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# Short Communications

## Subcellular Localization of Pulmonary Angiotensin-Converting Enzyme (Kininase II)

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Goat antibodies to pig lung angiotensin-converting enzyme (kininase II) were conjugated to microperoxidase. Rat lung tissue, previously incubated with non-immune goat serum, was incubated with the antibody–microperoxidase conjugate and then with $H_2O_2$ and 3,3′-diaminobenzidine. Electron microscopy revealed reaction product on the plasma membrane and caveolae of endothelial cells, especially those of capillaries and venules. These results support the hypothesis that angiotensin I and bradykinin are metabolized by enzymes on the luminal surface of pulmonary endothelial cells.

Previous studies have suggested that circulating angiotensin I and bradykinin are metabolized by enzymes on the luminal surface of pulmonary endothelial cells (Ryan *et al.*, 1972; Smith & Ryan, 1973; Ryan, 1974). Dorer *et al.* (1972, 1974) have purified to homogeneity a lung enzyme capable of metabolizing bradykinin and angiotensin I to yield metabolic products like those produced by intact lungs. Angiotensin I is converted, by removal of its *C*-terminal dipeptide, into its more potent lower homologue, angiotensin II. Bradykinin is inactivated by the removal of its *C*-terminal dipeptide (Dorer *et al.*, 1974). In terms of its actions on bradykinin and angiotensin I, the enzyme appears to be a dipeptidyl carboxypeptidase and is comparable with activities known previously as angiotensin-converting enzyme and kininase II (Dorer *et al.*, 1972; Yang *et al.*, 1971). The data suggest that the enzyme isolated by Dorer *et al.* (1972) may account in part for the processing of bradykinin and angiotensin I by intact lungs. Thus in the present study we have prepared antibodies to the enzyme and have used the antibodies, coupled to microperoxidase (a haem–undecapeptide isolated from cytochrome *c* after proteolysis by pepsin) (Feder, 1971), in an effort to determine the subcellular sites of the enzyme in intact lungs.

Two adult female mongrel goats were immunized with pig lung angiotensin-converting enzyme isolated as described by Dorer *et al.* (1972). Enzyme solution (0.1ml; 1.0mg/ml) was emulsified with 0.4ml of complete Freund's adjuvant (Difco Laboratories, Detroit, Mich., U.S.A.), and 0.25ml of the emulsion was injected intramuscularly into each hind leg, monthly for 3 months. Then 1 month later 0.25ml of the enzyme was emulsified with 0.25ml of complete Freund's adjuvant and was injected as described above. A final booster injection (0.2ml of enzyme plus 0.2ml of the adjuvant) was made on the fifth month. At 1 week after the last injection, the serum of one of the goats reacted with the immunogen to give a strong single precipitin band on Ouchterlony immunodiffusion plates (Ouchterlony, 1967).

Goat antiserum (50ml) was treated with solid $(NH_4)_2SO_4$ to give a final concentration of 1.9M-$(NH_4)_2SO_4$ (Stelos, 1967). The mixture was left at 4°C for 1h, and then the precipitate was collected by centrifugation (Sorvall centrifuge RC2-B, 12000*g* at 4°C for 30min). The supernatant was decanted and the precipitate was washed once with 1.9M-$(NH_4)_2SO_4$. The final precipitate was dissolved in 50ml of 150mM-NaCl, and the solution was dialysed for 48h against 10litres of 5mM-sodium phosphate, pH7.5, containing 10mM-NaCl. The dialysis buffer was changed twice, once at 16h and again at 36h. The non-diffusible material (18mg of protein/ml) was applied to a column (3.4cm×26cm) containing DEAE-cellulose (DE-52; Whatman Biochemicals Ltd., Maidstone, Kent, U.K.) equilibrated with the same buffer. The antibody was eluted from the cellulose at a conductivity between 2 and 3mmhos after application of a linear gradient of NaCl (10–300mM) in the starting buffer. The partially purified antibody preparation was tested with rabbit anti-goat serum and with rabbit anti-goat globulin (Sycco Co., Millburn, N.J., U.S.A.). The goat anti-(pig lung angiotensin-converting enzyme) showed a single precipitin band with the two rabbit antisera in Ouchterlony immunodiffusion plates and on im-

munoelectrophoresis. The rabbit antisera showed no precipitating activity for the enzyme. The same purification procedure was used for fractionating non-immune goat serum. Further purification of the antibody by immunoabsorption was not feasible because the supply of pure pig lung converting enzyme had been exhausted.

