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ACTION OF BETAMETHASONE DISODIUM PHOSPHATE ON THE METABOLISM OF COLLAGEN AND NONCOLLAGEN PROTEIN IN RAT CARRAGEENIN GRANULOMA

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Abstract—The mode of action of betamethasone disodium phosphate, a steroidal anti-inflammatory drug, on the metabolism of collagen and non-collagen protein in rat carrageenin granuloma was studied. The inhibitory effect of the steroid on the incorporation of [^{3}H]proline into collagen and noncollagen protein was progressively increased with an increase in dose and reached a maximum at a level of 0·2 mg/rat. The effect of the steroid on the degradation of collagen and noncollagen protein labeled with [^{3}H]proline was investigated. Betamethasone disodium phosphate inhibited the degradation of collagen *in vivo* and markedly reduced the amount of dialyzable hydroxyproline formed by the degradation of collagen during incubation of the minced granuloma *in vitro*. The degradation of noncollagen protein, in contrast with the degradation of collagen, was apparently not affected by the steroid treatment. It seems likely that betamethasone disodium phosphate, a steroidal anti-inflammatory drug, causes the resorption of pre-existing granulomatous tissue through a strong inhibitory action on protein synthesis without apparently affecting the degradation of noncollagen protein, even though the degradation of collagen is inhibited.

THE EFFECT of glucocorticoids on protein degradation has not been well studied, although the steroids have been found to inhibit protein synthesis. Houck *et al.*[1–5] found that the administration of cortisol resulted in a decrease in collagen concentration of rat skin, and collagenolytic and proteolytic activities were induced by anti-inflammatory drugs including cortisol. In contrast, Kivirriko *et al.*[6,7] and Smith and Allison[8] reported that the administration of cortisone decreased the excretion of hydroxyproline in the urine of rats, suggesting that: (1) the action of cortisone on collagen metabolism was mainly antianabolic, and (2) the catabolism of collagen was not affected.

In order to investigate the action of glucocorticoids as anti-inflammatory drugs on collagen metabolism, an inflamed tissue should be used as the experimental material, since collagen of inflamed tissue turns over at a considerably more rapid rate than the collagen of normal tissues.[9,10] In previous papers[11–13] it was shown by experiments using the carrageenin granuloma, an inflamed tissue, that glucocorticoids had a strong inhibitory effect on the synthesis of collagen and noncollagen protein, as well as a slight enhancement of the degradation of noncollagen protein. However, the effect of the steroids on collagen breakdown of the granuloma is not well understood. In the present study, therefore, the mode of action of betamethasone disodium phosphate, a steroidal anti-inflammatory drug, on the degradation of

[^{3}H]proline-labeled collagen and noncollagen protein of pre-existing carrageenin granuloma was investigated by injecting the steroid into the granuloma pouch daily for 3 days and analyzing the amounts and radioactivities of proteins. The inhibitory effect of the steroid on the synthesis of collagen and noncollagen protein was also studied by measuring the incorporation of [^{3}H]proline into proteins after a single injection of various doses of the steroid.

EXPERIMENTAL

Young male rats of the Donryu strain weighing 100–120 g were used in groups of seven or eight. A granuloma pouch was produced subcutaneously on the back of the rats by injecting carrageenin according to the procedure previously described,[12] with a slight modification using Seakem 202 carrageenin (Marine Colloid Inc., Springfield, N.J., U.S.A.) instead of TS-36 carrageenin.

Dose–response relationship for betamethasone disodium phosphate. Various doses of betamethasone disodium phosphate (Δ^1, 9α-fluoro, 16β-methyl-cortisol-17-disodium phosphate; 0·02, 0·07, 0·2 or 2·0 mg/rat in 0·2 ml of 0·9% NaCl) were injected i.v. on day 8 (8 days after carrageenin injection), while control animals were given 0·9% NaCl i.v. [^{3}H]L-Proline (30 μc/rat) was injected i.v. 4·5 hr after the steroid treatment, and 30 min later the animals were sacrificed. From the granulomatous tissue harvested, collagen and noncollagen protein were isolated, and their specific activities were measured according to the procedure described in a previous paper.[13]

