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Evidence for the inhibition of protocollagen biosynthesis by an anti-inflammatory steroid in rat carrageenin granuloma

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STEROIDAL anti-inflammatory drugs have been shown to inhibit collagen synthesis in granulation tissues.[1–5] The precise mechanism of the inhibition, however, is not yet known. It has been demonstrated in a previous paper[5] that betamethasone disodium phosphate, a steroidal anti-inflammatory drug, affected neither the process of hydroxylation of protocollagen nor the activity of protocollagen proline hydroxylase in rat carrageenin granuloma, even though the steroid strongly inhibited collagen synthesis in the granuloma. These data suggested indirectly that the steroid inhibited the synthesis of protocollagen, the proline rich polypeptide precursor of collagen. The present study was designed to obtain direct evidence for the inhibition of protocollagen biosynthesis by betamethasone disodium phosphate in rat carrageenin granuloma.

Experiments were done to establish a direct assay method for the rate of protocollagen synthesis in rat carrageenin granuloma. A granuloma pouch was induced in male rats of the Donryu strain weighing 100–120 g by injecting a 2 per cent solution of Seakem 202 carrageenin s.c. according to the procedure described previously.[1] On day 8 after the carrageenin injection, α,α'-dipyridyl (10 mg/rat) was administered into the granuloma pouch, and then 30 min later [^{3}H]proline (50 μc/rat; 29 c/m-mole, generally labeled) was injected i.v. The granuloma (3·5–4·5 g in wet weight) was harvested 30 min after the [^{3}H]proline injection and homogenized in 30 ml of ice-cold 1% L-proline with a Vir-Tis 45 homogenizer at maximum speed for 2 min. The homogenate was dialyzed against running tap water overnight and then against five liters of Tris-HCl buffer (50 mM, pH 7·6) containing 100 mM KCl with stirring at 4° for 2 days. The buffer was changed every day. The dialyzed homogenate was placed in a boiling water bath for 5 min[6] and centrifuged at 100,000 *g* for 60 min at 10°. The supernatant was used as the [^{3}H]proline-labeled protocollagen fraction of the granuloma. An aliquot of the supernatant was incubated with protocollagen proline hydroxylase as described in a previous paper.[5] In brief, the incubation medium consisted of the supernatant ([^{3}H]proline-labeled protocollagen fraction, 2·5–10·0 mg protein), an excess of a hydroxylase fraction obtained from the 8-day-old granuloma (100,000 *g* supernatant of its homogenate containing 38·8 mg protein), 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 7·0 ml. The mixtures were incubated with shaking at 37° for 90 min in air. Reactions were stopped by adding 7 ml of 12 N HCl, and the samples were hydrolyzed at 105° for 16 hr. The total radioactivity (dis/min) of [^{3}H]hydroxyproline in the hydrolysate was determined by the procedures described in a previous paper.[5] The radioactivity of collagen hydroxyproline formed during the incubation was obtained by subtracting the radioactivity of [^{3}H]hydroxyproline existing in the hydrolysates of 0-time incubation samples from the radioactivity of [^{3}H]hydroxyproline in the hydrolysates of respective incubation products. As shown in Fig. 1, the radioactivity of [^{3}H]hydroxyproline formed during the incubation was proportional to the amount of [^{3}H]proline-labeled protocollagen added to the incubation medium, indicating that the amount of labeled protocollagen can be measured in terms of the radioactivity of [^{3}H]hydroxyproline formed in the present incubation procedures. In addition, efficiency of the extraction procedure for the labeled protocollagen was examined. The residue of the first extraction of the labeled protocollagen from the granuloma homogenate was again placed in a boiling water bath with 30 ml of the extraction buffer for 5 min and centrifuged. The hydroxylation of the second extract gave [^{3}H]hydroxyproline less than 5 per cent of the first extracts, demonstrating that the efficiency of a single extraction was sufficient for the present assay method. Another series of experiments were done as illustrated in Fig. 2, in order to determine the time course of the accumulation of labeled protocollagen in the granuloma of α,α'-dipyridyl-treated rats. The results indicated that labeled protocollagen was accumulated linearly as a function of time up to 45 min after the injection of [^{3}H]proline. Therefore, the time suitable for killing animals was selected to be 30 min after the intravenous injection of [^{3}H]proline in order to determine the rate of labeled protocollagen synthesis in the α,α'-dipyridyl-treated granuloma.