The antibody fraction eluted from DEAE-cellulose was conjugated to microperoxidase (Sigma Chemical Co., St. Louis, Mo., U.S.A.) by the method of Avrameas (1969). The microperoxidase appeared to be homogeneous, yielding a single red spot, reactive with ninhydrin and chlorine–*o*-tolidine reagents, on paper electrophoresis (Whatman no. 1) at pH 2.0 and 5.0 (Ryan *et al.*, 1971). The conjugate was dialysed against 30mM-sodium phosphate buffer, pH 7.4, containing 150mM-NaCl, to remove conjugated microperoxidase (mol. wt. 2000).

Lung tissue was obtained from Sprague–Dawley rats anaesthetized with an intraperitoneal injection of chloral hydrate (300mg/kg body wt.). The lungs were ventilated mechanically and were perfused until the venous effluent became free of blood, as described previously (Ryan *et al.*, 1971). The lungs were then perfused with paraformaldehyde–picric acid (Stefanini *et al.*, 1967) for 10min. Peripheral portions of the fixed lungs were cut into cubes (about 2mm each side), which were then immersed in fresh fixative. The blocks of tissue were cut to smaller pieces under a dissecting microscope and then incubated successively in each of the following reaction mixtures: (1) fractionated non-immune goat serum, diluted 1:30 in 100mM-sodium phosphate buffer, pH 7.4, containing 150mM-NaCl, for 16h; (2) the antibody–microperoxidase conjugate, diluted 1:100 in 50mM-Tris–HCl, pH 7.1, containing 150mM-NaCl and 1% gelatin, for 3.5h; and (3) 3,3′-diaminobenzidine (0.125mg/ml of 50mM-Tris–HCl, pH 7.1, containing 150mM-NaCl) for 1h. $H_2SO_2$ (0.005%) was added for the final 15min of step 3. Between each step, the tissue was washed with 50mM-Tris–HCl, pH 7.1, containing 150mM-NaCl. The tissue blocks were then immersed in $OsO_4$ (1% in 30mM-veronal acetate buffer, pH 7.4) for 0.5h. The osmicated tissue was dehydrated and embedded as described previously (Smith & Ryan, 1970). Control incubations omitted one of the following: antibody–microperoxidase conjugate, diaminobenzidine, or $H_2O_2$. In other experiments, microperoxidase, not conjugated to antibody, was used in place of the conjugate. Grey or silver-grey sections (less than 80nm in thickness; Peachey, 1958) were examined unstained in a Philips EM301 electron microscope (Philips Electronic Instruments, Mount Vernon, N.Y., U.S.A.).

Sections of tissue treated in series with the full set of reaction mixtures showed electron-dense deposits, presumably oxidized diaminobenzidine, along the luminal surface of endothelial cells and their caveolae. Heaviest reactions occurred on endothelial cells of capillaries and venules (see Plate 1). Some deposits occurred along the plasma membrane of type I alveolar epithelial cells, but these deposits did not require antibody, the deposits on epithelial cells occurring also in tissue treated with unconjugated microperoxidase. Under the conditions of our experiments, an intrinsic peroxidase activity of lung tissue was not discernible. Tissue incubated in step 1 with fractionated non-immune goat serum coupled to microperoxidase showed heavy reactions on all cell types of the alveolar–capillary unit. No reactions were discernible in tissue incubated with non-immune goat serum and then incubated with non-immune serum coupled to microperoxidase. These findings probably are due to goat natural antibodies reactive with rat tissue antigens.

