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INHIBITION OF RAT AND HUMAN PROLIDASES BY CAPTOPRIL

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Abstract—We examined the effects of captopril on prolidase activity of crude homogenates of various tissues in the rat and in the human. It was found that captopril caused significant inhibition of prolidases from liver, kidney, and intestine in both species, whereas it showed minimal inhibitory effect on erythrocyte prolidase. In the rat, K_i for the inhibition of kidney and liver prolidases was in the range of 25–35 μM while, in the human, the value was considerably higher. The nature of inhibition was found to be competitive both in the rat and in the human. Oral administration of captopril (daily dose, 40 mg/kg) for 7 days in the rat resulted in increased urinary excretion of peptide-bound 4-hydroxy-L-proline compared to controls, indicating *in vivo* inhibition of tissue prolidases by captopril.

Captopril (D-3-mercapto-2-methyl propanoyl-L-proline, SQ 14,225) is an orally active drug used in the control of hypertension and congestive heart failure. It is a powerful competitive inhibitor of angiotensin-converting enzyme [1, 2]. The drug was designed to bind specifically to the active site of the converting enzyme and inhibit its catalytic activity. The rigid ring structure of the L-proline moiety in the drug locks the carboxyl group into a conformation highly suitable for interacting with a positively charged residue at the active site of the enzyme. Angiotensin-converting enzyme contains Zn^{2+} at its active site and the metal ion is necessary for the catalytic activity of the enzyme [3]. The thiol group of the drug strongly interacts with this Zn^{2+}. By virtue of its structure, the drug is a potent inhibitor of the converting enzyme, its K_i value being as low as 1.7 nM. However, captopril does not inhibit the activities of other Zn^{2+}-containing enzymes such as alcohol dehydrogenase or carbonic anhydrase [4]. At 1 mM concentration, it causes less than 50% inhibition of carboxypeptidases A and B, trypsin and chymotrypsin [2]. On the other hand, leucine aminopeptidase, another Zn^{2+}-containing enzyme, is inhibited rather strongly by captopril, 50% inhibition being produced by 4 μM inhibitor [2].

Structurally, there is a striking similarity between captopril and a dipeptide having L-proline as the C-terminal residue. Dipeptides of the x-pro type are substrates exclusively for prolidase. This dipeptidase is present in all tissues and plays an important role in the recycling of proline derived from collagen. There is evidence that this enzyme contains Zn^{2+} at its active site [5]. Because of the structural similarities between captopril and the substrates for prolidase, we designed experiments to determine if this dipeptidase is inhibited by captopril.

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MATERIALS AND METHODS

Preparation of enzyme solution. After the rats were killed by cervical dislocation, liver, kidney, and small intestine were removed. The intestine was washed with ice-cold KCl (0.154 M) and cut open longitudinally. The mucosa was scraped off and stored on ice until used. A 20% homogenate of the intestinal mucosa, liver or renal cortex was prepared in 50 mM Tris/HCl buffer, pH 8.0, in a loose-fitting Dounce glass homogenizer using 20 strokes. The homogenate was filtered through four layers of cotton gauze, and the filtrate was centrifuged at 105,000 *g* for 1 hr. The supernatant fraction was used as the enzyme source for the assay of prolidase. Human tissues were obtained at autopsy and processed similarly.

Preparation of enzyme from erythrocytes. Human or rat venous blood was collected in heparinized tubes. Erythrocytes were harvested by low-temperature centrifugation and washed three times with an equal volume of normal saline. Washed erythrocytes were hemolyzed with 4 vol. of ice-cold 5 mM Tris/HCl buffer, pH 8.0, and stirred at 0° for 15 min. The hemolysate was centrifuged at 105,000 *g* for 1 hr and the supernatant fraction was used as the enzyme source.

