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ALTERATION OF PROLINE HYDROXYLASE ACTIVITY BY GLUCOCORTICOIDS*

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Abstract—The biochemical mechanism by which glucocorticoids affect collagen metabolism was investigated. Triamcinolone, hydrocortisone and methylprednisolone significantly decreased liver proline hydroxylase activity *in vivo* to an extent unrelated to the decrease of protein in the 15,000 *g* supernatant. Triamcinolone inhibited liver proline hydroxylase in a dose-dependent manner *in vivo*. An observed elevation of liver proline hydroxylase activity in adrenalectomized animals was decreased to control levels by hydrocortisone treatment. Subdermal implants of polyvinyl sponges were used to induce granuloma growth as a model system of inflammation. The anti-inflammatory activity of corticosteroids was correlated with inhibition of proline hydroxylase activity in granuloma tissue. The data indicate that corticosteroids decrease collagen synthesis by inhibiting the proposed rate-limiting enzyme. This inhibitory effect on collagen metabolism is correlated with observed anti-inflammatory activity.

DRAMATIC effects of glucocorticoid therapy have been reported[1,2] in patients with various collagen diseases, i.e. those disorders characterized firstly, by degenerative changes and necrosis, and secondly, by inflammatory and reparative processes. Although much is known about various metabolic and biochemical changes which occur in connective tissue at intervals after glucocorticoid treatment, little is known about the molecular and biochemical mechanism of action of this group of frequently used drugs on connective tissue metabolism. The ability of this class of drugs to impede the progress of connective tissue diseases is believed to be primarily due to the anti-inflammatory effect of these steroids.[3] Since collagen synthesis plays an essential role in the inflammatory process,[4,5] it is reasonable to assume that this would constitute a biochemical site of anti-inflammatory activity of glucocorticoids.

A glucocorticoid-mediated decrease in collagen content in normal connective tissue[6] and in inflamed tissue[7,8] is well documented; however, the mechamism is not known. The decrease in collagen content observed after corticoid treatment in animal and man could be explained by a decreased rate of collagen synthesis or an increased rate of collagen degradation.

Houck *et al.*[6] reported a decrease in cutaneous insoluble collagen in cortisol-treated rats with the appearance of cutaneous extracellular, free collagenolytic activity. These authors felt that the observed loss in collagen was the result of collagen catabolism through the induction of collagenase activity in skin fibroblasts.[9] Kivirikko *et al.*[10] studied the action of cortisone on the metabolism of collagen by investigating the

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effect of this drug on the specific activity and total activity of [^{14}C]hydroxyproline in urine and in skin fractions. These workers showed that the specific activity and total activity of [^{14}C]hydroxyproline in the soluble collagen fraction of skin were decreased and correlated this with decreased hydroxyproline excretion. These results suggest that corticoids decrease collagen synthesis.

Glucocorticoids are well known inhibitors of granuloma formation,[7] a model system of inflammation; however, the biochemical mechanism of this anti-granuloma activity has not yet been clearly elucidated. Fukuhara and Tsurufuji[7] showed that betamethasone inhibited collagen synthesis in carrageenin granuloma. Juva[11] showed that changes in protocollagen proline hydroxylase activity paralleled the rate of collagen synthesis in sponge-induced granuloma tissue. Since various workers feel that proline hydroxylase is the rate-limiting step in collagen biosynthesis,[12] the regulation of this enzyme may be the mechanism by which glucocorticoids affect collagen synthesis and granuloma formation.

The above-cited studies indicate that some confusion exists as to the biochemical mechanism of action of the glucocorticoids. The present study was conducted to establish the relationship between the anti-inflammatory activity and the effect of the glucocorticoids upon collagen synthesis.

MATERIALS AND METHODS

Intact 100–150 and 250 g adrenalectomized male albino Sprague-Dawley rats were supplied by the Charles River Breeding Laboratories, Wilmington, Mass. The efficacy of the commercial adrenalectomy was determined at autopsy.

