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Effect of Cortisone on Collagenolytic Activity in the Rat[1] (38654)

R. L. CRUESS AND K. C. HONG

Orthopedic Research Laboratories, Royal Victoria Hospital and McGill University, Montreal, Quebec, Canada

The action of cortisone on bone collagen and collagenolytic activity has been investigated. Rats were injected subcutaneously with cortisone acetate and the collagen content, the incorporation of ^{14}C-proline into ^{14}C-hydroxy-proline, and the collagenolytic activity of the control and cortisone-treated rat bones were determined. The effect of cortisone on bone collagenolytic enzyme was also assayed.

It was observed that cortisone administration caused a reduction of the collagen content and a decreased incorporation of ^{14}C-proline into ^{14}C-hydroxyproline. The collagenolytic activity of the bone was reduced and cortisone has also an inhibitory effect on bone collagenolytic enzyme. These results suggest that cortisone not only inhibits the biosynthesis of bone collagen but diminishes the collagenolytic activity of rat bone.

Cortisone and bone collagenolytic activity. Cortisone is known to affect the metabolism of several constituents of connective tissue (1, 2) and it has been repotred that the resorption of bone tissue is accompanied by degradation of bone collagen, presumably due to a collagenolytic enzyme liberated during bone resorption (3). It is well established that cortisone decreases the synthesis of the organic constituents of bone matrix including collagen. There are conflicting reports concerning the effect of glucocorticoids on bone resorption. There is some indication that the rate of bone resorption may be increased (4–7) depending upon the experimental model studied. Thus, far, no direct measurements of collagenolytic activity of rat bone following cortisone administration have been reported. The following experiments were carried out in order to determine whether any alterations in collagenolytic activity of rat bone were present.

Materials and Methods. Animals. Male Wistar rats weighing 30 g were injected subcutaneously with 1 mg and 5 mg of cortisone acetate suspended in 0.4 ml of saline. After 3 days of daily injections, the animals were sacrificed by decapitation. The femora and tibiae were removed immediately after sacrifice and dissected from tissue and periosteum. The bone marrow was removed by flushing with an ice-cold saline solution. The epiphyses were discarded and the metaphyses were separated, pooled and lyophilized. The lipids were extracted and the lipid-free bones were used for analysis of collagen. The incorporation of ^{14}C-proline into ^{14}C-hydroxyproline was assayed as follows. The animals received an intraperitoneal injection of 4 μCi uniformly labeled ^{14}C-L-proline in saline solution and they were sacrificed 4 hr after injection. The metaphyseal bones were separated as mentioned above, and used for the determination of the specific activity of ^{14}C-hydroxyproline. In order to determine the collagenolytic activity of the bone, other animals were sacfiiced and the tibiae were removed immediately after sacrifice and dissected from soft tissues and periosteum, and the metaphyseal portions were separated and pooled. These bones were used for culture.

Preparation of ^{14}C-glycine labeled guinea pig skin collagen. Guinea pigs weighing 200 g were injected intraperitoneally with 150 μCi glycine/animal, 6 hr before sacrifice. The skins were removed in a cold room and kept cold during mincing. Neutral salt-soluble collagen was extracted with cold 1 *M* NaCl solution, 0.05 *M* Tris, pH 7.4, for 48 hr and purified by the method of Kaufman *et al.* (8). Purified collagen was tested with trypsin and the release of soluble radioactivity was less than 5% total counts added (50 μg of trypsin at 37° for 17 hr). The purified product was freeze-dried and stored in a desiccator at −20° until use.

Preparation of cultures. Purified, neutral soluble guinea pig skin collagen labeled with ^{14}C-glycine (5200 dpm/mg) was dissolved

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as 0.2% solution in Gay's solution and clarified by ultracentrifugation in a Beckman Spinco Model L ultracentrifuge at 30,000 rpm for 3 hr. 500 μl of 0.2% solution of the collagen was introduced in a culture tube and the minced metaphyseal bones of the tibiae were placed in the tube at 4° (9). The mixture was then incubated at 35° for 3 days. After incubation, the tissue culture media were brought to 2° and trichloroacetic acid added to a final concentration of 15% to precipitate any solubilized collagen or high molecular weight peptides (8). The solution was clarified by centrifugation at 15,000 rpm for 1 hr in a Beckman Spinco Model L ultracentrifuge. A 500 μl aliquot of the supernatant was dissolved in 10 ml of scintillation fluid and counted.

