Short Communication

Metabolism of Angiotensin I in the Pulmonary Circulation

By J. W. RYAN, J. M. STEWART, W. P. LEARY and J. G. LEDINGHAM

The Howard Hughes Medical Institute and the Department of Medicine, University of Miami, Miami, Fla. 33136, U.S.A., and the Department of the Regius Professor of Medicine, University of Oxford, Oxford OX1 3QT, U.K.

(Received 14 August 1970)

There is a growing body of evidence suggesting that angiotensin I is converted into angiotensin II during passage through the pulmonary circulation. The major finding in support of this hypothesis is that the venous effluent of lungs perfused with angiotensin I has a greater capacity to contract the rat colon and raise systemic arterial blood pressure than can be explained by the concentration of angiotensin I entering the pulmonary artery (Ng & Vane, 1967, 1968a, b; Biron & Huggins, 1968). These results are obtained by using lungs perfused with blood or Krebs–Henseleit solution (Krebs & Henseleit, 1932), indicating that the postulated conversion cannot be entirely accounted for by enzymes in blood. Bakhle, Reynard & Vane (1969) have also shown that the enhanced biological effects are not due to release of endogenous catecholamines or prostaglandins E_2 or $F_{2\alpha}$.

The change in biological activity of angiotensin I after passage through the lung could be due to conversion of the decapeptide into angiotensin II by tissue enzymes. However, this possibility is not proved by the bioassay of unfractionated perfusate. The biological assay preparations that have been used are highly selective in terms of known compounds, but previous studies have not ruled out the possibility that angiotensin I acts on the lung to stimulate the release of an unknown compound capable of contracting the rat colon and raising arterial blood pressure. The possibility also exists that metabolic products of angiotensin I potentiate the effects of the parent compound, perhaps as the C-terminal pentapeptide of bradykinin potentiates the effects of bradykinin on the guinea-pig ileum (Garbe, 1967).

Conversion of angiotensin I into angiotensin II in the pulmonary circulation could be of major physiological importance and therefore an attempt has been made in this study to obtain biochemical evidence of any such conversion.

Perfusion studies were performed by using female Sprague–Dawley rats, weighing 0.2–0.4 kg, fed on a standard small-animal diet (Purina Laboratory Chow) and given unrestricted access to water. Three rats were given tap water and three were given tap water containing 2% (w/v) NaCl. The

latter three rats received the salt water for 10–12 days before use. All rats were anaesthetized by an intraperitoneal injection of chloral hydrate (300 mg/kg body wt.). Lungs were perfused *in situ* as described by Leary & Ledingham (1969) by a method developed by F. Woods & W. P. Leary (unpublished work).

All glassware except the chromatography column was coated with silicone (Siliclad, Clay–Adams; Inc., New York, N.Y., U.S.A.). Krebs–Henseleit solution, aerated with $O_2 + CO_2$ (95:5) and heated at 37°C, was pumped through the lungs at a rate of 5–6 ml/min until the venous effluent was free of blood (2–3 min). Then (10-L-[^{14}C]leucine)-angiotensin I (25 $\mu Ci/\mu mol$, lot no. 6901; Schwarz Bio-Research Inc., Orangeburg, N.Y., U.S.A.) was added to the Krebs–Henseleit solution to a concentration of 700 μg (about 0.5 nmol)/ml and the perfusion rate was maintained at 6 ml/min. Venous effluent from the perfused lung was collected over the next 3 min into an Erlenmeyer flask containing 20 ml of cold 10% (w/v) trichloroacetic acid stirred constantly with a magnetic stirring device.

This perfusate was centrifuged at 1690g for 10 min at 4°C to remove a small (0.2 ml) brown precipitate. The supernatant was extracted five times with 2 vol. of diethyl ether. The aqueous layer was evaporated to dryness in a rotary evaporator. Its residue was dissolved in 1.1 ml of 1M-pyridine, and 1.0 ml of the solution was chromatographed on a Bio-Gel P-2 column (98 cm $\times$ 1.2 cm). The column was eluted with 1M-pyridine, and 50–100 2 ml fractions were collected. The column was calibrated previously to determine elution volumes of angiotensin I, angiotensin II, histidyl-leucine and leucine (Fig. 1a).

