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Anti-inflammatory Steroids and Collagen Metabolism: Glucocorticoid-Mediated Decrease of Prolyl Hydroxylase

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SUMMARY

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Administration of triamcinolone diacetate to rats results in a decrease of prolyl hydroxylase activity in organs from animals of different ages. The response of the enzyme activity is dose-dependent, reversible, independent of endocrine function (except for the response of the liver enzyme in hypophysectomized rats), and dependent on the number of daily injections. The decrease in enzyme activity is paralleled by a decrease in the amount of enzyme protein as measured by immunoassay. These results indicate that prolyl hydroxylase is decreased in a wide spectrum of tissues following administration of anti-inflammatory steroids. Furthermore, these findings suggest that one of the effects of this class of therapeutically used drugs on connective tissue metabolism may be mediated by a decrease in the prolyl hydroxylation step of collagen biosynthesis.

INTRODUCTION

The administration of pharmacological doses of anti-inflammatory steroids produces profound effects on collagen metabolism. Topical application of various fluorinated anti-inflammatory steroids produces atrophy of the skin and telangiectases in humans (1), two adverse effects which probably result from alteration of connective tissue metabolism in normal skin. Administration of anti-inflammatory steroids to weanling rats results in a marked decrease in urinary excretion of hydroxyproline (2). Although investigators are generally in agreement that the anti-inflammatory steroid-mediated alteration of collagen metabolism is mainly anti-anabolic in nature (3), the biochemical mechanism of this effect is unknown.

The anti-anabolic effect of anti-inflammatory steroids may result from a decrease in prolyl hydroxylase, the enzyme catalyzing the hydroxylation of certain prolyl residues in collagen. Administration of triamcinolone diacetate to rats has been reported to result in a decrease of prolyl hydroxylase activity in liver and granuloma tissue (4). However, there have been other reports that the incorporation of proline

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into proteins of both normal and inflamed connective tissue decrease shortly after administration of steroids to the whole animal or to organ cultures of chick tibia and granuloma tissues (5, 6). Since this decreased incorporation occurred at a time when prolyl hydroxylase activity was unaltered, it was concluded that this steroid-induced decrease of proline incorporation into total cellular protein is responsible for the anti-anabolic effect of this class of drugs on collagen biosynthesis. Furthermore, Nakagawa and Tsurufuji (5, 7) reported that incorporation of labeled proline into collagen of granuloma tissue is inhibited without alteration of proline hydroxylation.

The data presented here demonstrate that administration of triamcinolone to rats results in a decrease of prolyl hydroxylase activity in many tissues from animals of different ages. Furthermore, the effect of the steroid on this enzyme is dose-dependent, reversible, independent of endocrine function (except for the response of the liver enzyme in hypophysectomized rats), and dependent on the number of days of drug administration. The percentage decrease in prolyl hydroxylase activity in the tissues is equal to the percentage decrease in the total amount of protein antigenically related to prolyl hydroxylase. These findings indicate that the effect of anti-inflammatory steroids on prolyl hydroxylase is of primary importance in the alteration of collagen metabolism.

MATERIALS AND METHODS

Intact rats (1 day, 4 and 8 weeks), castrated (4 weeks), hypophysectomized (4 and 8 weeks), thyroidectomized, parathyroidectomized (4 weeks), and adrenalectomized (4 weeks) animals were obtained from Carworth Farms, Rockland, N. Y. The efficacy of thyroidectomy, castration, and adrenalectomy was determined at autopsy. The efficacy of hypophysectomy was determined by weight gain postoperatively. Radioisotopically labeled [3,4-^{3}H] proline and [4-^{3}H] proline were purchased from New England Nuclear and Schwarz/Mann, respectively. Powdered triamcinolone diacetate was kindly supplied by Dr. J. M. Smith of Lederle Laboratories, Pearl River, N. Y. A suspension was prepared in 0.9% NaCl solution. Control animals received 0.9% NaCl.

Prolyl hydroxylase assay. At death each organ was weighed and 10% (w/v) tissue homogenates were prepared in cold 0.25 M sucrose using a Polytron ST homogenizer. Prior to homogenization the skin (cleaned free of muscle, fat tissue, and hair) and aorta were frozen in liquid nitrogen and pulverized. The homogenates were centrifuged at 16,000 × *g* for 20 min, and the resulting supernatant solution was used as the source of enzyme activity. Polytron homogenization solubilizes the majority of cellular enzyme activity (8).