Chemical analysis. Proline was measured on a 6 *N* HCl hydrolysate at 100° for 17 hr according to the method of Troll and Lindsley (10) and hydroxyproline according to the method of Stegeman (11). The specific activity of ^{14}C-hydroxyproline was determined as follows. In order to separate ^{14}C-proline from ^{14}C-hydroxyproline, the hydrolysate was evaporated in vacuo at 25° and the dried material was dissolved with 80% ethanol. The insoluble material was separated by filtration. The filtrate was condensed in vacuo and applied on Whatman No. 1 filter paper with butan-1-ol:acetic acid:water (4:1:2, v/v/v) solvent for 20 hr. Standard proline and hydroxyproline were developed on the same paper. After separation, the area of hydroxyproline was cut out and eluted with distilled water and then condensed in vacuo. An aliquot of the solution was dissolved in 15 ml of Aquasol (New England Nuclear, Boston, MA) and the radioactivity of ^{14}C-hydroxyproline was determined by a liquid scintillation counter. The degree of quenching was estimated by the method of internal standardization (12) and the data corrected. Another aliquot of the solution was used for the determination of hydroxyproline content according to the method of Stegeman (11).

Preparation of bone collagenase. The tibiae of 30 g male Wistar rats were removed and dissected free of soft tissue and periosteum. The bone marrow was flushed out with a sterile ice-cold saline solution and the metaphyseal portions of the tibiae were separated and then placed in a culture tube containing 2 ml of tissue culture medium accordning to Shimizu *et al.* (13). The cultures were incubated for 5 days at 37°. The bones were removed and the pooled media were centrifuged. The supernatant was then dialyzed against distilled water at 2°. This crude enzyme was partially purified by the precipitation with $(NH_4)_2SO_4$. The partially purified enzyme was used in the present study. This enzyme showed negligible proteolytic activity using casein as substrate (14).

The effect of cortisone on bone collagenolytic enzyme was assayed on the ^{14}C-glycine labelled collagen by the release of soluble radioactivity from collagen fibrils. The reaction was carried out at 4° in tubes by mixing 100 μl of 0.2% solution of the ^{14}C-glycine labelled collagen in 0.4 *M* NaCl and 300 μl of the collagenolytic enzyme solution (30 μg of the enzyme) in 0.05 *M* Tris–HCl buffer, pH 7.6 containing 5 m*M* $CaCl_2$ with 0.5 μg and 5 μg of cortisone acetate in 50 μl of Tris–HCl buffer, pH 7.6 and this mixture was incubated at 37° for 17 hr. After incubation, 50 μl of 0.4 *M* EDTA, pH 7.5 was added to block the activity of the collagenolytic enzyme. The tubes were then immediately centrifuged at 15,000 rpm for 15 min in a Beckman Spinco Model L ultracentrifuge. 200 μl of the supernatant was dissolved in 15 μl of Aquasol and counted in a liquid scintillation counter. The blank values were obtained by parallel incubation made by addition of 50 μl of 0.4 M-EDTA, pH 7.5. The assays were done in duplicate for each culture tube.

Results. The administration of cortisone caused a significant reduction in the hydroxyproline content of metaphyseal bone (Table I). The incorporation of ^{14}C-proline into ^{14}C-hydroxyproline of the metaphysis was inhibited by cortisone treatment and this inhibitory effect increased with the larger dose and was statistically significant (Table II). The collagenolytic activity of bone was diminished by the cortisone treatment and this also was statistically significant (Table II).

