Characterization of Antibodies to Canine Renal Renin

Studies of Interspecies Homology of Renin Using Antibodies as Probe

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SUMMARY Antibodies raised to pure canine renal renin were used to probe homology of renin from other species. Goat, rabbit, and mouse antibodies exhibited similar properties and were specific for renin as confirmed by immunodiffusion, immunoelectrophoresis, and selective inhibition of renin enzymatic activity. Goat antibody also inhibited the enzymatic activities of rat, rabbit, hog, and bovine renin. Immunologic cross-reactivity was further confirmed by direct binding and competition assays. Goat anti-(canine renin) antibody did not inhibit human or mouse renin activity, but readily bound to these enzymes, suggesting the presence of epitopes distant from the enzymatic site. Purified goat Fab retained only 10% to 20% of the anticalytic activity of antibody, but this activity was largely recovered when donkey anti-goat antibody was added to the Fab-renin mixture. Thus, the major fraction (90% to 90%) of goat antibody exerted its anticalytic activity by immune complex formation or steric hindrance and is not catalytic-site directed, whereas 10% to 20% binds an epitope at or near the catalytic site. (Hypertension 4: 341-347, 1982)

KEY WORDS • antirenin antibody • renin interspecies homology • renin Fab fragment

ENZYME renin (EC 3-4-99-19) is an aspartyl protease that acts at the aminoterminal end of renin substrate to release the decapeptide angiotensin I (AI) which is, in turn, cleaved by converting enzyme to angiotensin II (AII), the biologically active component of the renin-angiotensin system. Renin-specific antibody is a valuable tool in characterizing the physiology, pathophysiology, and biosynthesis of renin. In earlier studies, antibodies were raised against impure renin preparations, and consequently their specificity was in question.* We previously reported purification of canine renal renin to homogeneity.3 We now report the properties of rabbit, goat, and mouse antibodies raised against purified canine renal renin.

Materials and Methods

Purification of Renin

Canine renal renin was purified to homogeneity, as described previously.8 Partially purified renin from rat, hog, rabbit, bovine, human, and mouse kidneys and mouse submaxillary glands was obtained by a three-step procedure. The tissues were homogenized in 50 mM potassium phosphate buffer, pH 7.5, containing a mixture of protease inhibitors (5 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, and 5 mM sodium tetrathionate). Ammonium sulfate was added to achieve 70% saturation. The precipitate obtained after centrifugation was dissolved in 50 mM sodium acetate, pH 5.5, and dialyzed for 16 hours against 5 mM sodium acetate, pH 5.5. The dialysate was applied to a peptatin-Sepharose column prepared by the method of Murakami and Inagami.* The column was washed with 20 mM sodium acetate and 1 M sodium chloride, pH 5.0, and renin was eluted with a linear concentration gradient between 100 mM Tris-HCl, pH 7.5, and the same buffer containing 2 M lithium bromide. The resultant specific activities of the partially purified renins ranged from 1.0 to 6 × 104 ng A1/hr/mg protein (2 to 12 Goldblatt units/mg protein).
Partial Purification of Renin Substrate

Partial purification of renin substrate from several species was carried out on plasma from nephrectomized animals according to the method of Boucher et al. Plasma from nephrectomized animals was treated with EDTA and NaOH followed by sulfuric acid, titrating to a final pH of 5.3; 2.3 M ammonium sulfate was added. The precipitate containing semi-purified renin substrate was redissolved and dialyzed against cold distilled water.

Partial Purification of Canine Renal Cathepsin B- and D-like Enzymes

Cortices of canine kidneys were minced, lyophilized, pulverized, and then defatted by stirring with diethyl ether. After evaporation of the ether, the powder was extracted with 30% acetone-water mixture. The extract was concentrated by a DEAE batch procedure, acidified, and precipitated with ammonium sulfate (70% saturation) as previously described. The ammonium sulfate precipitate was dissolved, dialyzed, and applied to a carboxymethyl cellulose column equilibrated with 20 mM sodium acetate, pH 5.0. Elution was effected by linear concentration gradient of 0 to 100 mM potassium chloride in the above buffer. Cathepsin B activity was detected in the nonbound and wash fractions, whereas cathepsin D activity was eluted between 30 and 70 mM potassium chloride.