Prolyl hydroxylase activity was measured by the tritium release assay of Hutton *et al.* (9). Substrate was prepared using either [3,4-^{3}H] or [4-^{3}H]proline. During this investigation substrates having various specific activities were used in the enzyme assay. Therefore comparisons of enzyme activities can only be made within each table or figure. The amount of tritiated water formed was linearly related to the time of incubation, to the amount of supernatant enzyme assayed, and to the amount of hydroxyproline formed. Final radioactivity in the experimental samples was at least 4 times the amount of radioactivity in the blank (60 cpm) prepared by incubating substrate (120,000 cpm) and cofactors without enzyme. Each incubation contained substrate (120,000 cpm), 0.3 μmoles of ferrous ammonium sulfate, 4.9 μmoles of ascorbic acid, 0.6 μmole of α-ketoglutarate, 348 μmoles of Tris-HCl (pH 7.5), and 0.02–0.1 ml of 16,000 × *g* supernatant enzyme made up to a total volume of 1.0 ml with deionized water. The samples were incubated at 30° for 30 min, and the reaction was terminated by addition of 0.1 ml of 50% (w/v) trichloracetic acid. The tritiated water formed was collected by vacuum distillation, and 0.7 ml was counted in a mixture of 30% (v/v) Triton X-100 as solubilizer in toluene–2,5-diphenyloxazole–1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene at an efficiency of 25%.

Enzymatically inactive, cross-reacting

protein was determined by the enzyme immunoassay of Stassen *et al.* (10).

Protein determination. The amount of protein was determined by the method of Lowry *et al.* (11), using bovine plasma albumin as standard.

RESULTS

Administration of triamcinolone diacetate (1.5 mg/kg) decreased prolyl hydroxylase activity in liver, lung, aorta, and skin of 4-week-old male rats (Fig. 1). The peak effect in liver, lung, and aorta was observed at 15 mg/kg. In the skin, however, increasing the dose of drug above 15 mg/kg resulted in a further decrease of enzyme activity.

The effect of administration of triamcinolone diacetate on prolyl hydroxylase activity in various organs of rats, ranging in age from 1 to 63 days, was determined (Table 1). In all organs of all the animals tested, significant decreases of prolyl hydroxylase activity were noted except in the livers of 63-day-old rats. Prolyl hydroxylase activity in skin was decreased to the same extent in 63-day-old animals as in 1- or 28-day-old rats. A much lower basal level of enzyme activity in skin of control animals was observed in the older group.

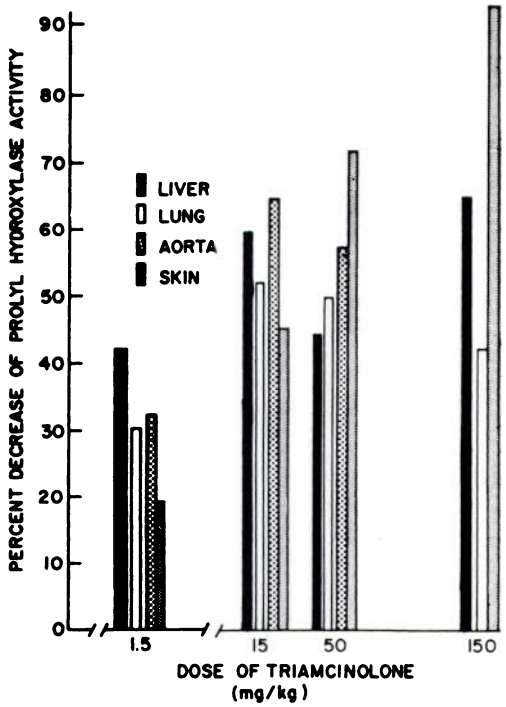

FIG. 1. *Effect of various doses of triamcinolone diacetate on prolyl hydroxylase activity of liver, lung, aorta, and skin of 28-day-old male rats.*

The percentage decrease of enzyme activity was calculated by dividing the mean enzyme activity of treated animals by that of control animals and subtracting the value obtained from 100. In all determinations the means of four to six animals in each control and drug-treated group were statistically different at $p \leq 0.05$.

