L-proline (29 c/m-mole, generally labeled) was injected i.v. at 0·5, 1, 2 or 4·5 hr, respectively, after the steroid injection and the animals were sacrificed 30 min later.

Analysis of collagen and noncollagen protein

The pouch of the granulomatous tissue was harvested. Collagen of the pouch was extracted as gelatin by autoclaving the tissue and hydrolyzed with 6 N HCl at 105° for 16 hr. The specific activity of hydroxyproline in the hydrolysate was measured after converting hydroxyproline into pyrrol[8] which was purified by passing through a silicic acid column.[9] The values were expressed as disintegrations per minute per microgram of hydroxyproline. The residue obtained by autoclaving the tissue was extracted once again in the autoclave to remove collagen completely. The collagen-free residue was homogenized in 8% perchloric acid by a glass homogenizer and then centrifuged. A homogeneous suspension of the precipitate in 5 ml water was made by vigorous shaking. The amount of noncollagen protein in an aliquot of the suspension was measured by the method of Lowry *et al.*[10] Another aliquot of the suspension was solubilized with the aid of 1 ml of Soluene 100 (Packard Co., Downers Grove, Ill., U.S.A.) and then 16 ml of a liquid scintillator (0·6% 2,5-diphenyloxazole in toluene) was added. The specific activity of the noncollagen protein was expressed as disintegrations per minute per microgram of protein equivalent to bovine serum albumin which was used as a reference standard.

The radioactivity was measured in a Packard liquid scintillation spectrometer model 3950 with adequate correction for quenching.

Total incorporation of [^{3}H]proline into collagen and noncollagen protein per granuloma should be in proportion to the specific activity of each protein, since the total amounts of the two proteins of a single granuloma would not have been changed significantly by the short-term treatment with the steroid used in the present experiment.

Incubation in vitro *of minced granuloma*

The granulomatous tissues from rats which had been given [^{3}H]proline were taken immediately after sacrifice and minced. The minced granuloma (1·5 g) was incubated under an atmosphere of 95% O_2—5% CO_2 at 37° in 30 ml of Krebs' saline serum substitute (KSSS)[11] containing 0·1 mM $FeSO_4$, 0·1 mM α-ketoglutarate, 0·2 mM

ascorbic acid, 0·2 mM L-proline and 3 mg each of penicillin and dihydrostreptomycin. At the end of the incubation, 50% trichloroacetic acid was added to give a 10% concentration and the flasks were chilled in ice water. The contents were centrifuged and the precipitate was washed with ethanol three times. Collagen of the precipitate was extracted as gelatin by autoclaving and the residue was used for the assay of noncollagen protein. The specific activity of hydroxyproline in gelatin and that of noncollagen protein were measured by the method described above.

Measurement of protocollagen proline hydroxylase activity

The substrate for the protocollagen proline hydroxylase was prepared from 8-day-old granuloma. The minced granuloma (35 g) was incubated at 37° for 2 hr under 95% O_2–5% CO_2 in 120 ml of KSSS containing 1 mM α,α′-dipyridyl and 500 μc [^{3}H]L-proline. After incubation, the medium was removed by decantation and the minces were washed with cold KSSS three times. The minces were suspended in 50 ml of 10% trichloroacetic acid and shaken at 70° for 1 hr and then the contents were filtered. The filtrate was thoroughly dialyzed against tris–HCl buffer (50 mM, pH 7·6) containing 100 mM KCl. The dialysate was used as [^{3}H]proline-labeled protocollagen substrate. The substrate solution contained $3·7 \times 10^5$ dis./min/ml, of which 1·2 per cent occurred as [^{3}H]hydroxyproline.

