Biochemical Pharmacology, Vol. 19, pp. 1–8. Pergamon Press, 1970, Printed in Great Britain

THE MODE OF ACTION OF HYDROCORTISONE ON THE PROTEIN METABOLISM IN RAT CARRAGEENIN GRANULOMA

TADAO OHNO and SUSUMU TSURUFUJI

Department of Physiological Chemistry, Faculty of Pharmaceutical Sciences, University of Tokyo, Tokyo, Japan

(*Received* 29 *April* 1969; *accepted* 11 *June* 1969)

Abstract—Involution of pre-existing carrageenin granuloma in rats was brought about by local injection of hydrocortisone acetate(HC). In *in vitro* incubation of minced granuloma removed from rats injected locally with HC 6 hr prior to sacrifice, the release of perchloric acid soluble tyrosine (Tyr) into the medium was significantly elevated, while non-steroidal anti-inflammatory drugs had no effect on the release of Tyr. The increase of degradation, in minced granuloma exposed *in vivo* to HC for 6 hr, of ^{3}H-Tyr-labeled tissue protein was suggested with a similar method as the incubation of non-labeled granuloma. Both *in vivo* and *in vitro* incorporation of ^{3}H-Tyr into tissue protein of the granuloma was reduced by HC administered *in vivo* 6 hr before. Thus, both the enhancement of catabolism and the reduction of anabolism were manifested as the effect of HC on the granuloma. The predominance of anti-anabolic effect was deduced from the result of incubation experiments in which puromycin or cycloheximide was used *in vitro* with or without HC-treatment.

ALTHOUGH it is well known that glucocorticoids have potent anti-granulomatic activity, much remains to be elucidated as to the biochemical mechanism of their action. In a previous paper[1] Fukuhara and Tsurufuji described some advantage of rat carrageenin granuloma as a model system for evaluating anti-inflammatory activity of a drug. They also reported that both steroidal and non-steroidal anti-inflammatory drugs effectively inhibited the formation of the granuloma. On the contrary when the drugs were given to animals holding already formed granuloma, only glucocorticoids brought a marked involution of the pre-existing granuloma while the non-steroidal drugs were ineffective. Such an involution of the granuloma induced by glucocorticoids may be accompanied by a disorder of protein metabolism. Thus, in the present study, the mode of action of hydrocortisone on the tissue protein metabolism of pre-existing carrageenin granuloma has been investigated and partly compared with that of some non-steroidal anti-inflammatory drugs by utilizing tyrosine as a marker of tissue protein.

MATERIALS AND METHODS

Carrageenin granuloma was induced subcutaneously on the back of male rats of Donryu strain, 42 ± 2 days of age, according to the procedure described in a previous paper[1] with a slight modification, i.e. 5 ml of air was injected subcutaneously 6 hr prior to the injection of carrageenin instead of 6 ml of air one day before. Granuloma pouch containing 4–10 ml of exudate inside was formed in a few days

after the injection. Throughout the experimental period rats had free access to water and food, a laboratory chow.

Aqueous suspension of hydrocortisone acetate (Nippon Merck-Banyu Co., Ltd., Japan) diluted with 0·9% NaCl to 10 mg/ml was injected into the granuloma pouch at a dose of 2 mg per rat at the designated time. Non-steroidal anti-inflammatory drugs: indomethacin (Nippon Merck-Banyu Co., Ltd., Japan), Phenylbutazone (Fujisawa Pharmaceut. Co., Ltd., Japan) and sodium salicylate (Koso Chem. Co., Ltd., Japan) were given as suspensions in 0·5% carboxymethylcellulose solution. Control groups received each vehicle of suspension. ^{3}H-L-Tyrosine (1000 mCi/m-mole, uniformly labeled; Daiichi Chem. Co., Ltd., Japan) was also given in 0·9% NaCl solution into the pouch in one series of experiments.