Immunization Procedures

Goats were immunized by initial intramuscular and intradermal injections of 250 μg of purified canine renin in 2 ml of 0.5 M sodium phosphate with 0.1 M sodium chloride, pH 7.5, emulsified with an equal volume of complete Freund's adjuvant. Monthly booster injections of 100 μg of renin were administered at multiple intradermal sites. Rabbits received an initial topoead injection of 100 μg of the pure canine enzyme in 0.6 ml of the same buffer, with or without 2% glutaraldehyde, mixed with an equal volume of Freund's adjuvant. Monthly booster injections of 50 μg canine renin in complete Freund's were given by multiple intradermal and toepad injections. A/J male mice were also immunized intraperitoneally with an initial 50 μg of pure canine renin in 4% glutaraldehyde and complete Freund's adjuvant in 0.1 Tris-HCl buffer, pH 7.4, followed by monthly boosters of 5 to 10 μg of the pure enzyme.

All animals were bled prior to and during the course of immunizations at monthly intervals, and the sera were tested for their ability to inhibit canine renal renin activity as described below.

Renin Activity Assay

Renin enzymatic activity in tissues or plasma was determined by the rate of generation of AI from substrate at 37° C, pH 7.4, in the presence of angiotensinase inhibitors (EDTA, 8 hydroxyquinoline, dimercaptopropanol). Dog, hog, rat, and human renins were incubated with either their respective homologous semipurified substrate or nephrectomized plasma. Plasma from nephrectomized dogs was used as substrate for rabbit, bovine, and mouse renins.

Determination of Renin Activity Inhibition

The reaction mixture contained 2 milliGoldblatt units of renin (50 μl) in 100 mM Tris, pH 7.5, and 100 μl of antiserum or antibody solution. After incubation at 37° C for 1 hour, residual renin activity of the reaction mixture was determined by incubation with renin substrate as described above. Maximal inhibition of enzymatic activity occurred within 30 minutes. Subsequent incubation did not result in a recovery of activity.

Immunoradiometric Assay

The concentration of renin was determined directly by an immunoradiometric assay modified from the method of Klinman et al. Microtiter wells of flexible polyvinyl plates (Dynatech Laboratories, Alexandria, Virginia) were filled with 10 ng (25 μl) of renin in 50 mM sodium acetate, pH 5.0, from different species, incubated at 4° C for 16 hours and the excess decanted. Then 10% horse serum was added and the plates incubated for an additional 2 hours at room temperature. Goat or mouse canine-(renin specific) antiserum or preimmune serum (50 μl, dilutions 1:200 to 5 × 10^4) was added to the wells for an additional 2 hours and the excess then decanted. 12B I antigoat or mouse Ig antibody (about 30,000 cpm) was then introduced into the wells and allowed to remain for 16 hours at 2° C. The wells were then washed thoroughly and counted in a Packard (Prias) gamma counter.

Competitive immunoradiometric assays were performed with the following modifications: 3 to 150 ng (0.01 to 0.3 Goldblattunits) of renin of different species was preincubated with canine-(renin specific) antiserum at 1:10^4 dilution or nonimmune serum (1:10^4) for 1 hour at 37° C. The resultant mixture was then added to the wells previously coated with fully purified canine renin (10 ng) and allowed to remain for 2 hours before the second radiolabeled antibody was added. Specific binding was calculated as the difference in radioactivity between the respective wells treated with antiserum and nonimmune serum. The immunoradiometric assay has the theoretical advantage of offering a greater sensitivity over the ordinary radioimmunoassay method. In the radioimmunometric assay, the antigen is reacted with an excess of specific antibody. Thus, the molar product of antigen-antibody binding is significantly increased. Practical problems, however, can occur with labeling and subsequently isolating the antibody without reducing its avidity. The background has been observed to fluctuate due to nonspecific binding of antiserum with the horse serum used to coat the wells. In this study we have not encountered such difficulties. All of the values reported are net binding, i.e., the difference between specific and nonspecific binding.
Isolation of IgG and Fab