On day 8 after carrageenin injection, betamethasone disodium phosphate (2 mg/rat) or 0·9% NaCl was administered i.v. and the animals were sacrificed 4·5 hr later. The granulomatous tissues were taken immediately after sacrifice and minced. The minced granuloma (0·8 g) was homogenized in 8 ml of ice-cold 0·1 M KCl, 0·05 M tris–HCl buffer (pH 7·6) with a Potter–Elvehjem homogenizer. The homogenate was centrifuged at 15,000 *g* for 90 min at 1°, and 1 ml of the supernatant was used for the assay of protocollagen proline hydroxylase activity on that day. The incubation medium contained 5–6 mg of the 15,000 *g* supernatant protein, [^{3}H]proline-labeled protocollagen substrate ($1·1 \times 10^5$ dis./min), 0·2 mM $FeSO_4$, 0·2 mM α-ketoglutarate, 1·0 mM ascorbic acid, 100 mM KCl and 50 mM tris–HCl buffer (pH 7·6) in a final volume of 5·0 ml. The mixtures were incubated with shaking at 37° for 60 min in air. Reactions were stopped by adding 5 ml of conc. HCl and the samples were hydrolyzed at 105° for 16 hr. The specific activity and the amount of hydroxyproline in the hydrolysate were then assayed by the methods described above, and the total radioactivity (dis./min) of [^{3}H]hydroxyproline in the hydrolysate was calculated. The radioactivity of collagen hydroxyproline produced during incubation was obtained by deducting the radioactivity of [^{3}H]hydroxyproline existing in the hydrolysate of [^{3}H]proline-labeled protocollagen substrate from the radioactivity of [^{3}H]hydroxyproline in the hydrolysate of the incubation products. The enzyme activity was expressed as the radioactivity (dis./min) of [^{3}H]hydroxyproline synthesized per milligram of 15,000 *g* supernatant protein.

RESULTS

Incorporation of [^{3}H]*proline into collagen and noncollagen protein* in vivo

The effects of betamethasone disodium phosphate on the incorporation of labeled proline into collagen and noncollagen protein are shown in Table 1. At 1 hr after the steroid treatment, its incorporation was significantly inhibited in both the proteins.

TABLE 1. EFFECT OF A SINGLE INJECTION OF BETAMETHASONE DISODIUM PHOSPHATE ON [^{3}H]PROLINE INCORPORATION INTO COLLAGEN AND NONCOLLAGEN PROTEIN OF CARRAGEENIN GRANULOMA*

Treatment	Time of [3H]-proline injection after treatment† (hr)	No. of animals	(A) Sp.act. of hydroxyproline (dis./min/μg hyp.)	(B) Sp.act. of noncollagen protein (dis./min/μg protein)	Ratio A/B
Control	0·5	8	5·249 ± 0·257	2·510 ± 0·141	2·109 ± 0·088
Betamethasone	0·5	8	5·997 ± 0·375 N.S.	2·618 ± 0·010 N.S.	2·241 ± 0·093 N.S.
Control	1·0	8	6·191 ± 0·201	3·139 ± 0·162	1·990 ± 0·065
Betamethasone	1·0	8	4·650 ± 0·241‡ (24·1)	2·604 ± 0·107§ (17·0)	1·787 ± 0·060§
Control	2·0	9	7·309 ± 0·512	3·947 ± 0·100	1·846 ± 0·105
Betamethasone	2·0	9	4·853 ± 0·364‡ (33·6)	3·088 ± 0·142‡ (21·8)	1·564 ± 0·082§
Control	4·5	8	7·352 ± 0·512	3·817 ± 0·711	1·929 ± 0·084
Betamethasone	4·5	8	2·794 ± 0·294‡ (62·0)	2·371 ± 0·446‡ (37·9)	1·167 ± 0·065‡

* Data are shown as means ± S.E. N.S. = not significant; hyp. = hydroxyproline. The per cent inhibition is shown in parentheses.

† At the time indicated, [^{3}H]L-proline (30μc/rat) was injected i.v. and the animals were sacrificed 30 min later.

‡ Difference from control is significant at $P < 0·01$.

§ Difference from control is significant at $P < 0·05$.

The inhibitory effect increased progressively with time and the inhibition of [^{3}H]-proline incorporation into collagen hydroxyproline was significantly higher than that into noncollagen protein.

Hydroxylation of protocollagen through incubation of minced granuloma

Incorporation of [^{3}H]proline into collagen hydroxyproline consists of two steps, its incorporation into protocollagen and hydroxylation of incorporated proline. In order to test the possibility of incomplete hydroxylation of protocollagen (protocollagen accumulation) in the granuloma treated with glucocorticoid, a series of incubation experiments were undertaken. Figure 1 indicates that hydroxylation of accumulated protocollagen appears to take place during incubation of minced granuloma which has been obtained from rats treated with α,α'-dipyridyl. Formation of [^{3}H]hydroxy-proline reached a plateau at 1 hr under the incubation conditions employed. Consequently, a 90-min incubation was used in all of the following incubation experiments.