Assay of prolidase-colorimetric method. Prolidase was assayed colorimetrically as described in Ref. 6, with minor modifications, using glycyl-L-proline as the substrate. The reaction mixture (250 μl) contained 50 mM Tris/HCl buffer, pH 8.0, 2 mM glycyl-L-proline and the enzyme. The enzyme was suitably diluted with 50 mM Tris/HCl buffer, pH 8.0, before use in the assay. The reaction was carried out at 37° for 30 min. At the end of incubation, 1 ml of 10% trichloroacetic acid was added to stop the reaction. The mixture was spun in a table-top clinical centrifuge for 10 min, and 0.5 ml of the clear supernatant

fraction was mixed with 1.5 ml glacial acetic acid and 1.5 ml Chinhard reagent (2.5 g ninhydrin dissolved at 70° in a mixture containing 60 ml of glacial acetic acid, 20 ml of 85% orthophosphoric acid and 20 ml of water). The mixture was heated at 90° for 10 min. After cooling the tubes to room temperature, the optical density was measured at 515 nm which was directly proportional to the amount of proline liberated from glycyl-L-proline by the enzyme.

Assay of prolidase—radiometric method. Prolidase was assayed radiometrically as described previously [7] using [1-^{14}C]glycyl-L-proline. The reaction mixture (250 μl) contained 50 mM Tris/HCl buffer, pH 8.0, 0.2 mM labeled glycyl-L-proline, and suitably diluted enzyme. The reaction was carried out at 37° for 30 min, and hydrolysis was terminated by heat inactivation of the enzyme (2 min in boiling water bath). Labeled glycine and labeled glycyl-L-proline were separated by paper chromatography (Whatman No. 1, 16 hr run) using *n*-butanol/glacial acetic acid/water (4:1:1, by vol.) as the solvent system. With the guidance of reference standards which were identified with Cu^{2+}-ninhydrin reagent [8], glycine and glycyl-L-proline spots were cut out and quantitated by liquid scintillation counting.

Captopril was dissolved in 50 mM Tris/HCl buffer, pH 8.0, and a freshly prepared solution was used for each assay.

Studies in vivo. Nine female Sprague–Dawley rats weighing approximately 200 g were individually housed in plastic metabolic cages (Fisher Scientific Co., Cat. No. 01-287). These cages separate feces from urine. The urine was collected in beakers containing thymol crystals and 0.1 ml of 12 N HCl to inhibit bacterial growth and to avoid hydrolysis of peptides by the enzymes which may be present in the urine. Twenty-four hour urine samples were collected from individual rats on days 7, 14 and 21, and the volume was measured and stored at −20° until analysis.

The rats had free access to Purina rat chow and water. Each day at 2:00 p.m., rats were given distilled water for 7 days by an esophageal feeding needle (18 gauge, Pepper & Sons, Inc., New Hyde Park, NY, U.S.A.) and the 24-hr urines collected on day 7 served as controls. Starting on day 8, the rats were given 40 mg/kg body weight of captopril (10 mg/ml water) in the same manner for 14 days.

Urine analysis. The urines were ultrafiltered in an Amicon centriflo CF25 membrane cone by centrifugation. Peptide hydrolysis was performed in 6 N HCl for 16 hr at 110°. Amino acid analysis was performed on a Beckman 120C amino acid analyzer equipped with a 0.6 × 24 cm column packed with W-3P cation exchange resin (Beckman Instruments, Palo Alto, CA, U.S.A.). The eluting buffers were: (1) 0.2 N lithium citrate, pH 2.83, for 50 min; (2) 0.3 N lithium citrate, pH 3.60, for 46 min; (3) 0.4 N lithium citrate, pH 3.57, for 50 min; and (4) 1.0 N lithium citrate, pH 3.80. The column temperature was changed from 40° to 66° at 80 min. Captopril does not react with ninhydrin and therefore its elution profile in the amino acid analyzer is not known. However, the presence of captopril did not alter the elution profile of any amino acid under the conditions employed.

