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Distribution and Disappearance of Exogenous [^{125}I] Big Renin in the Newborn Puppy

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Summary

The distribution and metabolism of infused exogenous [^{125}I] inactive plasma big renin, molecular weight 56,000, was studied in five newborn puppies. The animals were sacrificed and the organs removed and studied by chromatography, along with periodic blood samples taken during the 120-min study, for evidence of conversion of high-molecular-weight renin to low-molecular-weight renin. The decay curve suggested an initial rapid distribution (alpha) phase of 10 ± 1.5 min followed by a slower elimination (beta) phase of 40 ± 4.6 min. [^{125}I]-Big renin was taken up by the red blood cell and released slowly. The liver, kidneys, and lungs had the highest % of [^{125}I]-big renin at the termination of the study. There was no chromatographic evidence of a change in molecular weight of the [^{125}I]-big renin. These data show that big renin has a two-compartment disappearance curve and that there is no evidence of conversion of high-molecular-weight renin to low-molecular-weight renin systemically or in the tissues of the newborn canine puppy.

Abbreviations

CPM, counts per minute
MW, molecular weight
PRA, plasma renin activity
PRC, plasma renin concentration

Inactive renin of higher-molecular-weight, 56,000 daltons, is a normal constituent of human plasma. There is evidence to suggest that plasma inactive renin is a mixture of renin zymogen and active renin of similar weight (8). The lower-molecular-weight renin, 40,000 daltons, is found only rarely in human plasma of exceptionally high renin activity. Other evidence (10) leads to the idea that inactive renin is not a renin proenzyme, but an inhibitor-bound form of active renin. Plasma has 40–70% of its renin activity in the form of inactive renin (3, 11), whereas the proportion of inactive to active renin in hog, rat, and human kidneys (14) is less than 1%.

The purpose of the present study was to determine the metabolic clearance rate and organ uptake of exogenous [^{125}I]-inactive plasma big renin and to document whether high-molecular-renin was changed to lower-molecular-weight renin systemically or in the tissues of the newborn canine puppy.

MATERIALS AND METHODS

Inactive renin was purified from two sources: (1) the discarded blood from a newborn exchange transfusion and (2) human placental cord blood after birth. Human subjects approval was obtained. The inactive renin obtained from both sources had a molecular weight of 56,000 daltons, produced similar renin activity, and had similar Km.

The purification procedure for inactive renin consisted of four steps: (1) ammonium sulfate-fractionation; (2) gel filtration; (3) ion exchange chromatography; and (4) affinity chromatography, modified from Inagami and Murakami (9). All buffers used in the purification were prepared to contain 2 mM dipotassium ethylenediamine tetra-acetate, 5 mM *N*-ethylmaleimide, 5 mM O-phenahthroline, and 5 KIU/ml trasylol.

For ammonium sulfate-fractionation, the plasma is diluted 1:20 at 22°C. The plasma is adusted to 25% (w/v) with $(NH_4)_2SO_4$ for 2 h and separated by centrifugation. $(NH_4)_2SO_4$ is added to the supernatant to adjust to 55% (w/v) for 2 h and centrifuged. The precipitate is dissolved in one-fourth the original volume of plasma of 0.025 M sodium phosphate buffer, pH 7.0.

For gel filtration, the dissolved precipitate from salt fractionation is applied to a column of G-75 Pharmacia or Ultra Gel 44 (LKB) (2.5 x 140 cm) equilibrated with 0.025 sodium phosphate buffer pH 7.0 at 22°C, at a flow rate of 10–15 ml/h. Protein concentration is determined at 280 nm. PRC is determined by the radioimmunoassay method of Haber *et al.* (5, 13). Inactive renin is determined as PRC after trypsin activation (22). Sheep anephric serum is used as the angiotensinogen (renin substrate) (21). All fractions containing PRC are combined.

For ion exchange chromatography, the combined fraction from the gel chromatography step is applied to a DEAE cellulose column (5 x 40 cm). The active and inactive renins are eluted with a continuous gradient from 0.025 M sodium phosphate buffer pH 7.0 to 0.4 M sodium phosphate, 1.0 M NaCl buffer pH 7.0 at a flow rate of 30 ml/h at 22°C.

