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STUDIES WITH ^{14}C-PROLINE ON THE ACTION OF CORTISONE ON THE METABOLISM OF COLLAGEN IN THE RAT

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Abstract—The action of cortisone on the metabolism of collagen was studied by injecting ^{14}C-proline into rats and determining the effect of cortisone on the specific activity and total activity of ^{14}C-hydroxyproline in the urine and in the skin collagen fractions. In the first series of experiments the administration of cortisone was begun 10 days before the administration of ^{14}C-proline. Both the amount of hydroxyproline excreted and the specific activity of urine ^{14}C-hydroxyproline were lower in the cortisone-treated rats than in the controls. The total activity of urine ^{14}C-hydroxyproline was therefore still more reduced, and during some of the urine collection periods it decreased to almost half the value of the controls. The mean values for the specific activity and total activity of ^{14}C-hydroxyproline in the soluble collagen fraction of the skin were likewise lower in the cortisone-treated rats than in the controls. In the second series of experiments the ^{14}C-proline was injected 30 days before the start of cortisone administration. A small decrease occurred in the amount of hydroxyproline excreted in the urine in the cortisone-treated rats compared with the controls, but the total activity of urine ^{14}C-hydroxyproline remained unchanged. The total activity of ^{14}C-hydroxyproline in the skin collagen fractions was likewise not changed by the cortisone treatment. The results of the present study suggest that pharmacological doses of cortisone have an antianabolic action on the formation of soluble collagen, but no effect on the catabolism of the insoluble collagen fibres.

CORTISONE, at least in pharmacological doses, has an inhibitory effect on the metabolism of collagen. The nature of this inhibition, however, is still unknown. Several studies suggest that large doses of cortisone have an antianabolic action on the metabolism of collagen in the skin of animals[1–5] and in developing chick embryos[6–9], but some studies suggest that cortisone at the same time increases the catabolism of the collagen fibres.[2] In addition, it has been regarded as possible that an altered stability of collagen fibrils caused by changes in the mucopolysaccharides of the ground substance of the connective tissue might at least contribute to the changes observed after adrenalectomy.[4]

Our recent study[10] indicated that the administration of cortisone greatly decreased the excretion of hydroxyproline—the amino acid specific for collagen—in the urine of young rats, but not of older ones. These results supported the hypothesis that the action of cortisone on the metabolism of collagen is mainly antianabolic and not catabolic, for a catabolic action would have increased and not decreased the excretion of hydroxyproline. In the present study this question was studied further by injecting ^{14}C-proline into rats and analysing the specific activities and total activities of ^{14}C-hydroxyproline in the urine and in the collagen fractions of the skin.

MATERIAL AND METHODS

The experimental animals were male albino Wistar rats, 1½ months of age at the beginning of the experiments. They were fed *ad libitum* with a commercial pelleted diet (Hankkija Oy)* and allowed free access to water. Cortisone acetate (Adreson, Organon) was injected intraperitoneally. In the experiments on the antianabolic effect of cortisone the daily dose of cortisone acetate was 2 mg and in the experiments on the catabolic effect 4 mg per rat (about 2 mg/100 g body wt. in both experiments). ^{14}C-proline was injected subcutaneously in 1·00 ml of 0·9% sodium chloride solution. In the experiments on the antianabolic effect of cortisone 15 μc per rat and in the experiments on the catabolic effect of cortisone 25 μc per rat of uniformly labelled L-^{14}C-proline (12·6 μc/μM, The Radiochemical Centre, Amersham) was used.

Urine was collected under toluene during the periods indicated under Results. It was stored at +4° until hydrolysed with an equal volume of 12 N HCl for 3 hr at 124°, and used for the determination of the amount and specific activity of hydroxyproline excreted during the collection period.