Creatinine was determined by the method of Bonsnes and Taussky [9].

Materials. Captopril was a gift from Dr. D. W. Cushman, Squibb Institute for Medical Research, Princeton, NJ, U.S.A. Glycyl-L-proline was purchased from the Sigma Chemical Co., St. Louis, MO, U.S.A. [1-^{14}C]Glycyl-L-proline (sp. act. 6.4, mCi/mmole) was from the Radiochemical Center, Amersham, England.

RESULTS

Inhibition of rat tissue prolidases by captopril. The effect of increasing concentrations of captopril (0–250 μM) on the hydrolysis of glycyl-L-proline by prolidase from rat tissues (intestine, kidney, liver, and erythrocyte) was studied. The results are shown in Fig. 1. Prolidase from all tissues studied was strongly

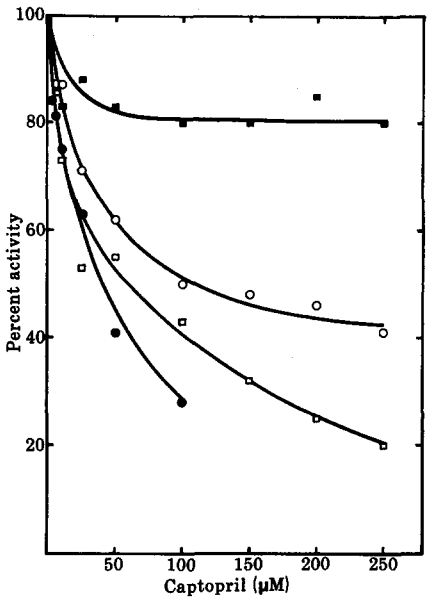

Fig. 1. Inhibition of rat tissue prolidases by captopril (0–250 μM). Key: (●—●) kidney, (○—○) intestine, (□—□) liver, and (■—■) erythrocyte. The final concentration of glycyl-L-proline in the reaction mixture was 2 mM.

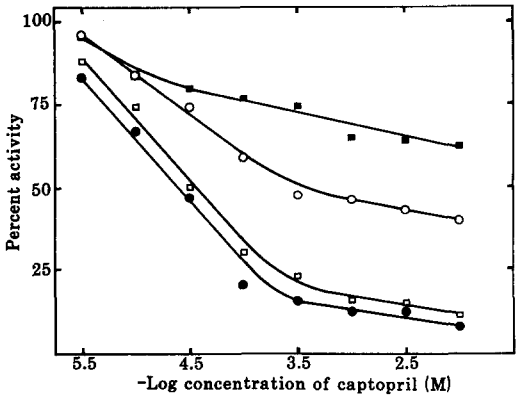

Fig. 2. Inhibition of rat tissue prolidases by captopril (0–10 mM). Key: (●—●) kidney, (○—○) intestine, (□—□) liver, and (■—■) erythrocyte. The final concentration of glycyl-L-proline in the reaction mixture was 2 mM.

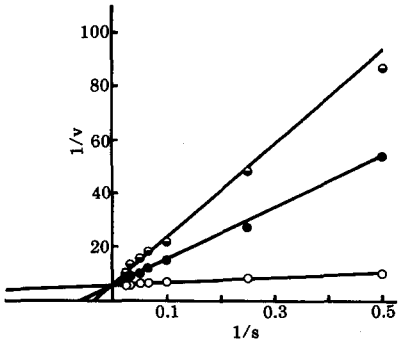

Fig. 3. Lineweaver–Burk plot for inhibition of rat renal prolidase by captopril. Key: (○—○) no captopril, (●—●) 100 μM captopril, (◑—◑) 250 μM captopril, (v) μmoles of glycyl-L-proline hydrolyzed · min^{-1} · (mg protein)$^{-1}$, and (s) glycyl-L-proline concentration in mM.

inhibited by captopril with the notable exception of erythrocyte prolidase. Renal prolidase was the most susceptible, followed by liver and intestinal prolidases in decreasing order. The erythrocyte prolidase was not inhibited appreciably. Studies with a wider range of captopril concentrations showed that, while prolidases from liver and kidney were inhibited about 90%, erythrocyte prolidase was inhibited less than 30% even at 10 mM captopril (Fig. 2). The K_i value for the inhibition of kidney and liver prolidases was in the range of 25–35 μM.

