Pulmonary Angiotensin-converting Enzyme

INTERSPECIES HOMOLOGY AND INHIBITION BY HETEROLOGOUS ANTIBODY IN VIVO*

JAMES M. CONROY, HELENE HOFFMAN, EDWARD S. KIRK, HEINZ O. HIRZEL, EDMUND H. SONNENBLICK, AND RICHARD L. SOFFER‡

From the Departments of Molecular Biology, Medicine and Physiology, Albert Einstein College of Medicine, Bronx, New York 10461

Angiotensin-converting enzyme (EC 3.4.15.1) is an exopeptidase which catalyzes cleavage of COOH-terminal dipeptidyl residues (for a recent review see Ref. 1). It participates in the control of blood pressure by catalyzing the generation of angiotensin II (2), a potent vasopressor octapeptide, and the control of blood pressure by catalyzing the generation of angiotensin II (2), a potent vasopressor octapeptide, and the vasodepressor effect of bradykinin was demonstrable (17). It was not possible to inhibit the vasopressor effect of angiotensin II and the vasodepressor effect of bradykinin were diminished and potentiated, respectively, in rats treated with anti-rabbit enzyme antibody. A smaller but significant immune-dependent inhibition of the vasopressor response to angiotensin II was also observed.

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‡ Faculty Research Associate of the American Cancer Society.

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immunized for at least 6 months prior to the bleedings for antibody preparations used in these studies. Preimmune and immune IgG fractions were obtained from the sera by heat treatment at 56°C for 30 min, precipitation at 50% saturation with crystalline ammonium sulfate, and chromatography on DEAE-cellulose (Whatman DE52) which was equilibrated and developed with 15 mM potassium phosphate, pH 8.0. Antibody solutions were concentrated in an Amicon pressure cell using a PM 10 filter and dialyzed extensively against 10 mM Tris-Cl, pH 7.4/0.15 M NaCl.

Immunodiffusion—Double diffusion in agar was performed as described by Ouchterlony (22).

Assays for Anticatalytic Activity—Reactions mixtures (100 μl) contained antibody (IgG fraction) and 89 milliunits of enzyme activity corresponding to 1.0 μg of the pure rabbit glycoprotein in 10 mM Tris-Cl, pH 7.4/0.15 M NaCl. After a 30-min incubation at 37°C aliquots were assayed for residual activity using Bz-Gly-His-Leu as substrate.

Radioimmunoassays—Pure rabbit lung converting enzyme (6 μg) was labeled with iodine-125 (New England Nuclear) as described by Hunter (23). Reaction mixtures for immunoprecipitation contained 5 μl of preimmune goat serum, 10 μl of [125I]-labeled enzyme (1.0 ng of protein (80 μCi)), 8 × 10^6 cpmp) and 10 μl of each of diluted antigen and goat antiserum as indicated. The final volume was adjusted to 0.2 ml with 20 mM Tris-Cl, pH 7.4/0.1 M NaCl containing 2 mg of bovine serum albumin per ml. In reaction mixtures containing detergent-solubilized extracts, the final concentration of Nonidet P-40 was 0.1% (v/v) and this amount of detergent was included in diluting and wash buffers. The characteristics of the immune precipitation of labeled enzyme, either itself or in competition with unlabeled pure rabbit enzyme, were identical in the presence or absence of detergent. Antibody and serum proteins were diluted in 20 mM Tris-Cl, pH 7.4/0.1 M NaCl containing 20 mg of albumin per ml. After incubation for 12 hours at 37°C, 50 μl containing 180 μg of donkey anti-goat IgG (Miles) was added to reaction mixtures to precipitate the antigen-antibody complex and incubation was continued an additional hour at 37°C, and then for 4 h at 0°C. The precipitate was collected by centrifugation at 4°C, washed twice with 1.0 ml of cold 0.05 M Tris-Cl, pH 7.4/0.1 M NaCl and its radioactivity was determined in a γ counter. When the various enzyme preparations were tested as competitive antigens, the amount of antiserum was that which precipitated approximately 50% of the radioactive enzyme in their absence.