Twenty-five ml of goat immune sera (GLN 816 or GLN 827) was dialyzed for 16 hours against 8 liters of 35 mM potassium phosphate buffer, pH 8.0, then applied to a DEAE-cellulose column (2.5 X 25 cm) equilibrated with the same buffer at a rate of 100 ml/hr. Elution was effected with the starting buffer. Fractions containing renin-inhibiting activity were pooled and applied to gel-filtration chromatography using AcA 34 ultragel (4 X 200 cm) equilibrated in 50 mM potassium phosphate with 150 mM sodium chloride, pH 7.0, at 50 ml/hr. The IgG fraction was recovered and subsequently concentrated by Amicon ultrafiltration cell to an appropriate volume (approximately 8-10 ml) and dialyzed against 100 mM phosphate buffer, pH 7.4. Papain digestion was performed in a reaction mixture containing 1 mM EDTA and 10 mM cysteine in the same buffer for about 90 minutes. Ten mg of papain was used for each gram of globulin. Fab was separated from uncleaved globulin and papain by gel filtration on a Sephadex G-100 column (2.5 X 200 cm) in 50 mM potassium phosphate, pH 7.0, and 150 mM sodium chloride.

Proteinase Assays

Nonspecific Proteolytic Activity

Bovine spleen cathepsin D (Sigma, St. Louis, Missouri) or partially purified canine renal cathepsin D-like enzyme was assayed by cleavage of 14C-labeled hemoglobin according to the method of Williams and Lin.

Cathepsin B Assay

Bovine spleen cathepsin B (Sigma, St. Louis) or partially purified canine renal cathepsin B-like enzyme was assayed using BZ-DL-Arg-2-naphylathamide at 37° C, pH 6.0, according to the method of Barrett.

Angiotensin-Converting Enzyme Assay

Purified canine pulmonary converting enzyme activity (kindly provided by Dr. R. Soffer) was measured by a radioassay with 3H-Hip-Gly-Gly (Ventrex Lab, Maine) as substrate by modification of the method of Ryan et al.

Pepsin Assay

Pepsin (Sigma, St. Louis) was assayed with hemoglobin as substrate at pH 2.2 and pH 5.0 according to the method of Anson and Williams and Lin.

Trypsin Assay

Trypsin (Boehringer, Mannheim, Indianapolis) was assayed according to the procedure of Kunitz using casein (Millipore, Inc., Freehold, New Jersey) as substrate. The effect of renin antiserum on these enzymes was studied by incubation of the above enzyme solutions with 100 μl preimmune serum (or immunoglobulin) or 100 μl of antiserum (or antibody) at 37° C for 1 hour prior to assay.

Immunodiffusion

Double immunodiffusion in agar was performed according to the method of Ouchterlony.

Immunoelectrophoresis

Immunoelectrophoresis was performed by the method of Grabar and Williams.

Protein Determination

Protein concentrations were determined by the Folin-phenol method of Lowry et al.

Results

The immune response to canine renin differed among the three species examined. Renin inhibitory activity was readily detected in goat sera 8 weeks after immunization with renin. By week 12, the serum of goat GLN 816 inhibited renin activity (2 milli-Goldblatt units) at a titer of 1:50,000. However, untreated canine renin resulted only in a slow and weak immune response in rabbits. Glutaraldehyde-aggregated renin, however, resulted in a titer of 1:5,000 after 20 weeks of immunization in rabbits R1700 and R1707. Immunization of mice with glutaraldehyde-aggregated renin resulted in a titer of 1:25,000 in A/J male mouse MB-1, and 1:5,000 in Balb/c male mouse MB-3 within 4 weeks. The inhibition of canine renal renin by goat (GLN 816), mouse (MB-1), and rabbit (R1700) sera is shown in figure 1.

Antibodies GLN816, GLN827 (goats), R1700, R1707, R1709 (rabbits), and MB1 (mice) were specific for renin. As shown in figure 2 A, a precipitin line of identity is apparent in Ouchterlony analysis between...
Crude canine renal extract, purified catepsin D or B, pepsin, trypsin, or Al-converting enzyme was incubated with renin antibodies or nonimmune globulin and the reaction mixture assayed for residual proteolytic activity. Inhibition of enzymatic activity was not observed.

The immunological relationships of renin from several species were examined. Double immunodiffusion in agar showed evidence of partial identity between renins from rat, hog, rabbit, and beef kidney with purified canine renin (data not shown). The renin activity from each of these species was inhibited by goat canine-(renin specific) antibody GLN 816 (fig. 3). A similar relative order of inhibition was demonstrated with rabbit and mouse canine-(renin specific) antisera. The relative amounts of goat canine-(renin specific) antiserum required to inhibit 50% of the activity of 2 milliGoldblatt units of renin from canine, hog, rat, rabbit, and beef kidneys were 1:10:18:20:20, respectively. None of these antibodies had an inhibitory effect on either human or mouse renin activity.