All chemicals used in this investigation were analytical reagent grade. Commercial corticoid preparations used were: methylprednisolone sodium succinate (Solu-Medrol; Upjohn Company, Kalamazoo, Mich.); hydrocortisone acetate (Hydrocortone; Merck, Sharp & Dohme, West Point, Pa.); betamethasone acetate (Celestone Soluspan; Schering Corp., Bloomfield, N.J.); and triamcinolone diacetate (Aristocort Forte). Saline suspensions of hydrocortisone were prepared by adding hydrocortisone powder to 0·9% (w/v) saline at a concentration of 30 mg/ml and mixing by use of a coaxial homogenizer with a Teflon-coated pestle until a smooth suspension was obtained. 3,4-^{3}H-l-Proline (specific activity, 5·2 c/m-mole) was purchased from New England Nuclear Corp. (Boston, Mass.).

Studies in vivo *on liver proline hydroxylase activity.* These studies were carried out to determine the effect of various glucocorticoids on liver proline hydroxylase activity. The animals received drug, either 100, 150 or 200 mg/kg, intraperitoneally once per day for 4 days and were sacrificed on the days specified. All animals were sacrificed by cervical dislocation followed by decapitation. Livers were perfused with cold 0·9% (w/v) saline and 10% tissue homogenates (w/v) were prepared in cold 0·25 M sucrose by use of a Polytron ST-10 system (Kinematica GmbH, Luzern, Switzerland). The homogenates were centrifuged for 15 min at 0° in an International model B-60 preparative ultracentrifuge at 15,000 *g*. The supernatant fraction which served as the enzyme source was stored at −15° until assayed.

Granuloma studies. Subdermal implants of polyvinyl sponges were used to induce granulation tissue growth. Sponges were cut into cylinders 10 mm in diameter and 42 mm in length that weighed 95·4 ± 4·7 mg. The sponges were washed in continuous

running water for 48 hr, sterilized for 15 min in an autoclave, and dried. Prior to implantation, the sponges were weighed and stored in sterile saline.

The animals were anesthetized with ether. A trocar was inserted and guided to the dorsothoracic region and the sponge was implanted. Daily local injections of steroids administered directly into the sponge were initiated on the first day after implantation. One-tenth ml of commercially prepared steroid preparation was diluted with 0·9% (w/v) saline to 1 ml. This was injected directly into the sponge at a dose of 0·6 mg betamethasone per day. Treatment was continued daily for the next 4 days and all animals were sacrificed on day 6 after sponge implantation.

The animals were killed by anesthetizing with ether and decapitation. The granuloma tissue was removed from the sponge. Tissue homogenates, 10% (w/v), were prepared with 0·25 M sucrose by use of the Polytron ST-10 system. The homogenates were centrifuged as described previously.

Proline hydroxylase assay. Enzyme activity in the 15,000 *g* supernatant was measured by the method of Hutton *et al.*[13] This procedure is based on the release of tritium into the incubation media when proline-rich polypeptide substrate is incubated with enzyme and cofactors. The stoichiometric formation of tritiated water and tritiated hydroxyproline, the cofactor requirements, and the enzymatic characteristics of the assay system when using crude tissue fractions have been reported in previous publications from this laboratory.[14,15] 3,4-^{3}H-Proline-rich, hydroxyproline-deficient peptide substrate was prepared from 10-day chick embryos as previously described.[13] Each incubation mixture contained 0·4 ml of labeled substrate (350,000 dis./min), 0·45 μmole of ferrous ammonium sulfate, 7·5 μmole of ascorbic acid, 0·9 μmole α-ketoglutarate, and 0·1 to 0·2 ml of 15,000 *g* supernatant made up to a total volume of 3·0 ml with 0·5 M Tris–HCl buffer. The samples were incubated aerobically at 30° for 30 min. The reaction was terminated by the addition of 0·3 ml of 50% (w/v) trichloroacetic acid while the samples were on ice. The resulting tritiated water was distilled and a 1·5 ml aliquot was counted in a mixture containing BBS-3 (Beckman, Fullerton, Calif.) as the solubilizer in a toluene–PPO–POPOP* mixture at 20% efficiency. Correction for efficiency was determined through automatic external standardization against an appropriate quench curve. Tritium release is linear over a 40-min period when liver supernatant is used as the enzyme source in a system containing substrate adequate for the detection of 10-fold increases over control values.

