Nature of the Pressor Substance in Rabbit Placenta

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(Received 12 April 1966)

1. The renin-like substance isolated from the placenta of the rabbit produces a prolonged increase of blood pressure in the nephrectomized rat. If incubated with renin substrate from ox serum, it forms a pressor substance that elevates blood pressure in exactly the same way as does angiotensin. 2. This angiotensin-like principle was concentrated by means of ion-exchange chromatography and compared with Val-5-angiotensins I and II in two paper-chromatography systems and in paper electrophoresis. 3. In all the three methods the unknown principle behaved like a mixture of the two reference compounds. 4. It is concluded that incubation of the renin-like substance of placental origin with substrate from ox serum gave a mixture of Val-5-angiotensins I and II. This is evidence that the enzyme isolated from the placenta is either closely related to, or identical with, renin.

Recently Gross, Schaechtelin, Ziegler & Berger (1964) described the extraction of a pressor principle from the placenta of pregnant rabbits as well as from the uterine wall of both pregnant and non-pregnant rabbits. Previously a renin-like substance had been claimed to be present in cat placenta (Stakemann, 1960). The pressor substance isolated by Gross *et al.* (1964) from rabbit placenta and uterus behaved in a way very similar to renin. It not only increased blood pressure by itself, but produced, when injected into a nephrectomized rat, a long-lasting increase in blood pressure typical of that caused by renin. In addition, it had an enzymic nature, forming from plasma substrate a pressor substance indistinguishable from angiotensin. These observations have been confirmed by other investigators (Bing & Fårup, 1966; Ferris & Mulrow, 1965). To find out whether the enzyme-like substance is renin and the compound formed by it identical with angiotensin, we undertook further purification experiments and tried, by means of comparative chromatography and electrophoresis, to obtain information on its physicochemical properties.

MATERIAL AND METHODS

Extraction of renin-like substance from the rabbit placenta. Uteri of pregnant rabbits were excised after 27 or 28 days of pregnancy and the foetuses, together with their placentae, removed from both uterine horns. Subsequently the placentae were isolated and roughly separated into the maternal and foetal parts.

Maternal placentae (342 g.) were homogenized with 3000 ml. of water and the homogenate was acidified to pH 2.8 by adding 2 N-H₂SO₄. After 30 min. it was neutralized to pH 7.3 by adding 2 N-KOH and then centrifuged for 15 min. at 4500 g. The supernatant was cooled to 0–4° and acidified with 2 N-H₂SO₄, under continuous stirring, to pH 3.5. Ammonium sulphate was then added to give 1.3 M final concentration. After 48 hr. the mixture was centrifuged in the same way as before. The resulting supernatant (4200 ml.) was acidified to pH 2.3 with 2 N-H₂SO₄, and then the concentration of ammonium sulphate was increased to 2.3 M. After 24 hr. the mixture was again centrifuged. The sediment was suspended in 150 ml. of water and dialysed against distilled water at 4° for 3 days. Freeze-drying gave 894 mg. of a dry powder containing the renin-like substance.

Preparation of substrate from ox serum. The substrate was prepared according to the method of Schales, Holden & Schales (1943). Ox serum (2920 ml.) was mixed with 852 ml. of distilled water and adjusted to pH 6.4 by adding 121.7 ml. of 0.4 N-H₂SO₄. Ammonium sulphate was added to give 1.25 M final concentration. The serum was then stored at 4° for 24 hr. Thereafter it was centrifuged at 4500 g for 10 min. Ammonium sulphate was added to the supernatant to give a total concentration of 1.67 M and a pH of 6.8. This mixture was stored at 4° for 24 hr. and then centrifuged again at 4500 g for 10 min. The resulting sediment was dissolved in 300 ml. of water and dialysed against distilled water for 3 days at 4°. The dialysed mixture was then centrifuged at 4500 g for 15 min. The resulting supernatant (350 ml.) contained the renin substrate.