Treatment in vivo *with betamethasone disodium phosphate.* A dose of 30 μc/100 g of body weight of [^{3}H]L-proline (266 mc/m-mole) was injected i.v. on day 6. Seven animals assigned for the initial control were killed on day 7 (7-day-control). The treated group was given the steroid (0·2 mg/rat) into the granuloma pouch daily from day 7 to day 9, and killed on day 10. L-Proline (2 mg/rat) was injected into granuloma pouches of both the steroid-treated and control rats every 12 hr from day 7 to day 10 in order to reduce the reutilization of radioactive-free proline, which was released through the degradation of metabolically labile proteins. The entire fluid in the pouch (designated as "pouch fluid") and the capsule of the granulomatous tissue were harvested and weighed immediately after sacrifice. Net body weight was calculated by subtracting pouch fluid and granulomatous tissue weights from the gross body weight.

Granulomatous tissue was minced into 1–2-mm pieces in an ice-cold Petri dish. The minced granuloma (800 mg) was incubated under an atmosphere of 95% O_2–5% CO_2 at 34° for 6 hr in 8 ml of Krebs saline serum substitute[14] (KSSS) containing 0·2 mM proline and 0·8 mg each of potassium penicillin-G and dihydrostreptomycin sulfate. At the end of the incubation period, cysteine was added to give a concentration of 10 mM and the mixture was frozen in ethanol–dry ice. Another 800 mg of the minced granuloma was frozen without incubation in 8 ml of incubation medium containing cysteine at a concentration of 10 mM (designated as "no incubation" flask). The frozen contents of the incubation and no incubation flasks were thawed and homogenized in a Potter–Elvehjem glass homogenizer at 3°. The homogenate was dialysed against 50 ml of distilled water with a small amount of toluene as a preservative. Dialysis was carried out at 4° for 5 days with occasional shaking. The amounts of free hydroxyproline and dialysable tyrosine in aliquots of the dialysate were measured by the methods of Kivirikko *et al.*[15] and of Ceriotti and Spandrio[16]

respectively. The amount of total dialysable hydroxyproline was measured by the method of Kivirikko *et al.*[15] after hydrolysis of an aliquot of the dialysate with 6 N HCl at 105° for 16 hr. Amounts of free and total dialysable hydroxyproline and dialysable tyrosine formed during incubation were calculated by subtracting the values of the no incubation flask from those of the incubation flask, respectively.

For the isolation of noncollagen protein, the dialysed content of the no incubation flask was heated in an autoclave at 110° for 2 hr and centrifuged. The precipitate was suspended in 20 ml of distilled water and once again heated in an autoclave to remove collagen completely. After centrifugation, the resulting collagen-free precipitate (i.e. noncollagen protein) was homogenized in 10 ml of distilled water in a glass homogenizer. The amount and radioactivity of noncollagen protein were measured by the method described in a previous paper.[13]

Collagen contained in the granulomatous tissue was extracted twice as gelatin by autoclaving at 110° for 2 hr from an aliquot of the minced granuloma. The extract was hydrolysed at 105° for 16 hr and the amount and radioactivity of collagen hydroxyproline were measured according to the procedure described in a previous paper.[13]

Treatment in vitro *with betamethasone disodium phosphate.* Granulomatous tissue was removed and minced immediately after sacrifice of rats bearing an 8-day-old granuloma. The minced granuloma (800 mg) was incubated under an atmosphere of 95% O_2–5% CO_2 at 34° for 6 hr with or without 2×10^{-5} M betamethasone disodium phosphate in 8 ml KSSS containing 0·8 mg each of potassium penicillin-G and dihydrostreptomycin sulfate. The reaction was stopped by freezing in ethanol–dry ice. The no incubation flask containing 800 mg of the minced granuloma and 8 ml KSSS was also placed in ethanol–dry ice. The amounts of free and total dialysable hydroxyproline formed during incubation by collagen degradation were determined by the procedures described above.

RESULTS

Incorporation of [^{3}H]*proline into collagen and noncollagen protein.* The inhibitory effects of various doses of betamethasone disodium phosphate on the incorporation of [^{3}H]proline into collagen and noncollagen protein of the carrageenin granuloma are summarized in Table 1. The steroid inhibited the incorporation of [^{3}H]proline into collagen hydroxyproline to a greater extent than that into noncollagen protein at all the doses used. This observation was in good agreement with the results reported in a previous paper.[13] The inhibitory effects of the steroid increased progressively with the increase in dose and reached a maximum at a dose of 0·2 mg/rat. Similar results were obtained for collagen synthesis.