A 20 μl sample of each fraction was applied to pieces (1 cm $\times$ 1 cm) of filter paper (Whatman no. 1) to detect radioactivity. The papers were dried and then placed in scintillation vials containing 18 ml of an 0.4% (w/v) solution of Omnifluor (New England Nuclear Corp., Boston, Mass., U.S.A.) in toluene. The radioactivities of vials were counted in a Unilux II liquid-scintillation counter (Nuclear–Chicago).

Fig. 1. (a) Molecular-sieve chromatography of angiotensin I and selected lower homologues. Angiotensin I and some of its homologues were chromatographed on a column (98 cm $\times$ 1.2 cm) of Bio-Gel P-2. Fractions (2 ml) were collected. Fractions containing (10-L-[14 C]leucine)-angiotensin I and (5-L-[14 C]isoleucine)-angiotensin II (45 μ Ci/ μ mol; Schwarz BioResearch Inc.) were located by scintillation counting. Histidyl-leucine and leucine were located with ninhydrin (E_{570}). $\circ$, Angiotensin I; $\bullet$, angiotensin II; $\triangle$, histidyl-leucine; $\blacktriangle$, leucine. (b) Molecular-sieve chromatography of the effluent of lungs perfused with (10-L-[14 C]leucine)-angiotensin I. The effluent was treated with trichloroacetic acid and extracted as described in the text. The extract was chromatographed on a column (98 cm $\times$ 1.2 cm) of Bio-Gel P-2 and fractions were collected as described above. Samples were tested for radioactivity and for ability to contract the rat colon and raise arterial blood pressure. $\circ$, Radioactivity; $\square$, blood pressure; $\bullet$, ability to contract rat colon.

In four experiments the fractions were recombined to correspond to peaks of radioactivity. In two experiments fractions were freeze-dried individually. Each residue was dissolved in 2 ml of

water and tested for effects on the rat colon (Regoli & Vane, 1964) and on the arterial blood pressure of the rat (Peart *et al.* 1966).

Further analysis of radioactive peaks was carried out by paper electrophoresis at pH 1.9 (formic acid-acetic acid-water, 6:13:81, by vol.), pH 5 (0.1 M-acetic acid adjusted to pH 5 with pyridine) or pH 9 [1% (w/v) $(\text{NH}_4)_2\text{CO}_3$]. Whatman no. 1 paper was used for analytical runs and Whatman 3MM paper was used for preparative electrophoresis. With any of these systems electrophoresis was carried out at 22 V/cm, 10–20 mA, for 1 h.

In all experiments the activity of the solution entering the lungs was compared with that of the venous effluent in terms of ability to raise mean arterial blood pressure. In none of these experiments was there a clear increase or decrease of activity. In contrast, the ability of the venous effluent to contract the rat colon was 2–5 times greater than could be accounted for by angiotensin I. On average, the rat colon was 15 times as sensitive to angiotensin II as to angiotensin I.

In the two experiments in which column fractions were assayed individually, as much as half of the pressor activity and well over half of the colon-contracting activity was found to be eluted much later than expected for angiotensin I or angiotensin II (Fig. 1b). The identity of the substance(s) of the second peak is not known. Control experiments were performed to rule out the possibility that the second peak of biological activity is a substance leached from the tubing of the perfusion circuit. Chromatography of the perfusate obtained by pumping angiotensin I through tubing but not through lungs showed a single peak of activity corresponding in elution to angiotensin I. Under the conditions of our chromatography an impurity of the column matrix would not be expected to be eluted as a peak.

The substance of the first biologically active peak was identified as angiotensin II. Its elution volume corresponded to that of authentic angiotensin II, being slightly less than that of angiotensin I. Fractions corresponding to the first peak of activity were combined and freeze-dried. The residue was dissolved in 0.5 ml of pyridine-acetic acid buffer, pH 5. The entire sample was applied to a strip of Whatman 3MM paper and electrophoresis was carried out at pH 5, 22 V/cm, 10–20 mA, for 1 h. Arginine and picric acid were included in the run as reference compounds. Radioactivity was located by strip counting (Actigraph III; Nuclear-Chicago). Then the paper was cut at 1.0 cm intervals from the origin towards the cathode. Each piece of paper was eluted with 2.0 ml of Tyrode's solution, and the eluates were tested for effects on the rat colon and arterial blood pressure. Biological activity was found as a single peak (R_{Arg} 0.18–0.27) correspond-

ing to the migration of authentic angiotensin II (R_{Arg} 0.24). This area of the electrophoretogram was devoid of radioactivity.