Protein Determinations—For enzyme preparations the method of Lowry et al. (24) was employed using crystalline albumin as the standard. Concentrations of IgG were estimated using an E 660, nm value of 14.0.

Experiments in Vivo—In a preliminary operation silastic rubber catheters (0.39 mm outside diameter, Dow Corning) were inserted into the right jugular vein and left carotid artery of male, white Sprague-Dawley rats weighing 300 to 350 g and were exteriorized on the back. After recovery from anesthesia the rats were placed in a plegixlas restraining cage and the arterial catheter was connected to a Statham P23 Db pressure transducer for recording phasic and mean blood pressures on an oscillograph (Grass Instrument Co.). Drugs were injected into the venous catheter with a Gilmont G3200 micrometer syringe. During interval periods the catheters were filled with heparin (1:1000), tied off, and the animals were returned to standard cages.

Globulin fractions were prepared by heat treatment of serum at 56°C for 30 min, followed by precipitation at 50% saturation with ammonium sulfate. The precipitate was taken up and dialyzed against 15 mM potassium phosphate, pH 8.0. Antibody solutions were concentrated in an Amicon pressure cell using a PM 10 filter and dialyzed extensively against 10 mM Tris-Cl, pH 7.4/0.15 M NaCl.

RESULTS

Experiments in Vitro—Studies by double immunodiffusion in agar revealed the presence, in solubilized pulmonary extracts of rat, guinea pig, and dog, of a protein which showed partial identity with the rabbit converting enzyme (Fig. 1). The reaction was immune-specific and was not seen with similar preparations from cow, frog, or chicken even when they were tested using a 10-fold excess (890 milliunits) relative to the reactive extracts. Guinea pig soluble activity (16,000 x g

Fig. 1. Double diffusion in agar. I, the center well contained 89 milliunits of pure rabbit lung converting enzyme and outer wells 1, 2, and 3 contained, respectively, 196, 125, and 146 μg of IgG fractions from goats 435, 447, and 448. II, III, and IV, the center wells contained 196, 125, and 146 μg, respectively, of IgG from fractions from goats 433, 447, and 448. The outer wells contained 89 milliunits each of pure rabbit lung enzyme (A) and Nonidet P-40 extracts of dog (B), guinea pig (C), and rat (D) lung particles.
**Fig. 2.** Anticatalytic action of anti-rabbit enzyme IgG on heterologous activities. The effect of antibody on 89 milliunits of activity was assayed as described under “Experimental Procedure.” The activities were rabbit Nonidet P-40 extract, O—O; guinea pig 16,000 × g supernatant fraction, Δ—Δ; dog Nonidet P-40 extract, □—□; and rat Nonidet P-40 extract, O—O. Panels A, B, and C represent results obtained with IgG fractions from goats 433, 447, and 448, respectively.

**Fig. 3.** Competition radioimmunoassays. Dilution of the radioiodinated rabbit glycoprotein was determined as described under “Experimental Procedure.” Panels A, B, and C represent antisera from goats 433, 447, and 448 which were used, respectively, at dilutions of 1:15,100; 1:6300; and 1:4000. Competing proteins were: A, pure rabbit lung enzyme; O, rabbit lung Nonidet P-40 extract; Δ, guinea pig 16,000 × g supernatant fraction; □, dog Nonidet P-40 extract.

the radioiodinated enzyme was 4.5, 6.1, and 5.8. Corresponding ratios of dog to rabbit activity were 8.1, 12.6, and 13.2. These results suggest that the guinea pig enzyme contains more extensive homology with the rabbit glycoprotein than does the dog enzyme. This homology apparently may not always be detected using inhibition by heterologous antibody, presumably because the anticatalytic parameter reflects a much smaller number of antigenic determinants than does the radioimmunoassay.