The skin specimens were fractionated for soluble collagen hydroxyproline and insoluble collagen hydroxyproline as follows: Two specimens of the dorsal skin of each animal were taken and analysed separately. The values for each animal are thus the mean values of two independent analyses. The specimens taken were rapidly dissected free of fascia and subcutaneous fat on ice-cooled trays, weighed and homogenized in cold (±0°) 0·45 M sodium chloride, 4 ml/g of skin, first with scissors and then with an Ultra-Turrax homogenizer (Janke-Kunkel) 5 times for 15 sec at 2 min intervals to prevent warming of the samples. The homogenates were extracted at +4° for 24 hr with occasional stirring, after which they were centrifuged at 60,000 *g* for 60 min. The supernatants obtained were precipitated with 4 vols of cold ethanol and after centrifugation extracted twice with 80% ethanol, twice with absolute ethanol, twice with ether and twice with warm ethanol–ether (1:2). The residues were gelatinized with distilled water at 124° for 3 hr and after filtration a sample of the gelatin solution was hydrolysed with an equal volume of 12 N HCl for 6 hr at 138° and used for the determination of the quantity and specific activity of the soluble collagen hydroxyproline.

The precipitates obtained after centrifugation at 60,000 *g* were washed 3 times with 0·45 M sodium chloride-solution and twice with distilled water, after which they were extracted with absolute ethanol, ether and ethanol–ether and gelatinized as above. A sample of the gelatin solution was hydrolysed with an equal volume of 12 N HCl for 6 hr at 138° and used for the determination of the quantity and specific activity of the insoluble collagen hydroxyproline.

The quantity of hydroxyproline was determined by the method of Prockop and Udenfriend[11] and the specific activity of ^{14}C-hydroxyproline by the method of Prockop *et al.*[12] Because of the high radioactivity of the ^{14}C-proline in the urine collected during the first 3 days after ^{14}C-proline administration, hydroxyproline was preliminarily separated from proline in these samples in a Dowex 50-X8 column with 2 N HCl as eluting agent.

* The food pellets, which contained powdered bone had a hydroxyproline content of about 0·05 per cent of the dry wt. The hydroxyproline excretion with this diet was within 10 per cent of that with a completely hydroxyproline-free diet.

RESULTS

Experiments on the antianabolic effect of cortisone on the metabolism of collagen

After preliminary experiments, an experiment comprising six rats in each of two groups was carried out. The administration of cortisone acetate was begun 10 days before the injection of ^{14}C-proline. The mean weight of the rats before the beginning of cortisone administration was 95 g in both groups. When ^{14}C-proline was injected, the mean weight of the controls was 118 g and of the cortisone-treated rats 109 g. Three rats of both groups were killed 8 hr after the ^{14}C-proline injection and the remaining three rats of both groups 5 days after the ^{14}C-proline injection.

The effect of cortisone on the urine hydroxyproline is shown in Table 1. The administration of cortisone reduced both the amount of hydroxyproline excreted in the urine and the specific activity of ^{14}C-hydroxyproline, compared with the controls. The total activity of ^{14}C-hydroxyproline was, therefore, still more reduced and during

TABLE 1. EFFECT OF CORTISONE ON THE URINARY EXCRETION OF HYDROXYPROLINE AND ON THE SPECIFIC ACTIVITY AND TOTAL ACTIVITY OF ^{14}C-HYDROXYPROLINE AFTER THE ADMINISTRATION OF ^{14}C-PROLINE

The administration of cortisone was begun 10 days before the ^{14}C-proline injection.

Collection period after ^{14}C-proline	Group	Specific activity of hydroxyproline (c.p.m./μg)	Excretion of hydroxyproline μg during collection	Total activity of hydroxyproline (c.p.m.)
0–8 hr	Controls	30·9 (26·8–36·0)	169 (155–186)	5200 (4620–6010)
	Cortisone	23·8 (23·1–24·9)	151 (142–158)	3600 (3540–3650)
0–12 hr	Controls	22·8 (20·5–25·3)	236 (206–293)	5440 (4260–7410)
	Cortisone	18·7 (15·5–22·4)	175 (161–189)	3250 (2710–3600)
12–24 hr	Controls	7·8 (7·4–8·0)	269 (219–348)	2090 (1760–2740)
	Cortisone	5·1 (4·9–5·5)	263 (182–306)	1350 (910–1690)
1½–2½ days	Controls	4·3 (4·0–4·9)	554 (389–666)	2410 (1610–2990)
	Cortisone	3·2 (2·9–3·6)	406 (287–508)	1290 (890–1500)
4–5 days	Controls	3·2 (3·0–3·5)	681 (540–806)	2270 (1660–2450)
	Cortisone	2·3 (2·2–2·6)	540 (512–571)	1260 (1170–1310)