For *in vitro* experiments, granulomatous tissue was taken out immediately after decapitation and minced into 1–2 mm pieces with small scissors in an ice-cold Petri-dish. The minced granuloma was washed gently and shortly with 5 volumes of cold medium, Kreb's saline serum substitute,[2] containing each 0·1 mg/ml of potassium penicillin-G and dihydrostreptomycin-sulfate. Each 1g of washed mince was placed in a 100 ml-Erlenmeyer flask, added with 10 ml medium, and then incubated at 37° under an atmosphere of 95% O_2–5% CO_2. Incubation period was usually set for 6 hr on the basis of the results of preliminary tests. After the incubation, the flasks were quickly chilled in an ice bath and the content was filtered through gauze. The filtrate was centrifuged at 1000 *g* for 10 min. The supernatant to be analysed as the incubation medium was fractionated, after the addition of cold perchloric acid (PCA) up to the final concentration of 8% (w/v), by centrifugation at 10,000 *g* for 20 min into PCA-soluble and PCA-insoluble fractions. Tissues remaining on the gauze were homogenized with 10 volume of water in a Potter–Elvehjem glass homogenizer and subjected to the same fractionation as the supernatant.

Tyrosine content in the PCA-soluble fraction was determined according to the method of Udenfriend and Cooper;[3] protein to that of Lowry *et al.*;[4] ^{3}H of each fraction to that of Mahin and Lofberg.[5]

RESULTS

Wet weight and protein content of the granuloma

Wet weight of the granuloma pouch wall reached the maximum at day 5 and then decreased gradually, whereas total protein content increased throughout the experimental period up to day 10. When 2 mg of hydrocortisone acetate was daily injected into the pouch from day 5 till day 10, rapid decrease in both the wet weight and the protein content was induced (Fig. 1a and 1b).

Catabolic effect of glucocorticoids on granulomatous tissue protein in vitro *incubation*

Such a rapid involution of the granuloma induced by hydrocortisone acetate as is shown in Fig. 1 may be accompanied by elevated degradation of its tissue protein. In order to investigate the catabolic effect, if any, of hydrocortisone on granulomatous tissue, *in vitro* incubation of minced granuloma was carried out using 8-day-old granuloma. Perchloric acid soluble tyrosine released into the medium was measured after 6 hr incubation of minced granuloma. Local injection of hydrocortisone acetate, 2 mg per rat, which was given into the granuloma pouch 6 or 24 hr before sacrifice increased significantly the release of PCA-soluble tyrosine into the incubation medium,

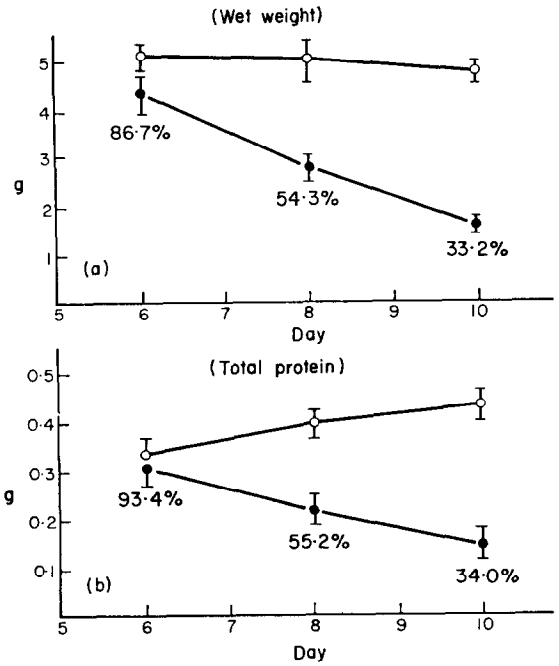

FIG. 1a. (upper) Effect of hydrocortisone acetate (HC) on the wet weight of carrageenin granuloma. Two mg of HC was injected daily from day 5 into the granuloma pouch (solid circles). 0·9% NaCl was injected in control (open circles). Vertical line is the S.E. of the mean of five animals. Figures are % of control.

FIG. 1b. (lower) Effect of HC on the total protein of carrageenin granuloma. Treatments were the same as Fig. 1a.