The nature of inhibition of prolidase by captopril was studied using rat kidney enzyme. The results, given in a Lineweaver–Burk plot (Fig. 3), show that captopril was a potent inhibitor of prolidase. The apparent K_m for the enzyme with glycyl-L-proline as the substrate was 1.8 mM in the absence of captopril. The K_m value increased in the presence of the inhibitor (16.7 and 28.6 mM at 100 and 250 μM captopril respectively), whereas V_{max} was virtually unaffected.

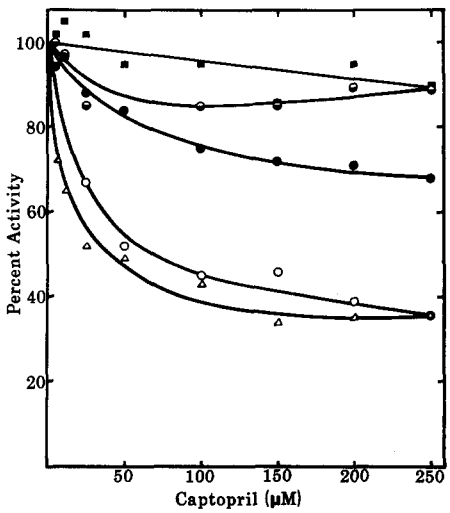

Fig. 4. Inhibition of prolidase by captopril in the human. Key: (■—■) erythrocyte, (◑—◑) liver, (○—○) intestine, and (●—●) kidney. The final concentration of glycyl-L-proline in the reaction mixture was 2 mM. The open triangles (△—△) represent kidney with 0.2 mM glycyl-L-proline.

Inhibition of human tissue prolidases by captopril. Similar inhibition studies with prolidase from human tissues showed that the human enzyme was also susceptible to inhibition by captopril, though to a lesser extent compared to the rat enzyme. Figure 4 shows that, in the human, the intestinal prolidase was the most susceptible followed by kidney, liver and erythrocyte prolidases in decreasing order. With 2 mM glycyl-L-proline as the substrate, the intestinal enzyme was inhibited 60% by 250 μM captopril, while prolidases from liver, kidney and erythrocyte were inhibited less than 30% under similar conditions. However, the extent of inhibition increased with decreasing concentration of the substrate. With 0.2 mM glycyl-L-proline as the substrate, prolidase from human kidney was inhibited 50% by 40 μM captopril.

The nature of the inhibition was studied using human kidney enzyme. The human enzyme was also inhibited competitively by captopril as was the rat enzyme (Fig. 5). The apparent K_m for the human

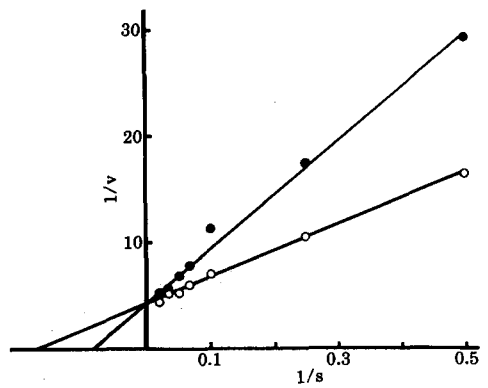

Fig. 5. Lineweaver–Burk plot for inhibition of human renal prolidase by captopril. Key: (○—○) no captopril, (●—●) 500 μM captopril, (v) μmoles of glycyl-L-proline hydrolyzed · min^{-1} · (mg protein)$^{-1}$, and (s) glycyl-L-proline concentration in mM.