some of the urine collection periods it was decreased to almost half the values of the controls. The results further show that maximal labelling of the urine hydroxyproline occurs very early, since in the controls the specific activity of the urine ^{14}C-hydroxyproline during 0–8 hr was considerably higher than during 0–12 hr, and the latter value was about three times as high as that during 12–24 hr.

The effect of cortisone on the hydroxyproline in the collagen fractions of the skin is shown in Table 2. The results indicate that cortisone slightly decreased the mean of the specific activity and of the total activity of the ^{14}C-hydroxyproline in the soluble collagen fraction of the skin. The deviation of the values at 8 hr was so great that the difference cannot be regarded as significant, but it is noteworthy that the direction of the changes was the same as would be expected from the values of the urine. At 5 days the changes were similar to those at 8 hr and probably the differences in the specific activities and total activities of the soluble collagen hydroxyproline can be

TABLE 2. EFFECT OF CORTISONE ON THE SPECIFIC ACTIVITY AND TOTAL ACTIVITY OF ^{14}C-HYDROXYPROLINE IN THE COLLAGEN FRACTIONS OF THE SKIN AFTER THE ADMINISTRATION OF ^{14}C-PROLINE

The administration of cortisone was begun 10 days before the ^{14}C-proline administration.

Time after ^{14}C-proline injection	Collagen fraction	Group	Specific activity of hydroxyproline (c.p.m./μg)	Content of hydroxyproline (μg/g of skin)	Total activity of hydroxyproline (c.p.m./g of skin)
8 hr	Soluble collagen	Controls	20·9 (18·8–24·1)	1,170 (1,120–1,210)	24,400 (21,000–28,100)
		Cortisone	18·3 (13·3–22·7)	1,100 (1,060–1,180)	20,300 (14,400–24,200)
	Insoluble collagen	Controls	0·18 (0·16–0·20)	17,400 (16,000–19,100)	3,100 (2,700–3,800)
		Cortisone	0·15 (0·09–0·18)	17,200 (14,000–20,000)	2,700 (1,300–3,600)
5 days	Soluble collagen	Controls	2·64 (2·55–2·75)	967 (814–1,070)	2,570 (2,080–2,940)
		Cortisone	2·09 (1·73–2·45)	903 (870–984)	1,890 (1,500–2,120)
	Insoluble collagen	Controls	1·64 (1·48–1·77)	20,600 (16,900–23,800)	33,900 (25,000–42,000)
		Cortisone	1·28 (0·98–1·48)	20,700 (17,300–23,600)	27,200 (17,700–34,900)

TABLE 4. EFFECT OF CORTISONE ON THE SPECIFIC ACTIVITY AND TOTAL ACTIVITY OF ^{14}C-HYDROXYPROLINE IN THE COLLAGEN FRACTIONS OF THE SKIN AFTER THE ADMINISTRATION OF ^{14}C-PROLINE

The administration of cortisone was begun 30 days after the ^{14}C-proline injection and the analysis was carried out 40 days after the ^{14}C-proline injection.

Collagen fraction	Group	Specific activity of hydroxyproline (c.p.m./μg)	Content of hydroxyproline (μg/g of skin)	Total activity of hydroxyproline (c.p.m./g of skin)
Soluble collagen	Controls	0·35 (0·32–0·37)	537 (484–617)	189 (164–228)
	Cortisone	0·44 (0·42–0·46)	416 (378–456)	185 (159–210)
Insoluble collagen	Controls	1·32 (1·27–1·38)	27,000 (22,300–30,800)	35,500 (30,800–40,000)
	Cortisone	1·41 (1·37–1·44)	27,200 (26,600–28,000)	38,400 (38,300–38,600)

regarded as significant at 5 days. Furthermore, the specific activity of hydroxyproline in the insoluble collagen fraction was lower in the cortisone-treated rats than in the controls at 5 days.