For affinity chromatography, a Spectra por stirred cell 76 mm with an anisotropic membrane is used with a molecular weight cut off of 20,000. Using nitrogen gas, at 22°C and 58 psi, there is a resulting flow rate of 40 ml/min. The volume is reduced and pumped onto a Sepharose-aminobutyl-pepstatin column (0.9 x 20 cm) at 22°C at a flow rate of 60 ml/h (9). The column was previously equilibrated with a 0.02 M sodium phosphate buffer, pH 6.0. The column is washed with 0.02 M sodium phosphate buffer pH 6.0 at 22°C, 1.0 M sodium phosphate buffer, 0.1 M NaCl, pH 6.0, 1.0 M sodium acetate, 0.2 g% glycerol buffer pH 4.8. The inactive renin is eluted with 0.02 M tris HCL, 2.0 M urea buffer pH 7.4. Ten-ml fractions are collected.

In Table 1, the final degree of purification was 17,107 [specific activity (fraction/specific activity of plasma)]. In Figure 1, the sodium dodecyl sulfate polyacrylamide gel electrophoresis shows the inactive renin band formed from the pepstatin agarose chromatography inactive renin peak. The gel was 8% acrylamide, 3% bisacrylamide run at 2 mA in 0.034 M sodium phosphate buffer, pH 7.0, 100 mg% urea.

The molecular weight, 56,000 daltons, was determined by Sephadex G-75 medium chromatography with the protein markers: myoglobin, 17,800 MW, alpha chymotrypsinogen, 25,000 MW, ovalbumin, 45,000 MW, and albumin, 67,000 MW. The eluting buffer was 0.1 M sodium phosphate buffer pH 6.9, 30 mg% *o*-phenanthroline, 0.9 g% sodium chloride.

Approximately 100 ng of purified inactive plasma big renin is labeled with [^{125}I] by the Chloramine-T method of Hunter and Greenwood (7). The iodinated big renin is purified by ACA-44 chromatography and its biologic activity assessed. The labeled big

Table 1. *Purification of inactive renin from newborn cord blood*

Fraction	Total activity big renin (ng/ml/h)	Total protein (mg)	Specific activity[1] (mg protein)	Degree of purification[2]	% Yield[3]
Plasma	4000	6500	0.615	1	100
$(NH_4)SO_4$ 25–55%	5200	4150	1.253	2.037	130
ACA 44 gel filtration	3840	24.72	155.339	252.6	96
DEAE sephecel	1114	1.3	856.92	1391.86	27
Agarose-pepstatin affinity	505	0.048	10520	17107	12.6

[1] Specific activity, total inactive renin activity/total protein (mg).
[2] Degree of Purification, specific activity (fraction)/specific activity (plasma).
[3] % yield, total activity (fraction)/total activity (plasma).

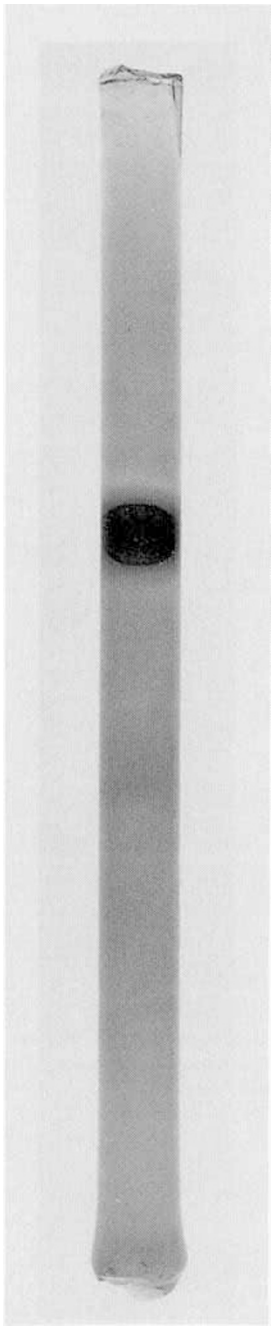

Fig. 1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis shows the inactive renin band formed from the pepstatin agarose chromatography inactive renin peak.