The temporal responses of prolyl hydroxylase activity of skin and liver to daily intraperitoneal injections of triamcinolone diacetate are presented in Table 2. In liver, in contrast to skin, a statistically significant decrease of prolyl hydroxylase activity was noted 12 hr after a single injection. This may be related to the route of administration of triamcinolone. Significant decreases of prolyl hydroxylase activity in both the liver and skin were noted three or four daily intraperitoneal injections of triamcinolone diacetate, with the maximum response in both organs being observed after four daily injections. The slight decrease of prolyl hydroxylase activity in skin 12 hr after a single intraperitoneal injection of drug and the marked and significant decrease of enzyme activity after multiple days of treatment offer a model system for exploring the relationship between drug-induced alteration of prolyl hydroxylase activity and hydroxyproline biosynthesis.

The triamcinolone-mediated decrease of prolyl hydroxylase activity in liver and skin of 4-week-old rats was reversible (Table 3). Triamcinolone diacetate was administered intraperitoneally at 15 or 100 mg/kg for 3 consecutive days. Treatment was then discontinued, and the remainder of the animals were killed 9 days later, or a total of 12 days after the initiation of drug treatment. Enzyme activity in the skin and liver tissue 9 days after discontinuation of drug treatment at 15 mg/kg was equal to or above the values for controls. However, at 100 mg/kg, while the level of enzyme activity in the liver was equal to the control value, the enzyme activity of skin was only partially restored. The enzyme activity in

TABLE 1

Prolyl hydroxylase activity in various organs of rats of different ages following administration of triamcinolone diacetate

The values represent the means ± standard errors of enzyme activity of four to six animals. Animals were treated daily for 4 consecutive days with triamcinolone diacetate (50 mg/kg intraperitoneally) except for the aorta groups, which received 15 mg/kg intraperitoneally. All animals were killed 24 hr after the last injection.

Age rats	Group	Prolyl hydroxylase activity				
		Liver	Lung	Heart	Aorta	Skin
days		*dpm × 10⁻³/mg protein*				
1	Control	5.02 ± 0.80[a]	7.04 ± 0.46[a]	ND[b]	ND	33.36 ± 0.86[a]
	Triamcinolone	2.38 ± 0.48[c]	3.48 ± 0.34[c]	ND	ND	9.03 ± 1.49[c]
28	Control	2.07 ± 0.30[a]	5.48 ± 0.54[a]	7.32 ± 0.92[a]	23.12 ± 2.51[a]	22.91 ± 2.46[a]
	Triamcinolone	0.95 ± 0.07[c]	3.44 ± 0.32[c]	4.05 ± 0.76[c]	9.37 ± 1.40[c]	3.58 ± 0.89[c]
63	Control	0.77 ± 0.10[a]	2.64 ± 0.21[a]	3.06 ± 0.21[a]	13.92 ± 0.24[a]	2.35 ± 0.40[a]
	Triamcinolone	0.78 ± 0.18	1.90 ± 0.05[c]	2.0 ± 0.10[c]	5.44 ± 0.87[c]	0.73 ± 0.13[c]

[a] Significantly different from other controls of different ages at $p \leq 0.05$.

[b] ND not determined.

[c] Significantly different from the control of the same age at $p \leq 0.05$.

TABLE 2

Time course of decrease of prolyl hydroxylase activity after single or multiple injections of triamcinolone diacetate

The values represent the means ± standard errors of enzyme activity from five to ten animals. Four-week-old animals were treated daily with triamcinolone diacetate (50 mg/kg intraperitoneally). The groups receiving a single injection were killed 12 hr afterward. Those groups receiving multiple injections were killed 24 hr after the last injection.

No. of Injections	Group	Prolyl hydroxylase activity	
		Liver	Skin
		dpm × 10⁻³/mg protein	
1	Control	2.02 ± 0.23	35.18 ± 3.88
	Triamcinolone	1.29 ± 0.17[a]	31.01 ± 4.03
3	Control	2.05 ± 0.19	35.50 ± 3.71
	Triamcinolone	1.20 ± 0.20[a]	15.17 ± 2.38[a]
4	Control	1.80 ± 0.17	31.38 ± 3.16
	Triamcinolone	0.71 ± 0.10[a]	5.38 ± 1.22[a]

[a] Significantly different from control at $p \leq 0.05$.

the skin of animals treated at the higher dose may be equal to that of control animals after a longer period of drug deprivation.