A second series of incubation experiments (Table 2) were done to demonstrate that the specific activity of hydroxyproline of the dipyridyl-treated group increased by six times during the incubation, while no increase was shown in the incubation of the control group. It is unlikely that *de novo* synthesis of collagen from free [^{3}H]proline remaining in the minced tissue occurred to an extent sufficient to elevate the specific

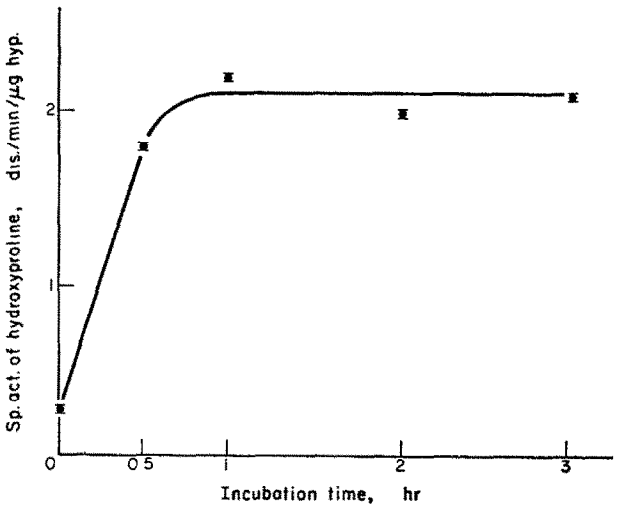

FIG. 1. Time course for the hydroxylation of [^{3}H]proline-labeled protocollagen in minced granuloma. α, α′-Dipyridyl (10 mg/rat) was injected into the pouch fluid of an 8-day-old granuloma 30 min before the intraperitoneal injection of [^{3}H]proline (30 μc/rat). The rats were sacrificed 1 hr after [^{3}H]proline injection and the minced granuloma was incubated under the conditions described in the text. Each point represents the mean ± S.E. of determinations.

activity of hydroxyproline, since no significant increase in the specific activity of noncollagen protein took place during the incubation. Therefore, it is evident that hydroxylation of protocollagen, if any accumulated in the granuloma tissue, may proceed effectually when minced granuloma is incubated under appropriate conditions such as those described above.

Based on the results of preliminary experiments described above, incubation of minced granuloma from groups of rats injected i.v. with betamethasone disodium phosphate was performed. The rats were given [^{3}H]proline i.v. 4·5 hr after the steroid injection. Some of the steroid-treated rats were also injected with α,α′-dipyridyl into the granuloma pouch fluid, 30 min before the [^{3}H]proline injection, in order to to cause the accumulation of labeled protocollagen in the tissue. Incubation of minced

TABLE 2. INCUBATION OF MINCED GRANULOMA FROM RATS INJECTED WITH α,α′-DIPYRIDYL*

Treatment of rats†	No. of rats	Sp.act. of hydroxyproline (dis./min/μg hyp.)		Sp.act. of noncollagen protein (dis./min/μg protein)	
		No incubation	90-min incubation	No incubation	90-min incubation
Control	6	6·474 ± 0·592	5·952 ± 0·585 N.S.	3·245 ± 0·103	3·673 ± 0·129 N.S.
α,α′-Dipyridyl (10 mg/rat)	6	0·489 ± 0·085	3·255 ± 0·371 P < 0·001	2·788 ± 0·243	3·289 ± 0·226 N.S.

* Results are shown as means ± S.E. See Table 1 for abbreviations.
† Treatment of rats and incubation conditions are described in legend to Fig. 1.

TABLE 3. INCUBATION OF MINCED GRANULOMA FROM RATS TREATED WITH BETAMETHASONE DISODIUM PHOSPHATE*

Treatment of rats	No. of rats	Sp.act. of hydroxyproline (dis./min/ μg hyp.)		P
		No incubation	90-min incubation	
Control	6	5·718 ± 0·272	5·566 ± 0·286	N.S.
Betamethasone	7	2·781 ± 0·253	2·793 ± 0·198	N.S.
Betamethasone + α,α′-dipyridyl†	5	0·167 ± 0·034	1·271 ± 0·092	< 0·001

* Results are shown as means ± S.E. See Table 1 for abbreviations.