Degradation of collagen and noncollagen protein. The results are summarized in Table 2. The body weight of steroid-treated rats was significantly lower than that of the controls. It seems likely that this body weight loss involves some change in protein metabolism of rats treated with the steroid. However, the protein metabolism of the granuloma may not be markedly influenced by growth retardation, since it was demonstrated in a previous paper[12] that a dietary limitation that induced body weight loss had little effect on the formation and maintenance of the carrageenin granuloma.

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

Dose	No. of animals	Collagen Sp. act. (dis./min/μg hyp.)	Collagen Per cent inhibition	Noncollagen protein Sp. act. (dis./min/μg prot.)	Noncollagen protein Per cent inhibition
Expt. 1					
Control	7	3·184 ± 0·126		1·993 ± 0·112	
0·02 mg/rat	7	2·255 ± 0·064†	29·2	1·696 ± 0·066‡	14·9
0·07 mg/rat	7	1·590 ± 0·161†	50·1	1·441 ± 0·046†	27·7
Expt. 2					
Control	7	3·516 ± 0·306		2·574 ± 0·144	
0·2 mg/rat	7	1·462 ± 0·171†	58·4	1·985 ± 0·072†	22·9
Expt. 3§					
Control	8	7·352 ± 0·512		3·817 ± 0·711	
2·0 mg/rat	8	2·794 ± 0·294†	62·0	2·371 ± 0·446†	37·9

* Data are shown as means ± S.E.
† Significantly different from control ($P < 0·01$).
‡ Significantly different from control ($P < 0·05$).
§ A dose of 30 μc/rat of [^{3}H]L-proline was injected in all the experiments. Specific activity of [^{3}H]L-proline: 266 mc/m-mole (The Radiochemical Centre, Amersham, England) for Expt. 1 and Expt. 2, 29 c/m-mole (Dai-ichi Kagaku Co., Ltd., Tokyo, Japan) for Expt. 3.

As shown in Table 2, the amount of total hydroxyproline in the entire granuloma of the control group increased markedly during the period from day 7 to day 10, while its radioactivity was significantly decreased. On the other hand, neither the amount nor the radioactivity of total hydroxyproline in the entire granuloma of the steroid-treated group changed significantly during the same period. These results suggest that betamethasone disodium phosphate inhibited both the synthesis and the degradation of collagen in the carrageenin granuloma. The inhibitory effect of the steroid on the degradation of collagen was further demonstrated by incubation experiments *in vitro* of the minced granuloma (Table 3). The amounts of both free and total dialysable hydroxyproline formed by the degradation of collagen during incubation were significantly less in the steroid-treated group than in the control groups.

Turning to the problem of the total noncollagen protein content of the entire granuloma, the control increased by 52 per cent from day 7 to day 10, whereas its radioactivity decreased by 41 per cent. On the other hand, in the steroid-treated group, the total noncollagen protein content did not increase, but decreased slightly, and its radioactivity decreased to an extent similar to that of the control. These observations seemed to be consistent with the data in Table 3 showing that the amount of dialysable tyrosine formed by the degradation of non-collagen protein during incubation of the minced granuloma was not significantly changed by treating animals for 3 days with the steroid, though the value of the 10-day control was not demonstrated. These results seemed to suggest that betamethasone disodium phosphate inhibited considerably the synthesis of noncollagen protein, but did not apparently influence the degradation of noncollagen protein.

TABLE 2. EFFECT OF LOCALLY INJECTED BETAMETHASONE DISODIUM PHOSPHATE ADMINISTERED DAILY FROM DAY 7 UP TO DAY 9 ON THE PREFORMED CARRAGEENIN GRANULOMA LABELED WITH [^{3}H]PROLINE*