Experiments on the catabolic effect of cortisone on the metabolism of collagen

Six rats weighing 118 g (116–121 g) received 25 μc of ^{14}C-proline. Three of these rats began to receive cortisone 30 days later. The mean weight of the controls at day 30 was 203 g and of the cortisone group 199 g. After 10 days' cortisone administration the corresponding values were 215 g and 198 g.

The effect of cortisone on the urine hydroxyproline is shown in Table 3. The two groups had about the same hydroxyproline excretion before the beginning of cortisone

TABLE 3. EFFECT OF CORTISONE ON THE SPECIFIC ACTIVITY AND TOTAL ACTIVITY OF ^{14}C-HYDROXYPROLINE IN THE URINE AFTER THE ADMINISTRATION OF ^{14}C-PROLINE

The administration of cortisone was begun 30 days after the ^{14}C-proline administration.

Collection period after ^{14}C-proline	Group	Specific activity of hydroxyproline (c.p.m./μg)	Excretion of hydroxyproline (μg/24 hr)	Total activity of hydroxyproline (c.p.m.)
19–20 days	Controls	2·05 (2·02–2·08)	415 (405–427)	851 (830–862)
	Cortisone*	1·96 (1·85–2·06)†	440 (408–470)	892 (817–967)†
29–30 days	Controls	0·99 (0·88–1·10)	512 (463–585)	501 (478–515)
	Cortisone*	1·10 (0·89–1·23)	510 (410–605)	524 (483–550)
34–35 days	Controls	0·98 (0·96–1·00)	522 (446–579)	507 (446–543)
	Cortisone	1·15 (0·91–1·55)	413 (318–495)	486 (290–678)
39–40 days	Controls	0·82 (0·77–0·88)	493 (436–537)	402 (384–413)
	Cortisone	0·96 (0·95–0·97)	420 (387–452)	404 (375–408)

* Before the beginning of cortisone administration.
† One sample lost.

administration. Thereafter there was a decrease in the urinary hydroxyproline excretion and a slight increase in the specific activity of ^{14}C-hydroxyproline in the cortisone-treated rats as compared with the controls, but no change in the total activity of ^{14}C-hydroxyproline. At 40 days the deviation of the values was very small within both groups. Thus the results exclude an increase in the total activity of ^{14}C-hydroxyproline in the urine of the cortisone-treated rats compared with the controls. In the same series of experiments a third group of rats received L-thyroxine. The total activity of ^{14}C-hydroxyproline in the urine of these rats increased greatly after the thyroxine administration (Kivirikko and Laitinen, to be published). This indicates that it would have been possible to establish an increased catabolism of the insoluble collagen fibres by the technique used in the present experiment.

The effect of cortisone on the hydroxyproline in the collagen fractions of the skin is shown in Table 4. The total activity of ^{14}C-hydroxyproline in the skin collagen fractions, like the total activity of ^{14}C-hydroxylproline in the urine, was not changed by the cortisone administration.

DISCUSSION

The studies of Lindstedt and Prockop[13] with ^{14}C-proline indicate that the urine hydroxyproline of young rats is derived from at least three pools of body collagen with half-lives of about 1 day, 5 days and 50 to 100 days. Comparison of these values with the specific activities of body collagen fractions suggested that the first two of these pools represented the soluble collagen fractions, *i.e.* fractions containing collagen not yet aggregated to the insoluble collagen fibres, and the third pool the insoluble collagen.[13, 14] The results of the present study indicate that the maximal labelling of the urine hydroxyproline occurs very early, as already suggested by Prockop,[14] for the specific activity of the ^{14}C-hydroxyproline in the urine of the controls collected during 0–8 hr was considerably higher than that in the urine collected during 0–12 hr and the latter value was about three times as high as that in the urine collected during 12–24 hr after the ^{14}C-proline injection.*