renin is activated using trypsin in the presence of big renin/renin-free substrate as described (8). The activated big renin is then incubated at an optimal pH in the presence of β-mercaptoethanol, 8-OH-quinoline, *o*-phenanthroline, and KEDTA at 37°C for 18 h for generation of angiotensin 1. (Studies in our laboratory have shown that the generation of angiotensin 1 by activated big renin in the presence of excess substrate is linear for 18 h). The generated angiotensin 1 is separated from the iodinated big renin on Dowex-resin. The biologic activity of exogenous big renin is assessed by its ability to generate angiotensin 1 and raise blood pressure *in vivo* in the canine puppy.

Five newborn puppies, 3–4 wk of age, 1.74 ± 0.17 kg weight (mean and S.E.) were studied. Femoral artery and vein and bladder catheters were placed 3 days before study and the puppies were returned to their mothers in the vivarium. The animals were studied unaesthetized and unstressed in a hammock apparatus.

Experiment. After two baseline samples for background CPM, sodium dodecyl sulfate polyacrylamide gel electrophoresis and PRA, 10 μg of [^{125}I]-inactive big renin was infused over 1 min. Blood samples were drawn at 2, 5, 15, 30, 60, and 120 min postinfusion. After the last blood sample, the animal was sacrificed and the organs removed. Each organ was finely minced, washed free of blood with saline over coarse filter paper. The organ was weighed, homogenized, and centrifuged at 19,000 revolutions per min, red cell fraction 40,000 at 4°C for 20 min. One ml was aliquoted for analysis by chromatography. Chromatography was performed on one ml of each plasma sample and the organ extract on two 10 x 100 mm columns. The first column was prepared with ACA-44 in the descending mode and the second is fitted with reverse flow adapters and filled with ACA-54. The column system is equilibrated with 200 ml of the buffer, calibrated against albumin, pepsin, trypsinogen, lactoglobulin, and blue dextran as molecular weight markers, and further calibrated against a plasma containing a known high activity of high (56,000 MW) and low 40,000 MW) renin. Retentions are calculated in reference to the blue dextran peak.

Big renin pharmokinetics were assessed by plotting plasma concentration of big renin *versus* time on semi-log paper and using single and multiple-exponential curve-fitting (12, 15). The half-life of elimination was determined from the equation:

$$t_{1/2} = \frac{0.693}{\text{alpha}} \qquad t_{1/2} = \frac{0.693}{\text{beta}}$$

where beta (B) is the first order constant of the elimination (B) phase. The clearance rate was calculated from the product of the apparent volume of distribution × (B). The "area" under the plasma concentration/time curve was calculated from time zero to infinity after a single dose. The disappearance curve expressed as the sum of two exponentials on the semilogarithm graph is described by the following general equation: $C(t) = Ae^{-at} + Be^{-bt}$, where C(t) is the plasma big renin at time t, A, and B are the Y intercepts of the straight line of the fast and slow components,

respectively, and alpha(a) and beta(b) are their slopes. Total CPM were their slopes. Total CPM were determined for whole blood, plasma, and red blood cell, plus the hematocrit for each sample.

RESULTS

To test whether the [^{125}I]-big renin was activated by the iodination procedure, trypsin-activation (10) was performed on the labeled big renin and chromatography performed on two 10 x 100 mm columns (Fig. 2). The first column was prepared with ACA-44 in the descending mode and the second is fitted with reverse flow adapters and filled with ACA-54. The column system is equilibrated with 200 ml of 0.025 sodium phosphate buffer, pH 7.0 at 22°C. It is calibrated against albumin, pepsin, trypsinogen, lactoglobulin, and blue dextran as molecular weight markers, and further calibrated against a plasma containing a known activity of high (56,000 MW) and low (40,000 MW) renins. The trypsin-activated [^{125}I]-big renin was eluted in a separate but partially overlapping fraction directly adjacent to the non-trypsin activated high-molecular-weight (56,000 dalton) [^{125}]-renin.