	7-Day-control	10-Day-control	Betamethasone treatment
No. of animals	7	7	7
Net body wt. (g)	122·2 ± 3·9	129·2 ± 4·2	106·9 ± 4·0†,‡
Pouch fluid (g)	7·5 ± 1·9	19·0 ± 5·5	5·8 ± 2·3§
Granulomatous tissue (g wet wt.)	4·25 ± 0·27	5·39 ± 0·32†	2·51 ± 0·13‡,‖
Collagen			
Total hyp. in entire granuloma (mg)	8·776 ± 0·924	14·487 ± 1·243‖	9·777 ± 0·921§
Hyp. (mg/g wet wt.)	2·050 ± 0·126	2·663 ± 0·111‖	3·900 ± 0·299§,‖
Sp. act. of hyp. (dis./min/μg hyp.)	89·124 ± 7·440	39·887 ± 2·335‖	71·953 ± 4·664‡
Radioactivity of total hyp. in entire granuloma (× 10^{-5} dis./min)	7·48 ± 0·34	5·68 ± 0·42‖	6·86 ± 0·47
Noncollagen protein			
Total noncollagen protein in entire granuloma (mg)	95·97 ± 5·59	145·77 ± 5·54‖	79·96 ± 9·89‡
Noncollagen protein (mg/g wet wt.)	22·75 ± 0·96	28·36 ± 1·42‖	32·11 ± 2·87‖
Sp. act. of noncollagen protein (dis./min/μg noncollagen protein)	14·682 ± 1·134	5·606 ± 0·356‖	12·218 ± 0·862‡
Radioactivity of total noncollagen protein in entire granuloma (× 10^{-5} dis./min)	13·85 ± 0·81	8·14 ± 0·49‖	9·56 ± 0·90‖

* Data are shown as means ± S.E.
† Significantly different from 7-day-control ($P < 0·05$).
‡ Significantly different from 10-day-control ($P < 0·01$).
§ Significantly different from 10-day-control ($P < 0·05$).
‖ Significantly different from 7-day-control ($P < 0·01$).

TABLE 3. FORMATION OF DIALYSABLE HYDROXYPROLINE AND TYROSINE DURING INCUBATION *in vitro* OF THE MINCED GRANULOMA FROM RATS TREATED WITH BETAMETHASONE DISODIUM PHOSPHATE*

	7-Day-control	10-Day-control	Betamethasone treatment
No. of animals	7	7	7
Free hyp. formed (μg)	17·34 ± 0·71	14·28 ± 1·06†	8·16 ± 0·85‡,§
Total dialysable hyp. formed (μg)	21·43 ± 2·02	13·06 ± 0·42‡	8·38 ± 1·99‡,‖
Dialysable tyrosine formed (μg)	185·8 ± 13·1		158·7 ± 19·5

* Data are shown as means ± S.E.
† Significantly different from 7-day-control ($P < 0·05$).
‡ Significantly different from 7-day-control ($P < 0·01$).
§ Significantly different from 10-day-control ($P < 0·01$).
‖ Significantly different from 10-day-control ($P < 0·05$).

Incubation of minced granuloma with betamethasone disodium phosphate in vitro. Incubation of minced granuloma was carried out at 34° for 6 hr in the presence or absence of 2 × 10^{-5} M betamethasone disodium phosphate in the medium. The results obtained are presented in Table 4. There was no significant difference between the control and the granuloma tissue incubated with the steroid in the amounts of both free and total dialysable hydroxyproline formed during incubation. Since the

TABLE 4. INCUBATION *in vitro* OF MINCED GRANULOMA WITH BETAMETHASONE DISODIUM PHOSPHATE*

Treatment	No. of flasks	Dialysable hydroxyproline formed (μg/flask)	
		Free hydroxyproline	Total hydroxyproline
Control	7	23·79 ± 1·95	24·95 ± 2·14
Betamethasone (2×10^{-5} M)	7	22·18 ± 1·69 (NS)	23·21 ± 1·74 (NS)

* Data are shown as means ± S.E. NS = not significant.

steroid, at the concentration used in the present experiment, did not affect the degradation of collagen *in vitro*, it seemed unlikely that collagenolytic activity was directly inhibited by the steroid in this type of experiment.