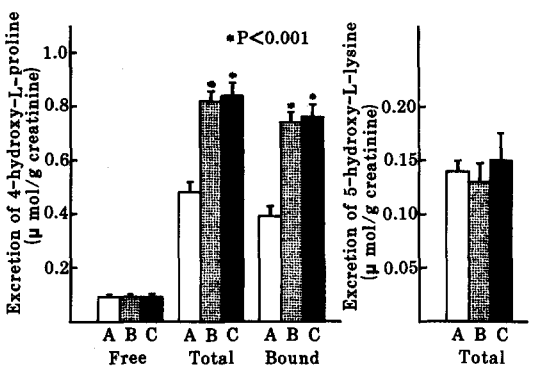

Fig. 6. Urinary excretion of 4-hydroxy-L-proline and 5-hydroxy-L-lysine before and after oral administration of captopril in the rat. In control experiments, distilled water was administered orally to rats daily by esophageal feeding for 7 days. On day 7, 24-hr urine collections were made which served as controls. Starting on day 8, captopril dissolved in distilled water (daily dose, 40 mg/kg) was administered orally in the same manner for 2 weeks. Twenty-four hour urines were collected at the end of week 1 and week 2 of captopril treatment. Key: (A) controls, (B) after 1 week of captopril treatment, and (C) after 2 weeks of captopril treatment.

kidney enzyme with glycyl-L-proline as substrate was 5.6 mM. The K_m value increased 2-fold in the presence of 500 μM captopril.

In vivo *studies in the rat.* During control experiments, rats were given distilled water using an esophageal feeding needle for 7 days, and 24-hr urine collection was made on day 7. These urine samples served as controls. Starting on day 8, captopril was administered daily in the same manner until day 21, and 24-hr urine samples were collected on days 14 and 21. Urines were analyzed for free and total 4-hydroxy-L-proline. This was done by estimating 4-hydroxy-L-proline before and after acid hydrolysis. The results are given in Fig. 6. There was no significant difference in the excretion of free 4-hydroxy-L-proline before and after captopril treatment. However, total 4-hydroxy-L-proline excretion was significantly greater after captopril treatment than prior to treatment. Peptide-bound 4-hydroxy-L-proline increased about 2-fold in the urine after administration of captopril ($P < 0.001$). Urinary excretion of total 5-hydroxy-L-lysine was the same before and after captopril treatment. Free 5-hydroxy-L-lysine was below detectable levels in the urines of both groups.

We also analyzed the urine samples for their glycyl-L-proline content, and we found that there was no significant difference between control and captopril-treated rats.

DISCUSSION

The results from *in vitro* studies demonstrate that captopril was a potent competitive inhibitor of prolidase from rat and human tissues and significant inhibition was observed at micromolar concentrations of captopril. The inhibition of prolidase may be even stronger *in vivo* than *in vitro* because the *in vivo* concentrations of prolidase substrates were extremely low compared to the concentration of glycyl-L-proline employed in *in vitro* studies. The fact that erythrocyte prolidase was resistant to captopril inhibition suggests that more than one form of this enzyme may be present in tissues and that these various forms of the enzyme are not equally affected by captopril. There is evidence for the presence of isoenzymes of prolidase [10]. Dipeptides of x-pro and x-hyp types are exclusively hydrolyzed by this enzyme, and accumulation of these peptides in blood can be expected during captopril treatment. We were not able to quantitate the urinary excretion of peptide-bound proline because, apart from peptides, proline was also released by acid hydrolysis from captopril present in the urine. However, the results with 4-hydroxy-L-proline clearly demonstrate that peptides of x-hyp type accumulate in blood as a consequence of captopril treatment.