The electrophoretogram contained two peaks of radioactivity, a small one (60 c.p.m.; background 6 c.p.m.) corresponding in migration (R_{Arg} 0.47) to angiotensin I, and a larger peak (700 c.p.m.), which migrated much faster (R_{Arg} 0.69). The second peak, judged from its early elution from the Bio-Gel P-2 column and its rapid migration, is probably a large C-terminal fragment of angiotensin I.

As shown in Fig. 1(b), over half of the radioactivity applied to the column was eluted in fractions expected for peptides of two to five amino acid residues. None of the radioactivity was accounted for as free leucine. This point rules out the possibility that angiotensin I is converted into angiotensin II by the stepwise removal of leucine and then histidine (Ng & Vane, 1967).

Subsequent analysis showed that virtually all of the radioactivity in fractions 28–40 was incorporated into histidyl-leucine. Fractions 28–32 and 33–40 were freeze-dried and analysed by electrophoresis at pH 5 and pH 9. Samples were run with or without the addition of carrier histidyl-leucine. Carrier was located after reaction with ninhydrin. At either pH value radioactivity of fractions 28–32 and 33–40 was not distinguishable from that of authentic histidyl-leucine (pH 5, R_{Arg} 0.71; pH 9, $R_{\text{picric acid}}$ 0.53).

Our results show that angiotensin I is converted into angiotensin II during a single circulation through rat lung washed free of blood. Conversion occurs by removal of the C-terminal dipeptide, histidyl-leucine, and not by the stepwise action of a carboxypeptidase. Much of the radioactivity associated with the large-molecular-size (mol.wt. about 900) material can be accounted for as a large C-terminal homologue of angiotensin I. This suggests that the converting enzyme of lung competes with an aminopeptidase. Angiotensin II

is not the only biologically active substance found in the venous effluent of lungs perfused with angiotensin I. There is a second substance, separable by chromatography on Bio-Gel P-2, that contracts the rat colon and raises arterial blood pressure. The relatively late elution of the second substance suggests that it either is of small molecular size or is adsorbed by the Bio-Gel matrix. Whether the second substance is an endogenous compound in lung or a lower homologue of angiotensin I is not known. The C-terminal heptapeptide of angiotensin II is known to have biological activity. However, this peptide would not be expected to separate from angiotensin I or angiotensin II during chromatography on Bio-Gel P-2. It remains to be determined whether smaller homologues are capable of contracting the rat colon and raising arterial blood pressure.

This work was supported in part by grants from the Florida Heart Association, the Population Council (M69148) and the National Institutes of Health (HE12843). W.P.L. was supported in part by the Nuffield Dominions Foundation, Oxford, and the Medical Research Council, Pretoria, South Africa.

- Bakhle, Y. S., Reynard, A. M. & Vane, J. R. (1969). *Nature, Lond.*, **222**, 956.
Biron, P. & Huggins, C. (1968). *Life Sci.* **7**, 965.
Garbe, G. (1967). *Arch. exp. Path. Pharmacol.* **256**, 112.
Krebs, H. A. & Henseleit, H. (1932). *Hoppe-Seyler's Z. physiol. Chem.* **210**, 32.
Leary, W. P. & Ledingham, J. G. (1969). *Nature, Lond.*, **222**, 959.
Ng, K. K. F. & Vane, J. R. (1967). *Nature, Lond.*, **216**, 762.
Ng, K. K. F. & Vane, J. R. (1968a). *Arch. exp. Path. Pharmacol.* **259**, 2.
Ng, K. K. F. & Vane, J. R. (1968b). *Nature, Lond.*, **218**, 144.
Peart, W. S., Lloyd, A. M., Thatcher, G. N., Lever, A. F., Payne, N. & Stone, N. (1966). *Biochem. J.* **99**, 708.
Regoli, D. & Vane, J. R. (1964). *Br. J. Pharmacol. Chemother.* **23**, 351.