Direct addition of the anti-inflammatory steroid to organ cultures of skin, lung, and liver and to established cell lines, including L-929 mouse fibroblasts human diploid fibroblasts, HeLa cells, and calvaria bone cells, failed to elicit a response in enzyme activity (data not shown). We therefore investigated the effect of ablation of various endocrine glands on the steroid-induced decrease of prolyl hydroxylase activity. Commercially available hypophysectomized male rats, operated on at 3 weeks of age, were maintained for either 1 week or 5 weeks before the initiation of drug treatment at 50 mg/kg intraperitoneally for 3 consecutive days. Prolyl hydrox-

TABLE 3

Reversibility of triamcinolone-mediated decrease of prolyl hydroxylase activity in liver and skin

The values represent the means ± standard errors of enzyme activity of three to five animals. Rats were treated daily for 3 consecutive days and killed 24 hr or 9 days after the last intraperitoneal injection with triamcinolone diacetate.

Dose	Day of death	Group	Prolyl hydroxylase activity	
			Liver	Skin
mg/kg			*dpm* × 10^{-2}/*mg protein*	
15	4	Control	1.72 ± 0.06	31.75 ± 1.47
		Triamcinolone	1.18 ± 0.03[a]	15.13 ± 2.91[a]
	12	Control	1.64 ± 0.09	24.33 ± 1.99
		Triamcinolone	1.91 ± 0.06	30.05 ± 0.48[a]
100	4	Control	1.44 ± 0.22	22.24 ± 2.11
		Triamcinolone	0.79 ± 0.15[a]	6.01 ± 0.64[a]
	12	Control	1.27 ± 0.16	17.68 ± 2.48
		Triamcinolone	1.30 ± 0.11	10.52 ± 2.42[a]

[a] Significantly different from control at $p \leq 0.05$.

ylase activity in the livers of 4-week-old hypophysectomized rats did not respond to the administration of anti-inflammatory steroids (Table 4). A significant decrease of enzyme activity was noted in the steroid-treated 8-week-old hypophysectomized rats as compared to control hypophysectomized rats (Table 4), whereas in intact rats the enzyme did not respond to steroid treatment (Tables 1 and 4). Thus the response of the enzyme of liver to hypophysectomy is dependent on the age of the animal. Enzyme activity in the lungs and skin of both 4- and 8-week-old hypophysectomized rats responded to steroid treatment, as it did in intact animals (Table 4). Enzyme activity in the lungs and skin of 4-week-old hypophysectomized rats and in the lungs of 8-week-old hypophysectomized rats was also decreased. The decreased levels of prolyl hydroxylase activity in skin of 4-week-old hypophysectomized animals may account for the decrease in proline hydroxylation of newly formed collagen of skin upon hypophysectomy reported by Valavaaro *et al.* (12).

Administration of triamcinolone diacetate to 3-week-old adrenalectomized, castrated, and thyroidectomized-parathyroidectomized male rats resulted in a decrease of prolyl hydroxylase activity of liver, heart, and skin (Table 5). The decreases in prolyl hydroxylase activity in various tissues were similar in endocrine ablated rats relative to intact animals. Prolyl hydroxylase activity of the skin was reduced in castrated and adrenalectomized rats as compared with intact controls.

Recently McGee *et al.* (13) demonstrated the presence of an enzymatically inactive protein from early logarithmic-phase L-929 fibroblasts that cross-reacts with antibody to purified prolyl hydroxylase. This protein (cross-reacting protein) appears to be an inactive precursor subunit of the enzymatically active protein, and enzyme activity is regulated by subunit aggregation (14, 15).

Administration of triamcinolone diacetate to 4-week-old rats resulted in a 70–80% decrease in enzyme activity and total antigenic protein as well as in the cross-reacting enzyme precursor (Table 6). The decrease in enzyme activity was found to be linearly related to the decrease in cross-reacting protein in both control and drug-treated animals (Fig. 2). Furthermore, in

TABLE 4

Triamcinolone-mediated decrease of prolyl hydroxylase activity in hypophysectomized rats

The values represent the means ± standard errors of enzyme activity of four to six animals. Commercially obtained, 4-week-old hypophysectomized rats were treated (50 mg/kg intraperitoneally) for 4 consecutive days or maintained until 8 weeks of age before initiation of drug treatment. All animals were killed 24 hr after the last injection. The values cannot be compared between the 4- and 8-week-old groups because different substrates were used to assay for enzyme activity.