Fig. 3 shows the two-compartment distribution and degradation curve for [^{125}I]-big renin in whole blood in the five newborn puppies. The decay curve suggested an initial rapid distribution (alpha) phase of 10 ± 1.5 min followed by a slower elimination (beta) phase of 40 ± 4.6 min (mean and S.E.).

Fig. 4 shows the 2-min and 60-min blood samples of [^{125}I]-big renin after infusion in the newborn puppy, on the same two column ACA-44, ACA-54 system as Fig. 2. The 2- and 60-min samples were eluted in the same fractions as (inactive-activated) big renin (Fig. 2). The pattern was similar for all tissues tested. The concentration of counts were present in the fraction containing renin, and therefore represent the distribution of labeled renin.

Table 2 shows the red blood cell uptake of [^{125}I]-big renin. It was slowly released from the red blood cell over the 120-min study period. Because the concentration of counts were present in the chromatographic fraction containing renin, the labeled material taken up by the red blood cells represented renin.

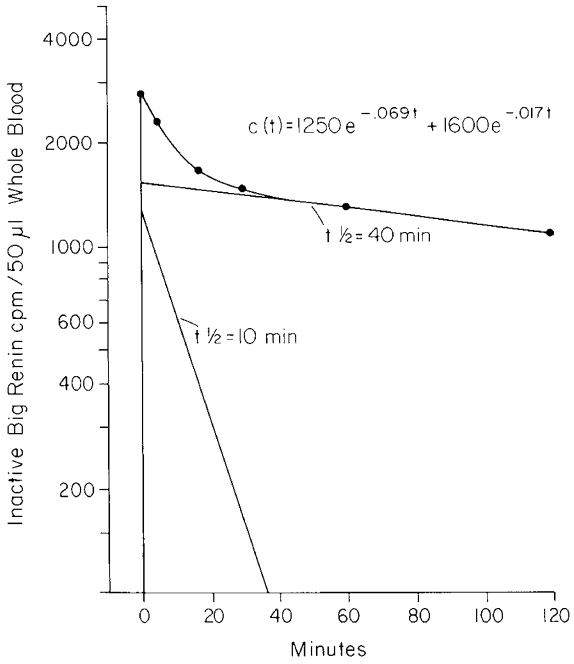

Fig. 3. The two-compartment curve for [^{125}I] big renin in whole blood in the newborn puppy shows an initial rapid distribution (alpha) phase of 10 min followed by a slower elimination (beta) phase of 40 min.