TABLE 1. EFFECT OF ANTI-INFLAMMATORY DRUGS ON THE RELEASE OF PERCHLORIC ACID (PCA) SOLUBLE TYR IN *IN VITRO* INCUBATION OF MINCED CARRAGEENIN GRANULOMA

Treatment	Time of treatment before sacrifice (hr)	No. of rats	PCA-soluble Tyr released into medium	
			μg/g wet tissue	% of control
control		8	193·9 ± 4·2‡	
Hydrocortisone acetate, 2 mg	6	6	249·4 ± 7·8†	129
Hydrocortisone acetate, 2 mg	24	6	209·8 ± 5·8*	108
control		5	200·2 ± 12·0	
Indomethacin, 1 mg	6	5	210·6 ± 9·7	105
Phenylbutazone, 15 mg	6	5	190·4 ± 6·2	95
Na-salicylate, 100 mg	6	5	211·8 ± 6·7	106

In all the experiments granuloma was taken 8 days after the injection of carrageenin. All the drugs were injected locally into the granuloma pouch. Doses are expressed as per rat.

* $P < 0·05$

† $P < 0·001$

‡ mean ± S.E.

whereas non-steroidal anti-inflammatory drugs, indomethacin, phenylbutazone and sodium salicylate, were ineffective when these drugs were injected locally into the granuloma pouch 6 hr before sacrifice (Table 1). The data suggest increased breakdown of granulomatous tissue protein in hydrocortisone-treated rats. To investigate whether glucocorticoids exert their catabolic effect directly on granulomatous tissue or not, an experiment for testing the effect of *in vitro* exposure of minced granuloma to glucocorticoids was designed. After 1 hr pre-incubation without glucocorticoid, minced granuloma was incubated for 6 hr in the medium containing 100 μg/ml of hydrocortisone sodium hemisuccinate (Upjohn Co., Ltd., U.S.A.) or 10 μg/ml of betamethasone disodium phosphate (Glaxo Lab. Ltd., England). The release of PCA-soluble tyrosine into the medium was increased significantly though not markedly (Table 2).

TABLE 2. EFFECT OF *IN VITRO* TREATMENT OF MINCED CARRAGEENIN GRANULOMA WITH GLUCOCORTICOIDS ON THE RELEASE OF PCA-SOLUBLE TYR DURING INCUBATION

	PCA-soluble Tyr released into the medium				
	control	HC-hemi		betamethasone	
Rat No.	μg/g wet wt.	μg/g wet wt.	% increase	μg/g wet wt.	% increase
1	176	179	1·7	187	6·3
2	174	180	3·4	185	6·4
3	146	175	19·9	174	19·2
4	133	151	13·5	146	9·8
5	149	160	7·4	148	− 0·7
mean	156	169*	9·2	168*	8·2

Minced granuloma, 10 days old, was transfered, after 1 hr pre-incubation in the control medium, into a medium containing hydrocortisone sodium hemisuccinate (HC-hemi; 100 μg/ml) or betamethasone disodium phosphate (betamethasone; 10 μg/ml) and incubated for 6 hr. In each experiment, tissues from the same rat were minced and divided into three flasks for control, HC-hemi and betamethasone respectively.

* $P < 0.025$, Statistical test has been performed in comparing the actual values by two way layout.

Incorporation of ^{3}H-tyrosine into the granuloma

It was repeatedly described that glucocorticoids inhibited the incorporation of labeled amino acids into the protein of peripheral tissues.[6–10] If glucocorticoids inhibit the protein synthesis in granulomatous tissue, it might play an important role in the involution of the granuloma. Therefore the effect of hydrocortisone acetate on the incorporation of ^{3}H-tyrosine into the granulomatous protein was examined by using 8-day-old granuloma. Rats were injected with 2 mg of hydrocortisone acetate into the granuloma pouch 6 or 24 hr prior to sacrifice. In *in vivo* experiments ^{3}H-tyrosine was given intravenously through femoral vein, and in *in vitro* incubation experiments of minced granuloma, ^{3}H-tyrosine was added into the medium at the beginning of the incubation or after 5 hr of pre-incubation. Results are summarized in Table 3. The incorporation of ^{3}H-tyrosine into granulomatous protein was reduced in all cases, both in the experiments *in vivo* and *in vitro*.