An examination of the binding of goat or mouse canine-(renin specific) antiserum to canine, rabbit, rat, and hog renins generally confirms the observations derived from renin activity inhibition (fig. 4 left). The relative effectiveness of antibody binding to each renin corresponds to relative effectiveness of enzyme inhibition. To confirm these findings, competitive immunoradiometric assays using mouse canine-renin antiserum and enzyme preparations from various species were performed. The results of the competition radioimmunoassay are similar and are shown in figure 4 right, although less differentiation is observed among weak-binding proteins.

Although anticanine-renin antisera were incapable of directly inhibiting human or mouse renin activities, it is of interest that the immunoradiometric assay demonstrated weak binding of goat or mouse anticanine-renin antisera to human (not shown) and mouse (fig. 4 left) renins. These observations were confirmed when a second antibody (donkey antigoat IgG) was added to the mouse or human renin-goat antibody reaction mixture resulting in sequestration of the enzyme and inhibition of enzymatic activity (table 1). Such a response was not seen with the nonimmune serum.

Properties of Purified Antirenin Fab Fragments

The results of purification of IgG and Fab fragments from goat antiserum GLN 816 are summarized in table 2. Despite complete recovery of protein subjected to papain digestion and gel-filtration on Sephadex-G100, the purified anti-renin Fab fragments only retained one fifth the anti-catalytic activity of the intact immunoglobulins or the antisera (table 2 and
FIGURE 4. Radioimmunometric assays. Left: Direct binding of serial dilutions of mouse canine-renin-antiserum (MB-1) to varying amounts (3 to 150 ng) of dog (Δ—Δ), rat (●—●), rabbit (○—○), and mouse (□—□) renins. Right: Competition assay. Renins from different species [rat (●—●), hog (○—○), and rabbit (□—□)] compete with dog renin (Δ—Δ) for mouse MB-1 canine-renin antiserum (1:10⁴).

TABLE 1. Effect of Antigoat-IgG Serum on the Inhibition of Human Renin Activity by Goat Canine-Renin-Antiserum (GLN 816)

<table>
<thead>
<tr>
<th>Dilutions of antidog renin serum (GLN 816) (100 µl)</th>
<th>Dilutions of donkey antigoat-IgG serum (100 µl)</th>
<th>0</th>
<th>1:1</th>
<th>1:10</th>
<th>1:10²</th>
<th>1:10³</th>
<th>1:10⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>290 (0%)*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>300 (0%)</td>
</tr>
<tr>
<td>1:1</td>
<td>300 (0%)</td>
<td>360 (0%)</td>
<td>360 (0%)</td>
<td>360 (0%)</td>
<td>20 (87%)</td>
<td>360 (0%)</td>
<td>320 (0%)</td>
</tr>
<tr>
<td>1:10</td>
<td>300 (0%)</td>
<td>280 (0%)</td>
<td>360 (0%)</td>
<td>360 (0%)</td>
<td>0 (100%)</td>
<td>280 (0%)</td>
<td></td>
</tr>
</tbody>
</table>

*Human renin activity expressed as ng A¹/ml/hr (% inhibition of activity).

There was no evidence of digestion of Fab since the major components were found in fractions having MW 150,000 or 50,000. Furthermore, incubation with papain for 1 or 8 hours yielded similar results.

When donkey antigoat-Fab antibody was added to the renin-Fab fragment (from GLN 816) incubation mixture, the residual renin activity was markedly diminished. In fact, near complete recovery of antienzymatic activity was observed when donkey antigoat-IgG antibody (1:10) was added to the goat Fab fragment (at all dilutions) incubated with 2 milli-Goldblatt units of canine renin (fig. 6). Such an enhancement of response was not observed with nonimmune Fab fragment or intact anti-renin immunoglobulin.