The effect of betamethasone disodium phosphate on protocollagen synthesis was investigated on the basis of the assay method described above. Betamethasone disodium phosphate (2 mg/rat; Δ^1, 9α-fluoro-16β-methylcortisol-17-disodium phosphate) was injected i.v. to granuloma-bearing rats 4 hr prior to the administration of α,α'-dipyridyl. Control animals were given i.v. 0·9% NaCl in place of the steroid. [^{3}H]proline was injected i.v. 30 min after the injection of α,α'-dipyridyl, and the animals

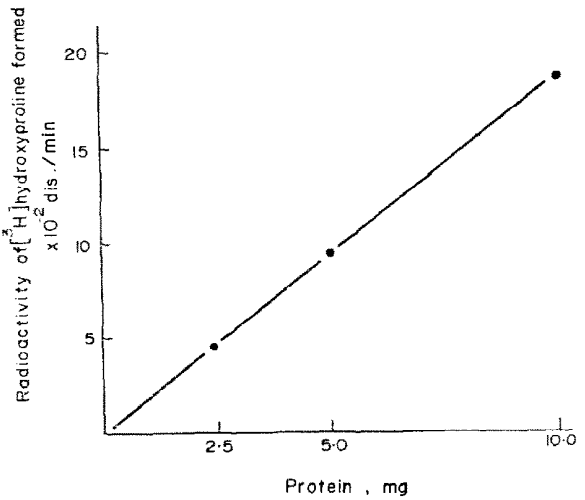

FIG. 1. Relationship between [^{3}H]hydroxyproline formed and the protein content of [^{3}H]proline-labeled protocollagen fraction added to the incubation mixture. Each point is the average of two determinations. Incubation conditions are described in the text.

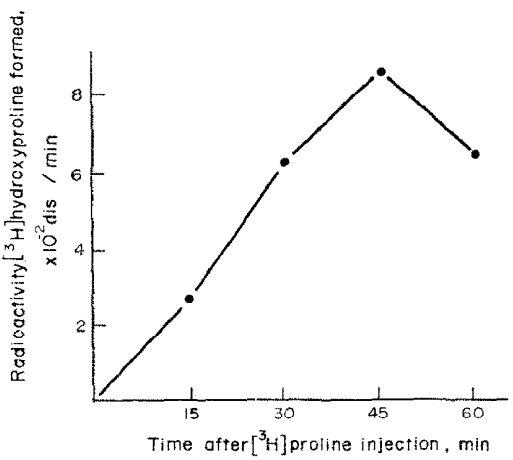

FIG. 2. Accumulation of [^{3}H]proline-labeled protocollagen in rat carrageenin granuloma. [^{3}H]L-proline (50 μc/rat) was injected i.v. 30 min after the injection of α,α'-dipyridyl (10 mg/rat) into the pouch. The animals were killed at 15, 30, 45 or 60 min after [^{3}H]proline injection. Each point is the average of two or three determinations. Labeled protocollagen fractions (6·4–8·8 mg protein) were incubated as described in the text.

were killed 30 min later. The amount of labeled protocollagen accumulated in the granuloma was determined according to the incubation method mentioned above. The results are summarized in Table 1. The incorporation of [^{3}H]proline into protocollagen was inhibited by 54 per cent at 4·5 hr after the steroid injection. Agreeing with the inhibition rate of 54 per cent in the present study, a previous paper[5] reported 62 per cent inhibition of the [^{3}H]proline incorporation into collagen hydroxyproline in the granulomas of rats treated with the steroid in the same manner as in the present study. Neither accumulation of protocollagen nor any change of protocollagen proline hydroxylase activity was also reported in the previous paper. It may be concluded, therefore, that the steroid inhibits the

TABLE 1. EFFECT OF BETAMETHASONE DISODIUM PHOSPHATE ON THE INCORPORATION OF [^{3}H]PROLINE INTO PROTOCOLLAGEN OF CARRAGEENIN GRANULOMA IN RATS*

Treatment	No. of rats	Radioactivity of [^{3}H]hydroxyproline		Radioactivity of [^{3}H]hydroxyproline formed (dis./min)	Inhibition (%)
		Before incubation (dis./min)	After incubation (dis./min)		
Control	7	108 ± 30	661 ± 53	553 ± 29	
Betamethasone	8	17 ± 8	271 ± 22	254 ± 26	54·0

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

synthesis of the collagen by inhibiting protocollagen synthesis without affecting the hydroxylation of protocollagen. The findings described above, however, do not rule out the possibility that the steroid inhibits [^{3}H]proline transport through the cell membrane and affects the proline pool size of the fibroblast, since all of the results described above were obtained from the experiments analyzing the incorporation of [^{3}H]proline into protocollagen or collagen hydroxyproline.

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Effect of oxytocin on Mg^{2+}-dependent ATPase

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IN ADDITION to various ATPases in erythrocyte membrane there is also present Mg^{2+}-dependent ATPase (adenosine triphosphate hydrolase, Mg^{2+}—EC 3.6.1.4) the function of which is not clear yet. Certain relation between this enzyme, the membrane contractile protein[1] and the contractile protein from smooth muscle is supposed. It was *viz* proved that the Mg^{2+}-dependent ATPase in erythrocyte membrane is activated by adrenaline or noradrenaline,[2] i.e. by the hormones which influence the contraction of smooth muscle. In the present paper an attempt was made to determine whether oxytocin which influences the contraction of some smooth muscles will activate Mg^{2+}-dependent ATPase in erythrocyte membrane, too.