Formation of angiotensin-like substance. The total amount of 894 mg. of powder containing the renin-like principle was dissolved in 150 ml. of distilled water and the solution adjusted with aqueous NH₃ solution to pH 7.3. This solution was incubated with the 350 ml. of renin substrate (pH 7.3) at 37° for 30 min. Subsequently the mixture was

boiled for 5 min., then cooled in ice and centrifuged for 10 min. at 4500 *g*. The concentration of angiotensin-like substance contained in the supernatant was tested in nephrectomized rats anaesthetized with urethane according to our standard procedure (Gross, Brunner & Ziegler, 1965) and the activity compared with that of synthetic angiotensin-amide (Val-5-angiotensin II-Asp-1- β -amide). It was calculated that 1 ml. of the supernatant contained an amount equivalent to 0.25 μ g. of angiotensin II-amide. The pressor activities (m μ g.) given in all the following experiments are based on the same comparison with angiotensin II-amide.

To isolate the pressor material, the incubation mixture was concentrated with the aid of an ion-exchange resin by a modification of the method described by Boucher, Veyrat, de Champlain & Genest (1964). To absorb the pressor material, 200 ml. of the incubation mixture was slowly filtered through a column (2.5 cm. $\times$ 20 cm.) of Dowex 50W (X2; NH₄⁺ form), then rinsed with 300 ml. of 0.2 M-ammonium acetate, pH 6, and subsequently with 300 ml. of water. The column was eluted with 300 ml. of 0.1 M-diethylamine followed by 300 ml. of aq. 0.2 N-NH₃. The eluate was collected in six 100 ml. fractions in silicone-treated flasks, each containing 0.8 ml. of acetic acid. These fractions were evaporated to dryness in a rotary evaporator at a bath temperature of 40° and subsequently kept under high vacuum at 45° until the ammonium salts had completely volatilized. The residues were weighed and the pressor substance contained therein was assayed in the nephrectomized rat (Table 1).

For the further experiments the most highly concentrated fraction (no. 2) was used. The powder was dissolved at 40° in 0.4 ml. of 0.5 N-acetic acid; a small amount of insoluble material was removed by centrifugation and the supernatant used as stock solution for the chromatographic and electrophoretic separation. A 10 μ l. portion of the stock solution contained approx. 600 m μ g. of pressor material.

Paper chromatography. Four 10 μ l. samples of stock solution were spotted at 1 cm. intervals on Whatman no. 1 paper sheets (25 cm. $\times$ 46 cm.). On either side of the central area a comparative solution containing 20 μ g. each of synthetic Val-5-angiotensin I (decapeptide) and Val-5-angiotensin II (octapeptide) was applied (see Figs. 3 and 4). The distance of 4 cm. separating the comparative samples from the 7 cm.-wide central area ensured that none of the

reference substances could diffuse into the central area, thus falsifying the evaluation. Descending chromatograms were run in the basic system no. 45 (butan-2-ol-aq. 3% NH₃; 25:11, v/v) and in the acid system no. 54 (butan-2-ol-propan-2-ol-monochloroacetic acid-water; 70:10:3:40, v/v/w/v). The running distance was 29 cm. Areas 7 cm. wide were cut from the centre of the dried chromatograms, and the lateral parts were stained with Pauly's reagent to determine the positions of Val-5-angiotensins I and II. The middle areas were divided horizontally into strips 1 cm. wide, one end of which was cut to a point and the other to a 'tongue' shape 8 mm. long and 6 mm. wide. These ends were used as wicks for the elution, being bent backwards and dipped into micro test tubes (6 mm. inner diam.) that were filled with 10% (v/v) acetic acid. The solvent was then run through for 2 hr., by which time about 0.5 ml. had collected in each of the silicone-treated flasks. These eluates were evaporated to dryness under reduced pressure at 50° and subsequently dissolved in 0.9% NaCl and assayed in the usual way in the nephrectomized rat (Gross *et al.* 1965).

Paper electrophoresis. As for the paper chromatography, four 10 μ l. samples of stock solution were placed on a Whatman no. 1 paper sheet (31 cm. $\times$ 44 cm.), and solutions of Val-5-angiotensins I and II were applied on both sides for comparison (see Fig. 5). In acetic acid-ammonium acetate buffer, pH 4.0 (11. contained 28.6 ml. of acetic acid and 7.7 g. of ammonium acetate), 1000 v was applied for 3 hr. with an electrode distance of 43 cm. The resulting electrophoretogram was divided and then assayed in the same way as the paper chromatograms.