**Experiments in Vivo**—A dialyzed 50% ammonium sulfate fraction from the antiserum of goat 433 was used to determine the effect of anti-rabbit enzyme antibody on the blood pressure response in the rat to vasoactive agents. The administered antibody was 6 times the amount required to inhibit the total activity in a homogenate from a pair of rat lungs by 50%. Preliminary experiments suggested that this quantity yielded close to the maximal immune-dependent alteration of the responses to exogenous vasoactive peptides. Preimmune or nonimmune globulins did not affect these responses and the immune fraction did not cause a change in the pressor activity of norepinephrine (not shown), indicating that its effect was not mediated by a generalized alteration in vascular reactivity.

All of the test animals survived the infusion, but died within 24 to 48 hours as did the controls. It is therefore not clear whether the deaths were due to an immune mechanism, to repeated administration of vasoactive peptides, or to catheter manipulations and dislodging of clots which often developed. There was no immediate immune-specific lethal reaction of the type which had been observed in rabbits (17).

The amount of vasoactive peptide required to elicit a standard response of 25 mm Hg was determined before and after administration of the globulin solutions using dose-response curves, as shown in Fig. 4. The dose of angiotensin I required to yield a blood pressure elevation of 25 mm Hg increased by an average of 8.8-fold after infusion of antibody with a range of 3.5- to 13.6-fold among the seven animals (Table I). Unexpectedly, the antibodies also inhibited the vasopressor effect of angiotensin II, although to a considerably lesser extent. The depressor effect of bradykinin was potentiated by an average of 13-fold in the presence of antibody with individual values ranging from 4- to 25-fold. Although the dose-response curves in the test animals were markedly shifted, they maintained their original slope (Fig. 4). The data shown are for pressor responses measured 1 h after infusion of antibody; however, similar results were obtained when the animals were restudied at 24 and 48 h. This duration is much longer than that reported for the action of venom peptide inhibitors in the rat (25). The anticatalytic action of the serum from one animal was measured as a function of time after administration of antibody. Serum obtained prior to infusion from one animal was measured as a function of time after administration of antibody. Serum obtained prior to infusion did not inhibit hydrolysis of Bz-Gly-His-Leu catalyzed by the pure rabbit converting enzyme. Assuming the blood volume to represent 7% of the animal’s weight, the anticatalytic action of its serum 1 h after infusion was equivalent to 49% of the total infused antibody. The corresponding values at 24 and 48 h were, respectively, 35% and 28%.

**DISCUSSION**

Because it is directly exposed to the circulation (12, 13), angiotensin-converting enzyme provides an unusual opportunity for examining the possibility that the action of an enzyme in vivo can be regulated by exogenous antibodies. The effectiveness of relatively short-lived, metabolically unstable venom peptide inhibitors in temporarily preventing the development of renin-dependent hypertension (10, 11) suggests that antibodies directed against the enzyme may ultimately be useful as diagnostic or therapeutic reagents. Goat antirabbit enzyme antibody is highly toxic when administered intravenously to rabbits (17). This lethal effect is presumably not related to anticatalytic action, since it does not occur with the
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Fig. 4. Effect of antirabbit converting enzyme antibodies on the rat blood pressure response to vasoactive peptides. The dose-response curves were obtained as described under "Experimental Procedure" before and 1 h after intravenous infusion of 180 mg of immune globulin from goat 433. Each point represents the mean of four determinations and is bracketed by the values corresponding to one standard deviation. The lines were fitted by the method of least squares.

TABLE I
Effect of immune globulin on rat response to vasoactive peptides

<table>
<thead>
<tr>
<th>Animals</th>
<th>Globulin*</th>
<th>Relative dose required to elicit standard test response*</th>
<th>Relative dose required to elicit standard test response*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Angiotensin I</td>
<td>Angiotensin II</td>
</tr>
<tr>
<td>7</td>
<td>Immune</td>
<td>8.8 (3.5-13.6)*</td>
<td>2.9 (1.8-3.9)*</td>
</tr>
<tr>
<td>2</td>
<td>Preimmune</td>
<td>0.85 (0.80-0.90)</td>
<td>0.95 (0.70-1.2)</td>
</tr>
<tr>
<td>4</td>
<td>Nonimmune</td>
<td>0.06 (0.74-1.1)</td>
<td>0.74 (0.60-0.95)</td>
</tr>
</tbody>
</table>

*The dose of each globulin fraction was 180 mg.