Age of rats	Group	Prolyl hydroxylase activity		
		Liver	Lung	Skin
wk		*dpm × 10⁻²/mg protein*		
4	Control	2.07 ± 0.30	5.76 ± 0.50	22.91 ± 2.46
	Triamcinolone	0.95 ± 0.07[a]	3.44 ± 0.32[a]	3.58 ± 0.89[a]
	Hypophysectomized	1.46 ± 0.15	3.74 ± 0.30[a]	10.11 ± 2.14[a]
	Hypophysectomized + triamcinolone	1.11 ± 0.21[a]	2.13 ± 0.21[a, b]	3.79 ± 2.11[a, b]
8	Control	3.37 ± 0.72	8.51 ± 0.92	3.01 ± 0.58
	Triamcinolone	2.66 ± 0.21	5.27 ± 0.61[a]	0.72 ± 0.15[a]
	Hypophysectomized	2.80 ± 0.39	5.12 ± 0.48[a]	4.18 ± 0.84
	Hypophysectomized + triamcinolone	0.51 ± 0.04[a, b]	3.69 ± 0.35[a, b]	0.74 ± 0.20[a, b]

[a] Significantly different from intact control at $p \leq 0.05$.

[b] Significantly different from endocrine-ablated animal at $p \leq 0.05$.

TABLE 5

Triamcinolone-mediated decrease of prolyl hydroxylase activity in adrenalectomized, castrated, and thyroid-parathyroidectomized rats

The values represent the means ± standard errors of enzyme activity of three to six animals. Commercially obtained, castrated, adrenalectomized, and thyroidectomized-parathyroidectomized 3-week-old rats were maintained for 1 week before initiation of drug treatment (50 mg/kg intraperitoneally) for 4 consecutive days. All animals were killed 24 hr after their last injection. Variation in controls resulted from the use of two different substrate preparations with different specific activities.

Group	Prolyl hydroxylase activity		
	Liver	Heart	Skin
	dpm × 10⁻²/mg protein		
Control	1.40 ± 0.17	4.63 ± 0.21	25.05 ± 1.71
Adrenalectomized	1.50 ± 0.27	5.56 ± 0.57	19.53 ± 1.40[a]
Adrenalectomized + triamcinolone	0.67 ± 0.15[a, b]	2.79 ± 0.19[a, b]	2.72 ± 0.59[a, b]
Castrated	1.53 ± 0.05	5.38 ± 0.27	15.70 ± 1.58[a]
Castrated + triamcinolone	0.79 ± 0.19[a, b]	2.27 ± 0.22[a, b]	2.18 ± 0.96[a, b]
Control	3.19 ± 0.10	7.10 ± 0.45	29.82 ± 2.39
Thyroidectomized-parathyroidectomized	2.84 ± 0.11[a]	6.01 ± 0.54	29.73 ± 2.88
Thyroidectomized-parathyroidectomized + triamcinolone	1.58 ± 0.08[a, b]	3.36 ± 0.31[a, b]	3.59 ± 0.88[a, b]

[a] Significantly different from intact control at $p \leq 0.05$.

[b] Significantly different from endocrine-ablated animal at $p \leq 0.05$.

TABLE 6

Effect of administration of triamcinolone diacetate on prolyl hydroxylase activity, total antigenic prolyl hydroxylase, and cross-reacting protein in skin

Four-week-old rats were treated with triamcinolone diacetate (50 mg/kg intraperitoneally) for 3 consecutive days and killed 24 hr after the last injection. Total antigenic protein and enzymatically inactive cross-reacting protein are expressed in activity units of a standard heat-inactivated rat skin prolyl hydroxylase which was separated from cross-reacting protein on a DEAE-Sephadex column (10). Values are the means ± standard errors of three animals.

Measurement	Control	Triamcinolone treated
	dpm × 10^{-3}/*mg protein*	
Prolyl hydroxylase activity	77 ± 6	20 ± 4[a]
Total antigen	457 ± 43	104 ± 4[a]
Cross-reacting protein	380 ± 49	84 ± 11[a]

[a] Significantly different from control at $p \leq 0.05$.

newborn rats the administration of triamcinolone diacetate resulted in proportional decreases of prolyl hydroxylase activity and cross-reacting protein in lung and skin (data not shown). These findings indicate that the triamcinolone-mediated decrease in prolyl hydroxylase activity is due to a decrease in the amount of enzyme precursor.