Degradation of granulomatous tissue protein in in vitro *incubation*

As to the possible cause for increased release of PCA-soluble tyrosine into the medium it should be taken into consideration that glucocorticoid reduces protein synthesis in granulomatous tissue as shown in Table 3. Release of free tyrosine may be elevated, without any increase of the degradation of tissue protein, simply by the decrease of reutilization in protein synthesis of free tyrosine liberated by physiologic breakdown of cellular protein. Therefore, in an attempt to obtain a direct evidence for increased breakdown of tissue protein in hydrocortisone-treated granuloma, an

TABLE 3. EFFECT OF HYDROCORTISONE ACETATE (HC) ON THE INCORPORATION OF ^{3}H-TYR INTO GRANULOMA PROTEIN UNDER THE VARIABLE CONDITIONS

Experiment No.	Time of HC treatment before sacrifice			
	Control dpm/μg protein	6 hr dpm/μg protein	24 hr dpm/μg protein	% of control
in vivo				
I	9·71 ± 0·51*(8)†	7·05 ± 0·10‡(6)		72·6
II	9·71 ± 0·51 (8)		7·82 ± 0·38§(6)	80·6
in vitro				
III	607 ± 21 (5)	462 ± 14§ (5)		76·2
IV	1032 ± 51 (5)	832 ± 32‡(5)		80·6

In all the experiments 8-day-old granuloma was used. HC, in the dose of 2 mg/rat, was directly injected into the granuloma pouch 6 hr (Expt. I, III and IV) or 24 hr (Expt. II) before sacrifice. In *in vivo* experiments, 100 μCi of ^{3}H-Tyr was given intravenously 1 hr before sacrifice and in *in vitro* experiments labeling of minced granuloma was carried out for 1 hr in the medium containing 10 μCi/ml of ^{3}H-Tyr. ^{3}H-Tyr was given into the medium at the beginning of the incubation (Expt. III) or after 5 hr pre-incubation (Expt. IV).

* mean ± S.E.

† Figures in parenthesis represent the number of rats.

‡ $P < 0{\cdot}02$

§ $P < 0{\cdot}001$

isotope tracer experiment was carried out. ^{3}H-tyrosine, 50 μCi/rat/day, was injected daily into the granuloma pouch for 4 days (day 4–day 7) in order to pre-label the granulomatous protein. On day 8, 10 mg of carrier tyrosine and 2 mg of hydrocortisone acetate were directly administered into the pouch 6 hr prior to the removal of the granuloma, and then minced granuloma was incubated for 6 hr in the medium containing non-radioactive tyrosine in the concentration of 0·6 mg/ml. The non-radioactive tyrosine was used for diluting free ^{3}H-tyrosine, which could have been liberated by the breakdown of labeled tissue protein. The radioactivity which became PCA-soluble during the incubation was measured and expressed as per cent of radioactivity of PCA-insoluble fraction of original minced granuloma. Results are summarized in Table 4. The radioactivity solubilized during the incubation in the hydrocortisone-treated group was significantly higher than that of the control group.

Incubation of minced granuloma with puromycin and cycloheximide

As mentioned above, the release of tyrosine into the medium from the incubated granuloma may be elevated by the decrease of reutilization in protein synthesis of free tyrosine which has been liberated by the degradation of tissue protein. Therefore, the following experiment was designed in an attempt to investigate the influence of the

TABLE 4. EFFECT OF HYDROCORTISONE ACETATE (HC) ON THE DEGRADATION OF PRE-LABELED PROTEIN OF CARRAGEENIN GRANULOMA

Treatment	Control	HC
No. of rats	6	7
Before incubation		
a: tissue PCA-insoluble	81·1* (54·6–97·6)	82·8 (71·9–99·2)
b: tissue PCA-soluble	13·1 (11·1–15·3)	12·8 (11·3–14·5)
After incubation		
c: medium PCA-soluble	14·8 (11·9–18·3)	18·3 (16·1–22·1)
d: tissue PCA-soluble	5·2 (4·5–6·0)	5·7 (4·3–6·6)
solubilized radioactivity (c + d − b)/a × 100 (%)	8·6 ± 0·9%†	13·6 ± 1·1%‡

^{3}H-tyrosine, 50 μCi/rat/day, was injected daily into the pouch during day 4–day 7. On day 8, 10 mg of carrier tyrosine and 2 mg of HC were injected into the pouch 6 hr prior to sacrifice. Minced granuloma was incubated for 6 hr in the medium containing 0·6 mg/ml carrier tyrosine.