DISCUSSION

It was reported in a previous paper[17] that the incorporation of [^{3}H]proline into collagen and noncollagen protein was inhibited by 90 and 74 per cent, respectively, when betamethasone disodium phosphate at a dose of 0·5 mg/rat was administered into the granuloma pouch daily from day 5 to day 8 after carrageenin injection. A preceding paper[13] also dealt with the same problem, analyzing the mechanism responsible for greater inhibition of collagen synthesis than of noncollagen protein by the steroid. In agreement with those previous papers, it was demonstrated in the present experiment that a single injection of the steroid in a variety of doses ranging from 0·02 to 2·0 mg/rat markedly inhibited [^{3}H]proline incorporation into collagen and noncollagen protein, interfering with collagen synthesis to a considerably greater extent. While all of these data suggest that the steroid inhibits collagen synthesis more selectively than noncollagen protein synthesis, there is always the possibility that the steroid has a selective effect on the proline pool size of the fibroblast, leading to less incorporation of [^{3}H]proline into collagen.

It was also found in the present experiment that the steroid has a great tendency to inhibit the degradation of collagen, since the radioactivity of collagen labeled with [^{3}H]proline was not significantly decreased by the steroid treatment (Table 2). This inhibitory effect of the steroid on collagen degradation was further demonstrated by an experiment *in vitro* (Table 3). Recently, Donoff *et al.*[18] have demonstrated that collagenase of granulation tissue obtained from healing open cutaneous wounds of the rabbit was inhibited by cysteine and EDTA. The amount of dialysable hydroxyproline formed during incubation, therefore, might be used as an index of collagenolytic activity in granuloma, since the formation of dialysable hydroxyproline was markedly inhibited by freeze-thawing of minced granuloma and by the addition of either cysteine or EDTA to the incubation medium at a concentration of 10^{-2} M (unpublished data). Although the precise mechanism of collagen breakdown to amino acids is not completely known, a hypothesis has been provided by Eisen *et al.*[19] which postulates that the collagen fiber is degraded into two large fragments by the initial action of an extracellular neutral collagenase, and subsequent denaturation of the cleaved collagen molecule permits the final degradation of the denatured products by commonly available tissue proteases and peptidases. Since almost all the dialysable hydroxyprolines are free hydroxyproline, there are the possibilities

that the production of free hydroxyproline by incubated minces of granuloma is due to the action of proteases and peptidases and the amount of substrate (partly degraded collagen) available to them is different in the control and the steroid-treated granuloma, and that the hydroxyproline might arise by oxidation of tissue proline in the 95% oxygen atmosphere of the incubation. As shown in Table 4, the amount of dialysable hydroxyproline formed during incubation of minced granuloma with the steroid was not changed, suggesting that the steroid did not directly affect the hydroxyproline-forming system of granuloma, which might involve collagenolytic activity. It is conceivable that the synthesis of a collagenolytic enzyme may be inhibited by the steroid, which interferes markedly with the synthesis of noncollagen protein. The results described above, therefore, seem to support the concept of Gross and Lapiere[20] and of Eisen and Gross[21] that collagenolytic activity depends either on *de novo* synthesis of collagenase or, alternatively, on its activation by some mechanism requiring protein synthesis. The degradation of noncollagen protein, in contrast with collagen degradation, was apparently not affected by the steroid treatment, suggesting that noncollagen protein was degraded by an enzyme which was stored in an active state in some manner such as lysosomal enzymes in the cells of granuloma.

Houck *et al.*[3–5] demonstrated that anti-inflammatory drugs such as cortisol induced collagenolytic and proteolytic activities *in vivo* in the rat skin or *in vitro* in human fibroblasts. It is possible that the discrepancy between our results and those of Houck *et al.* lies in the strong inhibitory effect of betamethasone on protein synthesis, since they found that inhibition of protein synthesis by treatment with puromycin or cycloheximide caused the profound depression of collagenolytic and proteolytic activities induced by anti-inflammatory drugs. The discrepancy might also be accounted for by differences in tissues analyzed, in doses of steroids used, and in time after the steroid treatment.

It was demonstrated in previous papers[11,12] that both steroidal and nonsteroidal anti-inflammatory drugs inhibited the formation of granuloma, but only steroidal drugs reduced the pre-existing granuloma. The experiments reported here suggest the following explanation for the steroid-induced reduction of pre-existing granuloma: betamethasone disodium phosphate markedly inhibits the synthesis of both collagen and noncollagen protein and considerably inhibits the degradation of collagen, whereas the degradation of noncollagen protein is not apparently affected, resulting in the resorption of pre-existing granuloma and the increasing concentration of collagen in granulomatous tissue.

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