The experiments on the antianabolic effect of cortisone on the metabolism of collagen were carried out during the first 5 days after the ^{14}C-proline injection, when the radioactivity of urine hydroxyproline is derived from the soluble collagen fractions.[13, 14] In the present study 0·45 M sodium chloride solution was used for the extraction of the soluble collagen, whereas Prockop[14] used a 0·15 M sodium chloride solution. The fraction extractable with 0·45 M sodium chloride includes the 0·15 M sodium chloride-soluble collagen but in addition small amounts of soluble collagen, which is an intermediate between the 0·15 M sodium chloride-soluble collagen and the insoluble collagen.[15] Because of the heterogeneity of the 0·45 M sodium chloride-soluble collagen fraction, it is not surprising that the specific activity of the hydroxyproline in this fraction was slightly lower than that of the urine hydroxyproline. Furthermore, it is possible that the specific activity of the hydroxyproline in the soluble collagen may have been somewhat higher in some other tissues than in the skin. Nevertheless, the results indicate that the specific activity of the urine hydroxyproline relatively closely paralleled the specific activity of the hydroxyproline in the 0·45 M sodium chloride-soluble collagen fraction during the 5-day experiment. By contrast, the specific activity of the insoluble collagen hydroxyproline was very low at 8 hr compared with that of the urine hydroxyproline and even at 5 days this value was only about half the value of the urine. Thus it seems evident that during the 5-day experiment the urine ^{14}C-hydroxyproline did not originate in appreciable amounts from the insoluble collagen.

In the cortisone-treated rats the specific activity and total activity of the urine ^{14}C-hydroxyproline were definitely reduced compared with the controls during this 5-day period, which suggests inhibition of soluble collagen formation. The determinations of the ^{14}C-hydroxyproline in the skin collagen fractions likewise revealed a slight decrease in the mean specific activity and total activity of the soluble collagen hydroxyproline in the cortisone-treated rats compared with the controls. The changes in the skin were considerably smaller than those in the urine, however. The results are in agreement with studies made by different analytical techniques, which suggest

* The ^{14}C-proline used in this study was assayed for ^{14}C-hydroxyproline radioactivity after a chromatography in a Dowex 50-X8 column with carrier hydroxyproline and found to contain 0·02 per cent ^{14}C-hydroxyproline. According to the experiment of Lindstedt and Prockop,[13] where ^{14}C-hydroxyproline was injected to rats, this impurity is too small to be an appreciable source for urine ^{14}C-hydroxyproline in our experiments even during the first urine collections.

an inhibitory action of pharmacological or massive doses of cortisone on collagen formation in the skin of animals[1–5] and in developing chick embryos.[6–9] Although some studies have suggested that the urine hydroxyproline might be mainly derived from bone collagen,[16] the results of Prockop[14] with ^{14}C-proline suggest that the urine hydroxyproline is derived from the collagen metabolism of several tissues. Thus the present results suggest an antianabolic action of cortisone on the formation of collagen in several tissues of the rat.

The experiments on the catabolic effect of cortisone on the metabolism of collagen were carried out during a period when the urine ^{14}C-hydroxyproline is known to be mainly derived from the insoluble collagen fibres.[13, 14] The results indicate that the total activity of ^{14}C-hydroxyproline excreted during the collection periods was not altered by cortisone administration. The small increase in the mean specific activity of the urine hydroxyproline in the cortisone-treated rats compared with the controls at 40 days was probably due to a decrease in the excretion of the unlabelled hydroxyproline derived from the recently synthesized collagen, as indicated by the small decrease in the amount of hydroxyproline excreted. Furthermore, the total activity of ^{14}C-hydroxyproline in the skin collagen fractions was not changed by the administration of cortisone. Thus, the results of the present study do not support the hypothesis that besides exerting an antianabolic action, pharmacological doses of cortisone also have a catabolic action on the metabolism of collagen.

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