* mean value (× 10^5 dpm/g wet wt.) of the group. Figures in parenthesis show the range of values in each group.

† mean ± S.E.

‡ $P < 0·02$

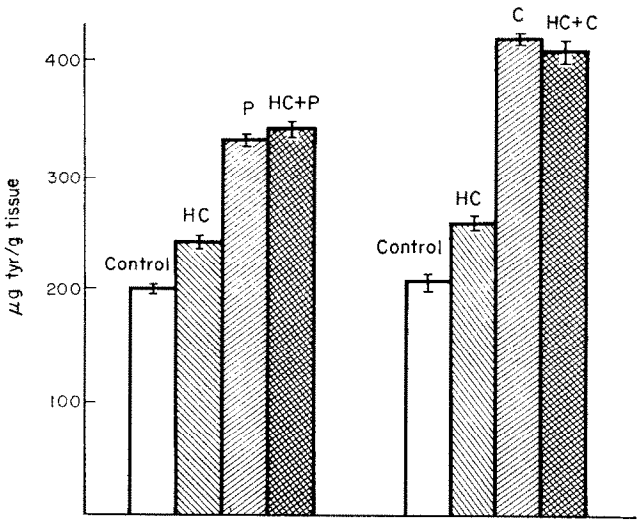

FIG. 2. Effect of puromycin and cycloheximide on the Tyr release of carrageenin granuloma *in vitro*. Puromycin-dihydrochloride (P:50 μg/ml) or cycloheximide (C:30 μg/ml) was added into the medium at the beginning of the incubation. Hydrocortisone acetate (HC:2 mg/rat) was injected into the granuloma pouch 6 hr before sacrifice. Incubation, 6 hr at 37°.

inhibition of protein synthesis on the release of acid soluble tyrosine into the incubation medium. As an inhibitor of protein synthesis, puromycin (50 μg/ml) or cycloheximide (30 μg/ml) was added into the medium at the beginning of the incubation period of 6 hr. For comparing the mechanism of the action of glucocorticoids to that of these antibiotics, interaction of hydrocortisone acetate with the antibiotics was also investigated. The results (Fig. 2) indicated that PCA-soluble tyrosine in the medium was increased to 165% of control by puromycin and 202% by cycloheximide. Pretreatment of the granuloma with hydrocortisone acetate *in vivo* gave no additive influence onto the effect of these antibiotics.

DISCUSSION

Although many papers have reported the inhibitory effect of glucocorticoids on granuloma formation, only a few works have been concerned with the application of glucocorticoids to already established granulation tissues.[11, 12] The present study revealed that hydrocortisone acetate was effective for reducing the wet weight and the total protein content of pre-existing rat carrageenin granuloma in good agreement with the results of our previous paper[1] reporting marked decrease in wet weight and total collagen content of the pre-existing granuloma under the influence of betamethasone disodium phosphate. Nocenti *et al.*[11] also reported that glucocorticoid treatment decreased the wet weight, the dry weight and the collagen content of preexisting cotton pellet granuloma, but no further attempt was made for elucidating the mechanism of glucocorticoid action.

The present experiments demonstrated that isolated granulomatous tissue exposed *in vivo* to glucocorticoid released PCA-soluble tyrosine into the incubation medium at an increased rate. On the other hand some non-steroidal anti-inflammatory drugs tested in the present experiment neither had influence on the release of acid-soluble tyrosine from minced granuloma (Table 1) nor stimulated involution of pre-existing granuloma in *in vivo* experiments as reported previously.[1] Sutherland and Haynes[13] reported that hydrocortisone increased the release of amino acids from thymus slices into the incubation medium and that this effect was specific for glucocorticoids. It is well known that glucocorticoids cause rapid thymic involution. Therefore, in both the granulomatous and the thymic tissues, the effect of glucocorticoids on the release of amino acids *in vitro* might have some relationship to *in vivo* involution induced by the steroids. However, there seems to be a difference in the mode of the action of glucocorticoids on the release of acid-soluble amino acid between thymus and granuloma. Sutherland and Haynes reported that it was impossible to induce an increase of the release of amino acids from the thymus slices by adding glucocorticoids *in vitro*, though in carrageenin granuloma hydrocortisone did act *in vitro* to accelerate significantly the release of acid soluble tyrosine. In rat carrageenin granuloma, therefore, at least a part of the action of hydrocortisone on the release of acid soluble tyrosine seems to be a direct work on the granulomatous tissue.