Paradoxically, there was no statistically significant difference in the urinary excretion of glycyl-L-proline before and after captopril administration. However, it is possible that, since glycyl-L-proline represented only a fraction of proline-containing dipeptides, increases in the urinary excretion of glycyl-L-proline after captopril administration were much smaller compared to increases in the excretion of total proline-containing peptides. Such a small increase might have been below the levels of detection under the conditions employed in the present study. Excretion of total 5-hydroxy-L-lysine was not affected by administration of captopril. This is expected because peptides containing this amino acid are not substrates for prolidase.

Captopril did not inhibit the activities of prolinase, which hydrolyzed dipeptides of pro-x or hyp-x type, and of dipeptidyl aminopeptidase IV, which released dipeptides of x-pro type from the amino terminus of large peptides (data not shown).

Dipeptides containing L-proline and 4-hydroxy-L-proline were released during collagen degradation, and these dipeptides were cleaved by prolidase and prolinase. Prolidase is present in all tissues and plays an important role in the recycling of collagen because more than 90% of proline derived from collagen is reused for collagen synthesis [11]. Prolidase deficiency is a relatively frequent genetic disease connected with defective collagen metabolism. The disease is associated with chronic ulcerative dermatitis, mental retardation and increased urinary excretion of imidodipeptides [12]. The clinical manifestations have been interpreted as a consequence of impaired recycling of collagen amino acids [13]. The dermatological features are the most important clues leading to the diagnosis of the disease because they are present in about 85% of the cases (for a review, see Ref. 14). The dermatologic manifestations include skin fragility, ulceration, photosensitivity, and maculopapular and purpuric lesions.

Captopril therapy is frequently associated with adverse side effects which include urticarial and maculopapular eruptions, reversible renal failure, nephrotic syndrome, proteinuria, agranulocytosis, and transient taste loss [15]. Cutaneous reactions are the most common among the side effects and these symptoms occur mostly at higher doses and resolve at lower doses. The exact mechanism underlying these dermatologic manifestations during captopril therapy is not known. However, various theories have been put forward to explain the mechanism of these side effects. Potentiation of the activity of kinins in the skin during captopril treatment has been suggested to be responsible for the cutaneous reactions [16]. Kininase II which inactivates bradykinin is identical with angiotensin-converting enzyme [17]. Inhibition of this enzyme by captopril would result in an increase in bradykinin levels. The captopril-induced eruptions may, therefore, be the result of potentiation of kinin-mediated skin reactions.

According to another theory, the captopril-induced skin manifestations are due to an immunologic mechanism rather than a pharmacologic effect [18]. This theory is based on the similarities between the side effects observed during treatment with captopril and penicillamine. The theory suggests that these compounds, because of their highly reactive sulfhydryl and carbonyl groups, cleave the macromolecules responsible for intercellular cohesion in a highly specific manner, producing antigens. The cutaneous reactions, according to this theory, are the result of immunologic reactions elicited by these antigens.

The sulfhydryl group in captopril forms the basis of yet another theory [19]. Some of the side effects of captopril therapy, such as cutaneous changes, taste loss, and alopecia, constitute the characteristic clinical symptoms of zinc deficiency. The sulfhydryl group in captopril can strongly bind to heavy metal ions including zinc. Therefore, it is possible that captopril treatment results in zinc deficiency which may be responsible for the side effects.

Our data presented in this paper suggest that the cutaneous manifestations during captopril therapy are possibly due to interference in collagen metabolism resulting from inhibition of prolidase by captopril. The skin lesions seen in patients with prolidase deficiency are strikingly similar to those observed during captopril treatment. Demonstration of prolidase inhibition by captopril *in vitro* in the rat and human and *in vivo* in the rat supports this hypothesis. It should be mentioned, however, that the dose of captopril administered in the rat was much higher than the therapeutic dose usually employed in the human. In spite of this limitation, the present study provides a strong basis for the theory that at least some of the side effects of captopril therapy in the human may be due to the inhibition of tissue prolidases by the drug.

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