† α,α′-Dipyridyl (10 mg/rat) was administered into granuloma pouch fluid 4 hr after i.v. injection of betamethasone disodium phosphate (2 mg/rat). [^{3}H]proline (30 μc/rat) was injected i.v. 30 min after α,α′-dipyridyl treatment. The animals were sacrificed 30 min later. The incubation was carried out as shown in the text.

granuloma from these double-treated rats indicated that accumulating protocollagen, if there was any, could be hydroxylated during the incubation in the steroid-treated (Table 3) as well as in the untreated granuloma (Table 2).

In the group treated with betamethasone only, the incubation did not increase the specific activity of hydroxyproline of granuloma collagen, indicating that the steroid did not exert an inhibitory effect on the hydroxylation of protocollagen which would account for the accumulation of protocollagen in the tissue.

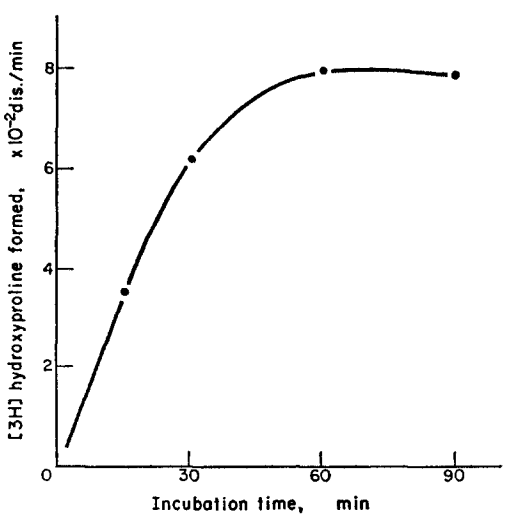

FIG. 2. Time course for the hydroxylation of [^{3}H]proline-labeled protocollagen. Incubation was carried out under the conditions described in the text, except that a Vir-Tis homogenizer was used for homogenization of the 8-day-old granuloma, and the 15,000 *g* supernatant protein (4·2 mg/flask) which had been stored at − 20° for 1 week was used.

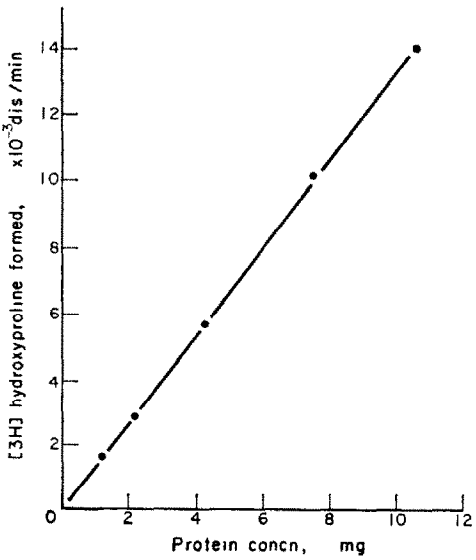

FIG. 3. Effect of enzyme concentration on the hydroxylation of [^{3}H]proline-labeled protocollagen. [^{3}H]proline-labeled protocollagen ($1{\cdot}1 \times 10^5$ dis./min) was incubated with varying amounts of the enzyme fraction from rat carrageenin granuloma (8 days old) under the conditions described in the text.

Assay of protocollagen proline hydroxylase activity of granuloma

The hydroxylation of [^{3}H]proline-labeled protocollagen reached a plateau at 60 min under the incubation conditions used (Fig. 2), and the amount of [^{3}H]hydroxyproline formed was proportional to the amount of 15,000 *g* supernatant protein added (Fig. 3). Consequently, a 60-min incubation and 5–6 mg of the 15,000 *g* supernatant protein were used for the assay of protocollagen proline hydroxylase activity of the granulomas.

Table 4 demonstrated that betamethasone disodium phosphate did not cause any decrease in the enzyme activity at 4·5 hr after the steroid treatment, although the incorporation of [^{3}H]proline into collagen hydroxyproline was markedly inhibited (62 per cent inhibition) by the steroid at that time (Table 1).

TABLE 4. PROTOCOLLAGEN PROLINE HYDROXYLASE ACTIVITY IN GRANULOMAS FROM CONTROL RATS AND RATS TREATED WITH BETAMETHASONE DISODIUM PHOSPHATE*

Treatment of rats	No. of rats	Hydroxyproline formed (dis./min/mg protein)	
Control	6	181·10 ± 4·45	
Betamethasone	6	186·70 ± 4·75	(N.S.)