Concerning the mechanism of glucocorticoid action for the elevated release of acid soluble tyrosine from minced granuloma, two possibilities, namely, increased degradation of tissue protein and decreased reutilization of free tyrosine which has been liberated by the breakdown of tissue protein need to be considered. Decreased incorporation of labeled tyrosine into granulomatous protein (Table 3) suggests that the latter contributes the elevated release of tyrosine to some extent. The increase in

the liberation of radioactivity which became acid soluble during the incubation with a large amount of carrier tyrosine (Table 4) gave an evidence to support the possibility of increased degradation. So that both mechanisms seem to be operating. When puromycin or cycloheximide was added to the incubation medium, marked increase in the release of acid soluble tyrosine from the minced granuloma was observed. This supports the possibility that inhibiting the reutilization of tyrosine by glucocorticoid greatly contribute to the increase of the tyrosine release. Moreover, no additive effect for accelerating the release of tyrosine was seen when hydrocortisone was used in combination with the antibiotics. It seems very likely, therefore, that anti-anabolic effect of glucocorticoid plays a major role in increasing the release of PCA-soluble tyrosine from the minced granuloma. It has been often discussed whether glucocorticoids are catabolic or anti-anabolic on peripheral tissues.[10, 14–18] The present experiments show both the mechanisms are actually responsible for glucocorticoid-induced involution of carrageenin granuloma, though anti-anabolic action seems to play a predominant role.

Acknowledgements—We thank Dr. S. Ishibashi for his very great kindness to review the manuscript, and the members of Department of Physiological Chemistry for their fruitful advice, technical assistance and discussion.

REFERENCES

1. M. FUKUHARA and S. TSURUFUJI, *Biochem. Pharmac.* **18**, 475 (1969).
2. H. A. KREBS, *Biophys. biochem. Acta* **4**, 249 (1950).
3. S. UDENFRIEND and J. R. COOPER, *J. biol. Chem.* **196**, 227 (1952).
4. O. H. LOWRY, N. J. ROSEROUGH, A. L. FARR and R. J. RANDALL, *J. biol. Chem.* **193**, 265 (1951).
5. D. T. MAHIN and R. T. LOFBERG, *Analyt. Biochem.* **16**, 500 (1966).
6. M. BLECHER and A. WHITE, *Recent Prog. Hor. Res.* **15**, 391 (1959).
7. H. J. MANKIN and K. A. CONGER, *Lab. Invest.* **15**, 794 (1966).
8. A. PENA, B. DVORKIN and A. WHITE, *J. biol. Chem.* **241**, 2144 (1966).
9. G. D. GRAY and O. J. KOEPPE, *Proc. Soc. exp. Biol. Med.* **122**, 950 (1966).
10. K. L. KIVIRIKKO, O. T. AER and T. HALME, *Biochem. Pharmac.* **14**, 1445 (1965).
11. M. R. NOCENTI, G. E. LEDERMAN, C. A. FUREY and A. J. LOPANO, *Proc. Soc. exp. Biol. Med.* **117**, 215 (1964).
12. A. ROBERT and J. E. NEZAMIS, *Endocrinology* **65**, 725 (1959).
13. E. W. SUTHERLAND, III and R. C. HAYNES, JR., *Endocrinology* **80**, 288 (1967).
14. O. K. SMITH and C. N. H. LONG, *Endocrinology* **80**, 561 (1967).
15. F. ALBRIGHT, *Harvey Lect.* **38**, 123 (1943).
16. Q. T. SMITH and D. J. ALLISON, *Endocrinol.* **77**, 785 (1965).
17. D. BELLAMY and R. A. LEONARD, *Gen. Comp. Endocr.* **5**, 402 (1965).
18. B. G. PALMER, *J. Endocrinology* **36**, 73 (1966).