DISCUSSION

Dramatic effects of administration of anti-inflammatory steroids on collagen metabolism have been reported in both normal and inflamed connective tissues. However, the mechanism of action of this class of therapeutic agents on collagen metabolism remains a mystery. Steroid treatment of animals results in a decrease in urinary excretion of hydroxyproline (2, 3, 16) which is accompanied by a dramatic decrease of newly formed collagen. In the present study administration of a synthetic anti-inflammatory steroid to rats resulted in a decrease of prolyl hydroxylase in a number of tissues in animals of different ages. The response was found to be dose-dependent, and the dose required to produce a significant decrease in enzyme was in the range used by other workers to produce observable changes in urinary hydroxyproline and soluble collagen hydroxyproline (2, 3, 16, 17).

The decrease in enzyme activity following steroid treatment is not due to a generalized non-reversible toxic effect of large doses of these drugs. When drug treatment was discontinued after three daily doses, the level of prolyl hydroxylase activity returned to control.

The dependence of prolyl hydroxylase activity on endocrine function was suggested by a related study in which the diurnal rhythmicity of enzyme activity in liver was reversed by adrenalectomy while the rhythmicity of enzyme activity in the skin was completely abolished by adrenalectomy and prior treatment with hydrocortisone (18). We thus determined whether the responsiveness of prolyl hydroxylase to steroid treatment would be altered by endocrine ablation. When triamcinolone was administered to 4-week-old hypophysectomized rats, no decrease in liver prolyl

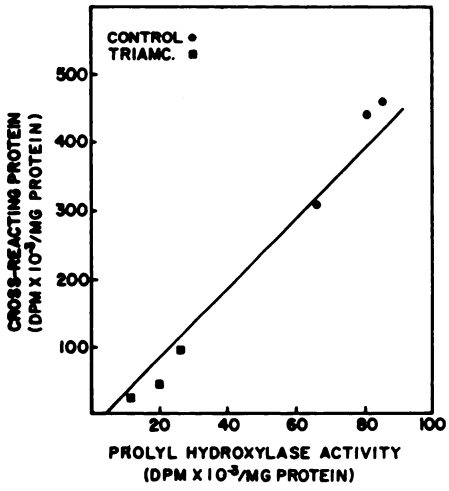

FIG. 2. *Correlation between amounts of proly hydroxylase activity and cross-reacting protein in control and triamcinolone diacetate-treated rats*

Rats (28 days old) were treated for 4 consecutive days with treamcinolone diacetate (50 mg/kg intraperitoneally) and killed 24 hr after the last injection. The statistics for the data are as follows: correlation coefficient = 0.99, slope = 5.6, y intercept = 33.8, and x intercept = 6.0. Each point represents data from one animal.

hydroxylase activity was observed as compared to hypophysectomized controls. The response of prolyl hydroxylase of liver to steroid treatment was dependent on the age of the animal. In 8-week-old animals, in which a response of enzyme activity in liver tissue following steroid treatment was not observed, hypophysectomy resulted in a response to drug treatment. The paradoxical effect of hypophysectomy on prolyl hydroxylase activity in the livers of animals of different ages following steroid treatment may result from different levels of factor(s) responsible for steroid action on liver prolyl hydroxylase activity during this development period. Although a diminished or increased responsiveness of prolyl hydroxylase activity in various organs to administration of anti-inflammatory steroids may be obtained by ablation of certain endocrine glands, there is no absolute requirement for a particular endocrine gland for a second positive messenger for the action of anti-inflammatory steroids on prolyl hydroxylase activity. However, these studies do not rule out the possibility that a factor(s) produced by an endocrine gland(s) may be required for maintaining the basal level of prolyl hydroxylase activity and that the suppression of secretion of this humoral agent(s) by treatment with anti-inflammatory steroids may result in a decrease of enzyme activity. For example, such a factor may exist in the hypophysis, since in hypophysectomized weanling rats a substantial decrease of prolyl hydroxylase activity in skin and lung tissues was observed.

Perturbations of the basal level of prolyl hydroxylase activity were observed in various tissues by ablation of certain endocrine glands. The basal level of prolyl hydroxylase activity in skin was decreased in 4-week-old hypophysectomized, adrenalectomized, and castrated animals. Enzyme activity in lung was also lower in hypophysectomized animals than in controls. Kao *et al.* (19) demonstrated that a decrease in prolyl hydroxylase activity in uterine tissue following ovariectomy was restored to the control level by treatment with either estradiol and progesterone or a combination of these hormones. The regulation of prolyl hydroxylase activity in the whole animal may involve hormonal interactions.

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