* Results are shown as means ± S.E. Treatment of rats and incubation conditions are described in the text.

DISCUSSION

The incorporation of [^{3}H]proline into collagen and noncollagen protein of rat carrageenin granuloma was significantly inhibited at 1 hr after the steroid treatment. The inhibitory effect increased progressively with time, although at 30 min after the treatment both proteins still remained unaffected. At all times longer than 30 min, the inhibitory effect of the steroid was significant and the extent of the inhibition was significantly greater in the case of collagen than in that of noncollagen protein (Table 1). This result indicates that the synthesis of individual proteins of the granuloma may be affected separately by glucocorticoids, eliminating the possibility of nonspecific inhibition of protein biosynthesis in the granuloma tissue. Another possible explanation for the difference in the degree of inhibition is to assume that the extent of the effect of glucocorticoids differs from one type of cell to another in the granuloma.

A similar result showing a greater inhibitory effect of a glucocorticoid on the synthesis of collagen than on that of noncollagen protein was also reported by Ebert and Prockop.[12] One possible mechanism responsible for this fact is, as they suggest, the inhibition by the steroid of a specific step in collagen biosynthesis such as hydroxylation of protocollagen. A possible vasoconstrictive effect of corticoids may produce tissue anoxia, which might inhibit the hydroxylation process, since this process requires molecular oxygen.[13–15] If hydroxylation of protocollagen in the granuloma tissue is inhibited by the steroid, accumulation of protocollagen would take place in the tissue, as in the case of the granuloma treated with α,α'-dipyridyl (Table 2).

Hydroxylation of [^{3}H]protocollagen accumulating in the granuloma tissue proceeds by incubating the minced granuloma in an appropriate medium. As shown by the bottom figures in Table 3, [^{3}H]protocollagen in the granuloma doubly treated with the steroid and α,α'-dipyridyl could be hydroxylated effectively. No increase, however, in the specific activity of collagen hydroxyproline was seen when minced granuloma from the rats treated with betamethasone disodium phosphate was incubated in a medium in which active hydroxylation would have taken place if any accumulation of [^{3}H]proline-labeled protocollagen had been present (Table 3). It may be concluded, therefore, that glucocorticoids do not inhibit the hydroxylation step of collagen biosynthesis.

As shown in Tables 2 and 3, two series of experiments were done with the aid of α,α'-dipyridyl. The data of these experiments show that groups treated with α,α'-dipyridyl gave lower specific activity for hydroxyproline even after completion of the hydroxylation reaction *in vitro* (55 and 47 per cent of their corresponding controls). For calculating this per cent specific activity for the experiment in Table 3, the value of the group treated with betamethasone only was used as control, because in this series of experiments α,α'-dipyridyl was given in combination with betamethasone. α,α'-Dipyridyl does not cause any significant decrease in the specific activity of noncollagen protein compared with the control. It is unlikely, therefore, that dipyridyl inhibits protein synthesis in general, agreeing with the report by Chvapil *et al.*[16] We can only speculate upon the cause for the lower specific activity of hydroxyproline after incubation in the dipyridyl-treated group: (1) the efficiency of hydroxylation *in vitro* may be lower; (2) protocollagen may degrade when it is accumulated because of its high susceptibility to tissue proteases;[17] or (3) accumulating protocollagen may inhibit hydroxylation or synthesis of protocollagen, or both, by some feedback mechanism. The fact that incubation of the granuloma from rats treated with both the steroid and

dipyridyl gave hydroxyproline with a specific activity that was 47 per cent of the control and that the group treated with dipyridyl only gave an equivalent specific activity (55 per cent of their own control) appear to support the concept that glucocorticoids do not affect hydroxylation of protocollagen.

Further evidence supporting this concept was obtained by direct assay of the enzyme activity of protocollagen proline hydroxylase of the granuloma treated with betamethasone disodium phosphate (Table 4). The enzyme activity was not influenced by the steroid treatment. All of this evidence suggests that the inhibitory effect of the steroid on [^{3}H]-proline incorporation into collagen hydroxyproline is caused by inhibition of protocollagen synthesis or by depression of [^{3}H]-proline transport through cell membrane, or by both.

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