Protein determination. Protein concentration in the 15,000 *g* supernatant was determined by the method of Lowry *et al.*,[16] using bovine plasma albumin (Calbiochem, Los Angeles, Calif.) as standard.

RESULTS AND DISCUSSION

Triamcinolone, hydrocortisone and methylprednisolone (150/kg) produced a significant decrease in the specific activity of proline hydroxylase in liver (Table 1). This decrease was significant when enzyme activity was expressed per gram of tissue equivalent or per liver. Triamcinolone was the most effective of the three in reducing liver proline hydroxylase activity *in vivo*. Neither hydrocortisone, methylprednisolone nor triamcinolone, when added directly into the incubation medium, inhibited enzyme activity. Attempts to demonstrate the presence of an inhibitory substance in the supernatants from the steroid-treated animals were not successful. A mixing experiment

* PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis-2-(4-methyl 5-phenyloxazolyl)benzene.

TABLE 1. GLUCOCORTICOID-INDUCED DECREASE OF RAT LIVER PROLINE HYDROXYLASE ACTIVITY

	N	^{3}H-water* (dis./min/mg protein)	Supernatant protein (mg/g tissue)
Control	5	700 ± 98	71·0 ± 1·6
Hydrocortisone†	4	294 ± 63‡	53·7 ± 4·8‡
Triamcinolone	5	132 ± 25‡	47·3 ± 1·6‡
Methylprednisolone	5	448 ± 58‡	67·3 ± 6·0

* Enzyme activity is expressed as the mean (± S.E.) of the amount dis./min of (^{3}H) H_2O formed from (3, 4-^{3}H) proline per milligram of protein per 30 min.

† Animals received 150 mg/kg of drug i.p. for 4 consecutive days and were sacrificed 18 hr after last injection.

‡ Significantly different from control at $P < 0·05$.

of control supernatant and triamcinolone supernatant gave greater than additive enzyme activity.

Steroid-induced changes of distinct metabolic activities in connective tissue may result from a general inhibition of protein synthesis[17] or from a nonspecific depression of a basic metabolic process.[18] It is unlikely that the inhibitory effect on proline hydroxylase activity is a manifestation of a decrease of protein in the 15,000 *g* supernatant, since protein concentration could be further significantly ($P \leq 0·05$) decreased when proline hydroxylase inhibition by hydrocortisone was maximal (Table 2).

TABLE 2. EFFECT OF HYDROCORTISONE ON LIVER PROLINE HYDROXYLASE ACTIVITY AND PROTEIN IN THE 15,000 *g* SUPERNATANT

	N	^{3}H-water* (dis./min/mg protein)	Supernatant protein (mg/g tissue)
Control	5	1345 ± 30	75·2 ± 3·1
Hydrocortisone			
100 mg/kg	6	899 ± 64†	65·2 ± 1·9†
200 mg/kg	6	892 ± 48†	57·3 ± 1·7†

* Enzyme activity is expressed as the mean (± S.E.) of the amount (dis./min) of (^{3}H) H_2O formed from (3,4-^{3}H) proline per milligram of protein per 30 min.

† Significantly different from control at $P < 0·05$.