TABLE 2. Purification of Renin-Specific IgG and Fab (25 ml Serum GLN 816)

<table>
<thead>
<tr>
<th>Total protein (mg)</th>
<th>Anticatalytic activity (units)</th>
<th>Specific activity (units/mg)</th>
<th>Recovery of activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>1430</td>
<td>21000</td>
<td>14.7</td>
</tr>
<tr>
<td>DEAE</td>
<td>1238</td>
<td>21000</td>
<td>17.0</td>
</tr>
<tr>
<td>AcA34 (IgG)</td>
<td>214</td>
<td>15000</td>
<td>70.0</td>
</tr>
<tr>
<td>Papain digestion</td>
<td>202</td>
<td>21000</td>
<td>10.4</td>
</tr>
<tr>
<td>G100 (Fab)</td>
<td>134</td>
<td>21000</td>
<td>15.7</td>
</tr>
</tbody>
</table>

*An anticatalytic unit is the reciprocal of the titer of the sample (100 µl), measured as 50% inhibition of 2 milli-Goldblatt units of canine renin activity.
Discussion

Antisera raised in response to immunization with renin-containing preparations have been used as a tool for studying the role of renin in cardiovascular homeostasis and pathogenesis of experimental hypertension for nearly 50 years. In light of information available from recent renin purifications, it is now apparent that only a small fraction of the immunogens used in these earlier studies was renin. Thus, the interpretation of these studies must be clouded by questions concerning the effects of antibodies to other renal proteins. The availability of purified canine renal renin now permits more specific antibodies to be raised. We have characterized these antibodies as a prelude to examining their effect as in vitro and in vivo probes into renin physiology and biosynthesis.

The eight antisera from three species examined in detail in this study bind renin but not other kidney proteins as evidenced by double immunodiffusion and immunoelectrophoretic studies. They had no inhibitory effect on the purified enzymes cathepsin D and B, pepsin, trypsin, angiotensin-converting enzymes, or the mixture of proteases present in crude renal extracts. Purified immunoglobulins retained the full enzyme inhibitory effect of the antisera. Since IgG can bind complement, form immune complexes and has a long half-life in vivo, Fab was prepared from GLN 816 and GLN 827 immunoglobulins for physiologic studies. Surprisingly, Fab fragments only retained one-fifth the anti-enzyme activity of the intact immunoglobulins in vitro. Substantial inhibitory activity was restored when donkey anti-goat antibody was added to the Fab-renin mixture. A possible explanation is that the immune response to renin is heterogeneous. Some antibodies are directed at the active site, whereas others bind to different epitopes that do not affect enzymatic activity. There is ample precedent for this in studies with antibodies to other enzymes. Intact IgG effectively removes the enzyme from solution in the form of immune complexes. Thus, antibodies to either the catalytic site or to other parts of the molecule may be equally effective in inhibiting enzymatic activity. Fab must bind to or near the catalytic site to inhibit enzymatic activity. When a second antibody (anti-Fab) is added, however, immune complexes form again, sequestering the enzyme.

The question of immunological relationships among various renins was first examined by Johnson and Wakerlin. Using bioassay techniques, they demonstrated that canine antisera raised to crude hog renin were capable of neutralizing impure renins from several species, but not from man. Lamfrom et al. extended these observations. They showed that antisera raised to various impure renins (rat, rabbit, hog, canine, beef, sheep) in different species (rat, rabbit, dog) were incapable of inactivating crude human renin. On the other hand, all the nonhuman animal renins could be neutralized with varying efficiency by anti-hog renin antiserum. Furthermore, anti-(human renin) sera failed to neutralize renin from other species. Goldblatt et al. also demonstrated that an-
tirenin produced in man by the administration of heterologous (hog) renin was ineffective against im-
pure human renin. Unfortunately, the impurity of the
renins used and the lack of characterization of the
specificity of the antisera under study raised many
questions as to the validity of these conclusions.

Now, about 30 years since the initial study,29 we are
able to address this issue with certainty using specific
antisera raised to homogeneous canine renin supported prior reports. Cross reactivity
questions as to the validity of these conclusions.

heterologous (hog) renin was ineffective against im-
tirenin produced in man by the administration of

response to immunization with purified homogenous
able to address this issue with certainty using specific
epitopes are distant from the catalytic site.

specificity of the antisera under study raised many

pure human renin. Unfortunately, the impurity of the

both by demonstrating enzyme inhibition when a sec-

are some cross-reactive epitopes between mouse and
human renins and the canine enzyme and that these
epitopes are distant from the catalytic site.

The demonstration of specificity of these antibodies for renin and interspecies homology of canine, hog,
rabbit, mouse, and human renins provides insight into
interspecies structural similarity between renins and enables us to use the renin-specific antibody or Fab
fragments for studies of the renin-angiotensin system in
various animal species.

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