The addition of cortisone to a culture medium containing the crude enzyme prepa-

TABLE I. EFFECT OF CORTISONE ON THE HYDROXYPROLINE CONTENT OF METAPHYSEAL BONE.[a]

	Hydroxyproline mg/100 mg bone
Control	1.71 ± 0.07
Cortisone 1 mg	1.59 ± 0.05*
Cortisone-5-mg	1.60 ± 0.05*

[a] Note: Results are given as the means ± standard deviations of six determinations on one group. (Six animals were used in each group.) Significant differences between control and treated group is indicated by: $*P < 0.01$.

TABLE II. EFFECT OF CORTISONE ON THE INCORPORATION OF ^{14}C-PROLINE INTO ^{14}C-HYDROXYPROLINE AND COLLAGENOLYTIC ACTIVITY.[a]

	Specific activity of hydroxyproline (dpm/μg)	DPM degraded ^{14}C collagen
Control	108.0 ± 7.9	1190 ± 79
Cortisone 1 mg	85.7 ± 7.6*	940 ± 23*
Cortisone 5 mg	54.3 ± 8.3*	676 ± 75*

[a] Note: Six animals were used in one group and two metaphyseal bones of tibiae used in each pooled sample. Each value represents the mean ± standard deviation of six samples with each group measured in duplicate. Significant differences between control and treated group is indicated by: $*P < 0.01$.

TABLE III. INHIBITORY EFFECT OF CORTISONE ON BONE COLLAGENOLYTIC ENZYME.[a]

Cortisone acetate	Degraded ^{14}C-collagen (cpm over Blank)
0	1172 ± 166
0.5 μg	785 ± 14*
5.0 μg	706 ± 25*

[a] Results are given as the means ± SD of three determinations. Each assay was done in duplicate. Significant differences between control and cortisone added samples is indicated by: $* P < 0.01$.

ration and prelabeled collagen inhibited the release of radioactive material (Table III).

Discussion. It is desirable to differentiate between the known effects of cortisone on bone resorption and formation rates and its effect upon the biochemical functions of osseous tissue. Bone formation is generally felt to be inhibited by cortisone administration (4, 15). The effect of cortisone upon bone resorption has been reported as one of enhancement (4, 5) or inhibition (6, 7, 16). There is no controversy over the effect of cortisone upon the synthesis rates of the various components of bone matrix. It has been clearly demonstrated that collagen and sulphated mucopolysaccharide synthesis is markedly diminished by cortisone administration (17) and this correlates well with the known effects of the hormone. No information is available on the effect of the hormone upon the enzymes which must be responsible for resorbing the organic portions of bone matrix.

Histologically, cortisone causes a plug of metaphyseal bone to form in the rat (18). The exact mechanism whereby this occurs is somewhat unclear although it has been demonstrated previously (17) that there is an inhibition of synthesis of collagen and mucopolysaccharides. The present experiment was undertaken in an attempt to correlate the histological events with collagenolytic activity, since it appeared that resorption must be inhibited if an accumulation of bone occurred in this region in the presence of diminished bone formation. The data on composition of the bone and the synthesis rates of collagen were carried out in order to be certain that a pharmacological effect had been achieved.

In the present study, both the collagen content and the incorporation of ^{14}C proline into ^{14}C hydroxyproline of bone samples were reduced by cortisone administration. Thus, decreased anabolism by bone cells was once more demonstrated. There was a statistically significant and dose related decrease in collagenolytic activity found in bone tissue. In addition cortisone added to a crude enzyme extract was shown to inhibit collagenolytic activity. The exact mechanism for this apparently direct inhibition needs further clarification.

The mechanism by which the hormone inhibits collagenolytic activity is unclear. It has been shown that hypocalcemia in the rat will decrease bone formation, bone mineralization, and bone resorption (19). This would account for the diminution in

resorption and in formation which was demonstrated and would correlate well with observations in cellular behavior carried out by Epker (20) in which he found fewer bone remodelling fossi as well as a decreased rate of function of osteoclasts and osteoblasts. In addition to this, it appears that cortisone is capable of directly inhibiting the activity of the enzyme extract, offering another explanation for the lack of resorption observed in this experimental animal. In addition, there could be interference with protein synthesis required for activation of a collagenase zymogen (21) or with the activation of a latent collagenase as found by Vaes (22).

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