Triamcinolone reduction of liver proline hydroxylase activity is linearly related to the number of daily doses (Fig. 1). A significant decrease of enzyme activity was observed 24 hr after drug treatment. This dose-dependent relationship suggests the possibility that proline hydroxylase is the biochemical target for glucocorticoid-induced alteration of collagen metabolism. Smith[19] administered cortisone for at least 4 days before any change in insoluble skin collagen was observed. Thus, the anti-anabolic effect is seen much earlier than a catabolic effect on the insoluble pool, as proposed by Houck *et al.*[6]

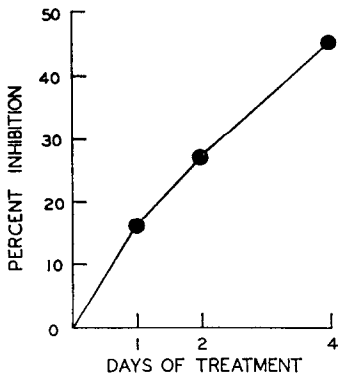

FIG. 1. Dose response of triamcinolone on liver proline hydroxylase activity. Animals were treated with distilled water or triamcinolone (150 mg/kg, i.p.) daily and a control group and treated group were sacrificed at the times specified. All drug-treated groups were significantly different from control group values.

Liver proline hydroxylase activity was elevated in adrenalectomized animals and was decreased to the control level by hydrocortisone treatment (150 mg/kg, i.p.) for 4 consecutive days (Table 3). These results may explain the reported increase in the amount of collagen in carrageenin-induced granulomas in adrenalectomized guinea pigs[20] and the potentiated granuloma development in adrenalectomized rats[21,22] as compared to intact controls.

TABLE 3. EFFECT OF HYDROCORTISONE ON LIVER PROLINE HYDROXYLASE ACTIVITY IN ADRENALECTOMIZED RATS

	N	^{3}H-water* (dis./min/mg protein)	Supernatant protein (mg/g tissue)
Control	6	902 ± 81†,‡	74·7 ± 2·8†,§
Hydrocortisone‖	6	511 ± 50‡,§,¶	58·5 ± 3·3‡,¶
Adrenalectomized	8	1248 ± 34†,§,¶	75·0 ± 3·9†,§
Adrenalectomized and hydrocortisone	5	834 ± 106†,‡	62·5 ± 2·2‡,¶

* Enzyme activity is expressed as the mean (± S.E.) of the amount (dis./min) of (^{3}H) H_2O formed from (3, 4–^{3}H) proline per milligram of protein per 30 min.

† Significantly different from hydrocortisone at $P < 0{\cdot}05$.

‡ Significantly different from adrenalectomized at $P < 0{\cdot}05$.

§ Significantly different from adrenalectomized and hydrocortisone at $P < 0{\cdot}05$.

‖ Animals received 150 mg/kg of drug i.p. for 4 consecutive days and were sacrificed 18 hr after last injection.

¶ Significantly different from control at $P < 0{\cdot}05$.

The pharmacological importance of the inhibitory effect of these steroids on proline hydroxylase activity as a possible biochemical mechanism of anti-inflammatory activity depends upon the effect of these compounds on enzyme activity in inflamed or diseased connective tissue. The production of experimental granulomas by artificial means is a

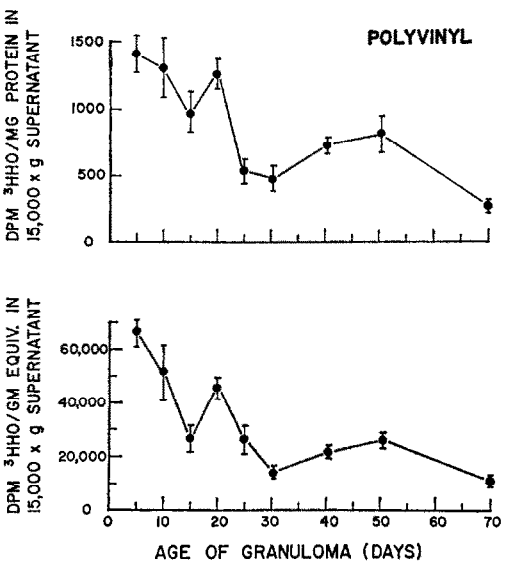

FIG. 2. Proline hydroxylase activity in the 15,000 *g* supernatant of granuloma tissue produced by subcutaneous implantation of polyvinyl sponges. Each point represents the mean enzyme activity ± S.E. of 4–9 granulomas of the same age.