The amount of peptide required to elicit a change in blood pressure of 25 mm Hg was determined before and 1 h after administration of globulin using dose-response curves of the type shown in Fig. 4. The data are expressed as ratios with the pre-infusion dose normalized to 1.0. Mean and extreme (denoted by parentheses) values are shown for each group. The actual mean dose of angiotensin I needed to elicit the standard test response increased from 63 ± 11 to 600 ± 180 ng after infusion of immune globulin. The corresponding values for angiotensin II were 32 ± 4 to 96 ± 19 ng and those for bradykinin were 4.4 ± 1.4 to 0.37 ± 0.17 µg.

*p < 0.001 as compared to corresponding pre- and nonimmune control values.

*F) < 0.001 as compared to corresponding values obtained with angiotensin II.

venom peptide inhibitors. However, it has precluded infusion of sufficient amounts of immune globulins to satisfactorily assess their effect on the metabolism of vasoactive peptides in vivo. Our studies on the immunologic relationship of enzymes in other species to that of the rabbit were initiated to find a heterologous recipient which might tolerate administration of enough anti-rabbit enzyme antibody to anticipate important immune-dependent effects on the blood pressure responses to vasoactive peptide substrates.

It has previously been shown that goat antibody against pure rabbit lung enzyme reacts with a constituent of vascular endothelial cells in other organs (12), that rabbit antibody against pure porcine lung enzyme recognizes a component of the luminal surface of rat pulmonary vascular endothelial membranes (18) and that rabbit antibody to a partially purified preparation of the porcine renal enzyme inhibits pig plasma and lung activities, but not that of human lung (6). Our present studies indicate that goat antibody to the rabbit lung glycoprotein interacts with pulmonary enzymes from rat, guinea pig, and dog, but not those from cow, frog, or chicken. An important observation is the variability in heterologous cross-anticatalytic action of antisera prepared in different goats. This variability may reflect the relatively small number of determinants which participate either directly or indirectly in catalysis, since it was not present using immunodiffusion and radioimmunoassay, procedures which encompass all antigenic determinants. The heterologous anticausal action of antibody from a single animal therefore appears to represent a poor criterion of immunologic homology. As a corollary, demonstration of homology by other criteria does not necessarily indicate that an antibody preparation will be useful in regulating catalysis.

The rat was found to be a satisfactory recipient animal in which to study the effect of anti rabbit enzyme antibody in...
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uivo. It was possible to administer without immediate toxic effect at least 6 times the amount of antibody required to inhibit the total enzyme activity of a pair of rat lungs by 50% in vitro. Under these conditions the pressor effect of angiotensin I and the depressor effect of bradykinin were inhibited and potentiated, respectively, by approximately 1 order of magnitude. These results provide strong evidence that the action of the enzyme on its two important substrates was inhibited in vivo. The immune-dependent inhibition of the vasopressor response to angiotensin II was unexpected, since this biologically active octapeptide is the product of the enzyme's action on angiotensin I. Angiotensin II receptors have been identified in plasma membranes of aortic (26) and uterine (27) smooth muscle cells and in a similar membranous fraction of adrenal cortical cells (28). If such receptors were also located on the plasma membrane of vascular endothelial cells in close approximation to the converting enzyme protein, then antibody attached to the latter might sterically inhibit access of angiotensin II to its effector site. It is interesting to note in this context that a renin-like activity has been reported as a component of vascular walls (29). It is therefore intriguing to speculate that renin, angiotensin-converting enzyme, and an angiotensin II receptor molecule may constitute an anatomic and physiologic unit of blood vessel surfaces.

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