The immunocytochemical results reported here support our hypothesis that circulating bradykinin and angiotensin I, like the adenine nucleotides (Ryan & Smith, 1971), are metabolized by enzymes on the luminal surface of pulmonary endothelial cells. The developing picture from the results of these and previous experiments is that of an enzyme anchored to the surface of the endothelial cells of pulmonary capillaries and venules. The enzyme, in solid phase, is perfused with its substrates in a continuously flowing liquid phase. The lungs receive the entire cardiac output and have one of the most extensive capillary networks of the mammalian body. A volume of 1 ml of blood may occupy more than 10 miles of capillary length, a circumstance that could well favour efficient metabolism of bradykinin and angiotensin I by relatively modest amounts of enzyme. The ability of a single lung enzyme to inactivate bradykinin, a substance that tends to lower blood pressure, while converting angiotensin I into angiotensin II, the most hypertensive substance known, suggests a role of the enzyme in blood-pressure homoeostasis.

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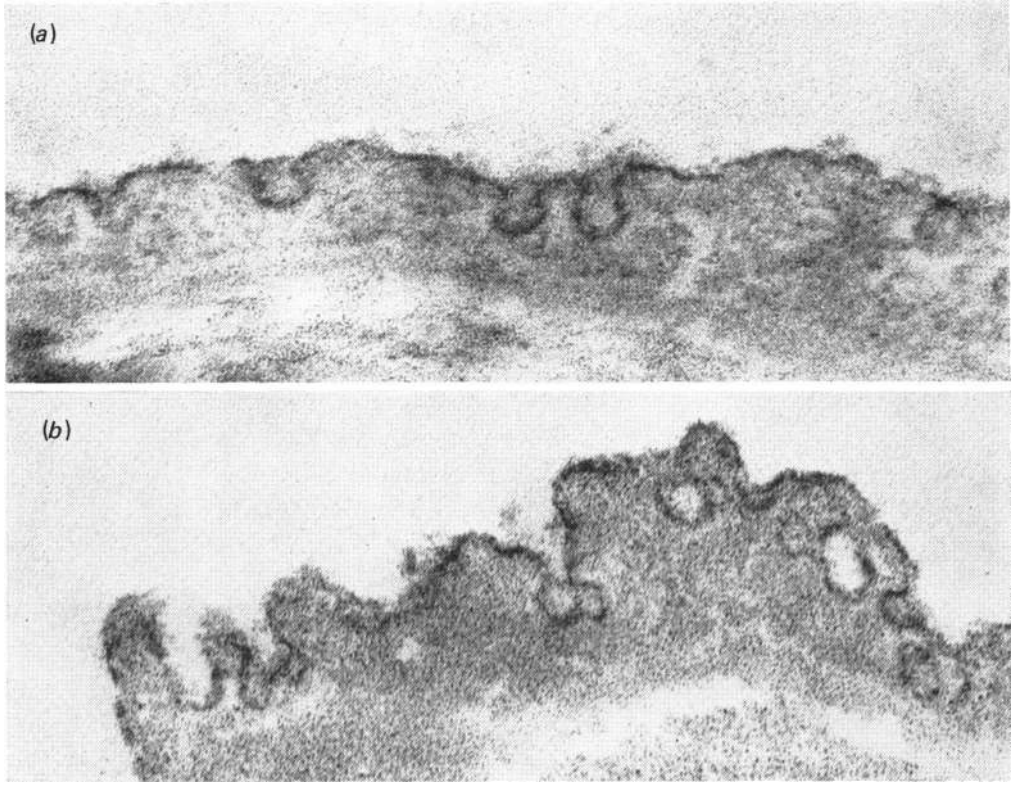


EXPLANATION OF PLATE 1

*Subcellular localization of angiotensin-converting enzyme*

Lung tissue, fixed in paraformaldehyde–picric acid, was incubated in succession with non-immune goat serum, antibody to converting enzyme coupled to microperoxidase, and then 3,3′-diaminobenzidine and $H_2O_2$. Electron-dense deposits, presumably oxidized diaminobenzidine, were localized on the luminal aspect of caveolae and plasma membrane of endothelial cells, especially those of capillaries and venules. The sections were not stained. In both photographs, the luminal surface is uppermost. Magnifications: (*a*) × 114000; (*b*) × 124000.

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