model system often used to study anti-inflammatory activity. Proline hydroxylase activity in granuloma tissue induced by polyvinyl sponge implants was highest on day 5 (when first samples taken) and decreased thereafter to the lowest value observed on day 70 after sponge implantation (Fig. 2). Proline hydroxylase has previously been reported to reach maximal levels on a protein basis on day 5 in carrageenin-induced granuloma tissue.[1,2]

Triamcinolone and betamethasone treatment decreased 5-day granuloma growth and proline hydroxylase activity in granuloma tissue induced by implantation of polyvinyl sponges for 5 days (Table 4.) The data suggest a biochemical mechanism for the previous report that betamethasone inhibits collagen synthesis in carrageenin-

TABLE 4. EFFECT OF TRIAMCINOLONE AND BETAMETHASONE ON GRANULOMA BODY WEIGHT RATIO AND GRANULOMA PROLINE HYDROXYLASE ACTIVITY IN THE RAT

	N	Granuloma (g/100 g body wt.)	^{3}H-water* (dis./min/mg protein)	Supernatant protein (mg/g tissue)
Control	8	0·231 ± 0·02	3153 ± 173	45·7 ± 2·8
Triamcinolone† (4·0 mg/rat)	8	0·128 ± 0·02‡	1335 ± 262‡	43·7 ± 4·0
Betamethasone (0·6 mg/rat)	8	0·145 ± 0·01‡	786 ± 105‡	35·3 ± 3·0‡

* Enzyme activity is expressed as the mean (± S.E.) of the amount (dis./min) of (^{3}H) H_2O formed from (3,4-^{3}H) proline per milligram of protein per 30 min.

† One polyvinyl sponge was implanted subcutaneously in the dorsothoracic region of each male rat on day 1. Drug was injected directly into the sponge daily for the next 4 days and all animals were sacrificed on day 6.

‡ Significantly different from control at $P < 0·05$.

inflamed tissue.[7] Some workers[18] have used the argument that, since steroids do not decrease total hydroxyproline but do reduce collagen synthesis as determined by labeled proline incorporation, the primary steroidal effect may be to depress a basic cellular metabolic process. However, an observable effect on total hydroxyproline of collagen resulting from a decrease in collagen synthesis will only be manifested when the existing collagen has had time to turn over.

Although some workers feel that the inhibitory effect of steroids on collagen may be explained by stabilization of lysosomes[23,24] or by the increased activity of collagenase,[9] the results of the present investigation indicate that these drugs decrease collagen synthesis by inhibiting proline hydroxylase. After administration of cortisol, Houck *et al.*[6] found that the loss of cutaneous collagen is at least 10-fold greater than normal loss of all tissue collagen from the whole rat. This author concluded that inhibition of collagen anabolism alone cannot explain the observed decrease in collagen concentration. However, in the overall synthesis of collagen proposed by Prockop and Kivirikko,[25] the conversion of the insoluble to one of the more soluble pools resulting from a depletion of the latter pool is quite feasible. Houck and Patel [26] observed a 22% decrease in the insoluble pool of collagen and a 3-fold increase in hydroxyproline concentration of soluble collagen, while the specific activity of hydroxyproline after a pulse label of radioactive proline decreased by 5-fold. Dilution of the soluble pool by unlabeled hydroxyproline from the insoluble pool alone does not account for the total decrease in hydroxyproline specific activity observed in the soluble pool. The effect of steroids on proteolytic and collagenolytic activities[9] may be the mechanism for rendering the insoluble pool more soluble. However, the ability of these corticosteroids to inhibit proline hydroxylase appears to be primarily responsible for the decrease in collagen content produced by these drugs.

Our results are in agreement with those of Kivirikko *et al.*[10] and Smith[19] that the glucocorticoids decrease collagen through a depressant effect on the synthesis of soluble collagen. Our data indicate further that the mechanism by which this is accomplished is the inhibition of proline hydroxylase, the proposed rate-limiting enzyme in the collagen biosynthetic pathway.

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