RESULTS

The bioassay of the renin-like substance isolated from the placental extract carried out in the nephrectomized rat according to the standard procedure (Gross *et al.* 1965) reveals a blood-pressure curve that is characteristic of renin of renal origin (Fig. 1). The extract was compared with known rabbit renin in the nephrectomized rat in which both the renin and the unknown substance gave the same blood-pressure response. Further, the substance

Table 1. Angiotensin-like pressor activity of various fractions from Dowex 50W (X2) chromatography of the incubation mixture of renin substrate from ox serum and placental extract

Pressor activity is given in terms of the equi-active weight of Val-5-angiotensin II-Asp-1- β -amide.

Fraction no.	Weight (mg.)	Pressor activity	
		(m μ g. total)	(m μ g./mg.)
1	125	13 600	110
2	27	24 000	890
3	16	5 400	310
4	20	2 800	150
5	10	3 000	300
6	5	2 800	600

Fig. 1. Renin-like effect of placental extract on blood pressure in a nephrectomized rat under urethane anaesthesia. A, Various doses (in m μ g.) of Val-5-angiotensin II-Asp-1- β -amide; R, renin-like substance (0.6 mg./0.1 ml.) from rabbit placenta. The blood-pressure response to the two doses of angiotensin after the injection of renin-like substance corresponds in its maximal height to the response to the same doses given before injection of placental extract.

displays another feature typical of renin in that injections of angiotensin given during the prolonged elevation of blood pressure after administration of renin produce a smaller pressor effect than when given before, but elicit exactly the same maximum response. This phenomenon was described by Gross, Bock & Turrian (1961) for angiotensin II-amide injected before and during the action of hog renin in nephrectomized rats.

The pressor effect exerted by the incubation mixture of renin-like substance of placental origin with substrate from ox serum is very similar to that seen when angiotensin II-amide is tested in the nephrectomized rat (Fig. 2). The results of these two assays therefore confirm the previous findings that a renin-like substance is present in rabbit placenta which, by incubation with substrate, forms a substance with angiotensin-like activity (Gross *et al.* 1964).

The evaluation of the paper chromatogram in solvent system no. 45 is shown in Fig. 3. The synthetic reference compounds Val-5-angiotensins I and II are well separated from each other, with mean R_F values 0.46 and 0.31 respectively. The assay of the pressor activity in the middle area containing the unknown substance also revealed two maxima at the same position as Val-5-angiotensin I (strips 12–14, in a total of approx. 300 m μ g.; the maximum pressor activity of the unknown substance is shifted about 1 cm. towards a lower R_F value as compared with the reference substance, in agreement with the concave shape of the line formed by the four reference spots) and Val-5-angiotensin II (strips 9 and 10, in a total of 100 m μ g.). The strips from the middle area for which no values are indicated contained no demonstrable quantity of pressor substance. A small amount of an unidentified substance with pressor activity was found on strip 19.

The result of the chromatography in system

Fig. 2. Angiotensin-like activity of the incubation mixture of renin-like substance from rabbit placenta and renin substrate from ox serum in a nephrectomized rat under urethane anaesthesia. A, Various doses (in m μ g.) of Val-5-angiotensin II-Asp-1- β -amide; T, incubation mixture (0.1 ml.).

no. 54 is given in Fig. 4. Here no separation of Val-5-angiotensins I and II can be obtained, the R_F values for both substances being about 0.70. In accordance with this finding, the evaluation of the pressor activity in the central area revealed only one maximum in strip 20.

In the electrophoresis experiment at pH 4.0 (Fig. 5) Val-5-angiotensins I and II were separated again. Under the experimental conditions chosen, the reference substances migrated towards the cathode,

Fig. 3. Paper chromatography of reference mixture of Val-5-angiotensins I and II and of the incubation product (fraction 2) in solvent system no. 45. A, Starting points of the reference mixture containing Val-5-angiotensins I and II; B, starting points of the incubation product, fraction no. 2; I and II, spots of Val-5-angiotensins I and II respectively, after development with Pauly's reagent. The numbers on the middle strips indicate the pressor activity (in m μ g.) found on elution.

Fig. 4. Paper chromatography of reference mixture of Val-5-angiotensins I and II and of the incubation product (fraction 2) in solvent system no. 54 (for details see Fig. 3).

Fig. 5. Paper electrophoresis of reference mixture of Val-5-angiotensins I and II and of the incubation product (fraction 2) at pH 4.0 for 3 hr. at 23 v/cm. (for details see Fig. 3).

with an average of 15 and 12 cm. respectively. From the position of the eight spots of the reference samples stained with Pauly's reagent it is evident that the reproducibility of the migration distance is somewhat less good than with the paper chromatography. Allowing for this fact, the maxima of the reference substances and of the unknown substance in the central area corresponded quite well in the electrophoresis experiment also (nos. 15-18 correspond to Val-5-angiotensin I, total approx. 1800 $\mu\text{g.}$; nos. 10-12 correspond to Val-5-angiotensin II, total approx. 230 $\mu\text{g.}$).

The incubation mixture therefore contains two substances that behave in these three different systems similarly to synthetic Val-5-angiotensins I and II.

DISCUSSION

Extracts of maternal parts of rabbit placenta contain a renin-like principle that increases blood pressure in the nephrectomized rat in the same way as renin of renal origin and forms, by incubation with substrate from ox serum, a substance with a pressor activity similar to that of angiotensin. Physicochemical characterization of this angiotensin-like substance by means of chromatography and electrophoresis showed that the angiotensin-like compound behaves very similarly to a mixture of Val-5-angiotensins I and II. The two chromatographic systems contrast in the sense that in one (no. 45) angiotensins I and II run differently, whereas in the other (no. 54) they have the same R_f value. While in the first system the incubation mixture has two pressor maxima and the activity spreads out over a relatively large area, in the second system it is more concentrated and has a sharp maximum at the place where the two angio-

tensins migrate. The activity found on strip no. 19 of system no. 45 could represent a degradation product of the original substance that still has some pressor activity.

The two methods that separate angiotensins I and II reveal a striking difference in the pressor activity ratio between the substance similar to angiotensin I and that similar to angiotensin II. Whereas paper chromatography in system no. 45 gives a 3:1 ratio, the corresponding ratio obtained by electrophoresis is 8:1. A possible explanation for this discrepancy could be a variable degree of completeness of elution from the paper strips, since such extremely small amounts of a peptide could easily be absorbed partially on the cellulose and released rather slowly to the elution solvent. The different composition of the solvents and buffers used in the two methods could favour such a variable degree of elution. Probably for the same reason the total amounts of pressor activity eluted from the two chromatographic experiments and from the electrophoresis also differ considerably. In addition, the ratio of angiotensins I and II found for fraction no. 2 of the Dowex column would not necessarily indicate the true value for the crude incubation mixture, since the two peptides could behave differently on elution from the column.

The results obtained can be interpreted as an indication that the substance which is formed by the renin-like principle extracted from rabbit placenta with substrate from ox serum is angiotensin I that is partly converted into angiotensin II by a converting enzyme present probably in the fraction containing the renin-like material. The result is a mixture of angiotensins I and II, possibly also with some degradation products that may have a residual pressor activity. Since the electrophoretic and chromatographic experiments provide strong evidence that the end product is angiotensin, it may also be assumed that the enzyme present in the placenta is either closely related to, or identical with, renin. Since no pure renin of renal origin is available so far, it is not possible to prove the identity of an enzyme with similar properties but from a different source than the kidney. It is therefore preferable to refer, for the time being, to a renin-like substance of placental origin.

The role played by the renin-angiotensin system present in the uterus and in the placenta and the way in which it is correlated with the corresponding factors of renal origin remain to be elucidated. The heat-stable pressor substance hysteronin formed in man by the action of a renin-like enzyme of decidual origin on a substrate in the amniotic fluid or plasma was claimed not to be identical with angiotensin (Hunter & Howard, 1960, 1961). Their findings, however, have not been confirmed in recent investigations (Gomel &

Hardwick, 1966). Our results as well as those of Brown *et al.* (1964), who found a renin-like substance in the human amniotic fluid, provide evidence that the renin-angiotensin system may not be confined to the kidney. Nothing, however, can be said about its role in normal pregnancy or in toxæmia of pregnancy.

The authors are indebted to Dr D. F. Elliott for his most valuable advice in preparing this manuscript.

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