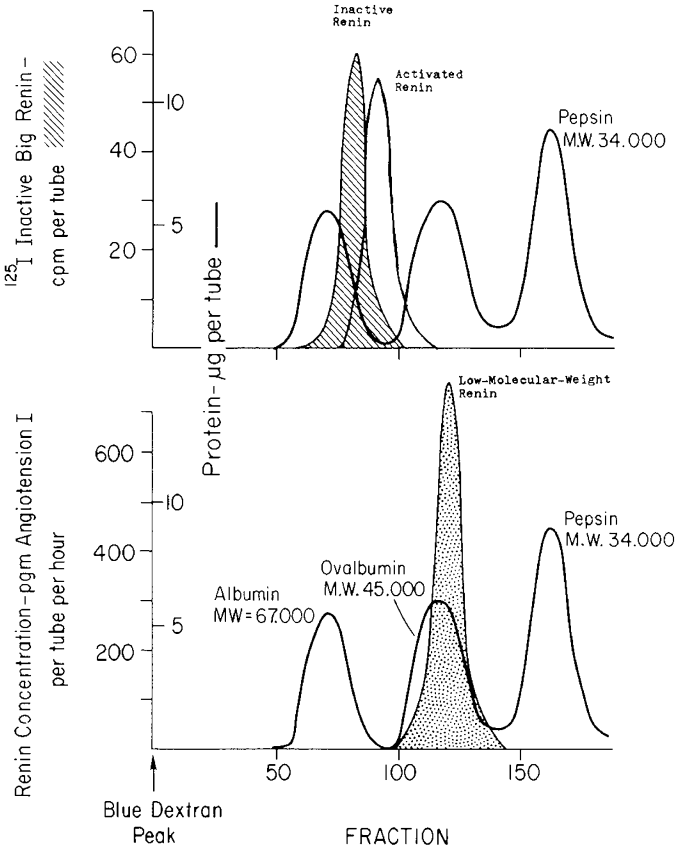

Fig. 2. Chromatography of labeled [^{125}I] inactive big renin and trypsin activated labeled [^{125}I] inactive renin on a gel filtration (ACA-44) column connected to a (ACA-54) column. Big renin (inactive and activated) elutes in a separate fraction from lower-molecular-weight renin.

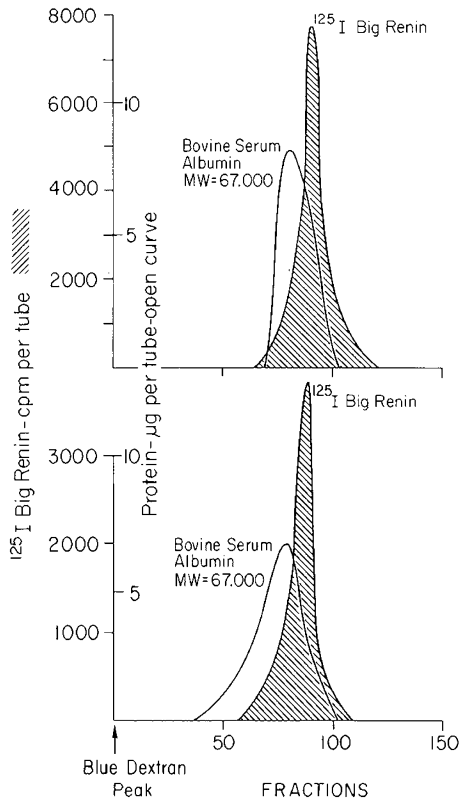

Fig. 4. Chromatography of the [^{125}I] big renin blood samples, 2 and 60 min after infusion on the same gel filtration columns as Figure 1. Elution occurs in the high-molecular-weight fractions.

Table 2. *Red blood cell (RBC) uptake of [^{125}I] big renin in the newborn puppy*

Time after injection (min)	Total counts per min bound by the RBC[1]				
	MPA[2]	MPB	MPC	MPD	MPE
0 (baseline)	0	0	0	0	0
2	45,213	54,175	19,170	37,812	14,168
5	4358	35,728	16,425	25,779	10,492
15	3781	28,614	12,337	17,837	7759
30	3491	22,029	11,826	14,034	6780
60	3510	15,488	11,005	16,358	2399
120	894	13,439	9301	14,056	7646

[1] $\frac{\text{Total counts injected}}{\text{min}} = 28\text{–}29{,}000{,}000$

[2] MPA, MPB, MPC, MOD, and MPE refer to puppies.

Table 3. *Organ uptake of [^{125}I] big renin in the newborn puppy*

Organs	% of total count injected bound per organ × 100[1]				
	MPA[2]	MPB	MPC	MPD	MPE
Heart	8.53	10.63	10.11	9.1	9.7
Brain	8.28	10.97	10.22	9.4	9.6
Liver	71.76	144.89	126.44	94.3	110.4
Spleen	7.32	5.26	5.0	6.2	6.5
Left kidney	18.78	20.36	20.1	19.4	19.3
Right kidney	12.91	12.39	12.0	12.2	13.1
Left adrenal	0.06	0.09	0.08	0.09	0.08
Right adrenal	0.05	0.09	0.08	0.07	0.08
Pancreas	0.67	0.92	0.87	0.75	0.88
Lungs	21.05	28.8	27.4	26.4	28.1
Aorta	0.45	0.63	0.60	0.58	0.61
Thigh muscle	0.04	0.03	0.03	0.03	0.04

[1] $\frac{\text{Total counts injected}}{\text{min}} = 28\text{–}29{,}000{,}000$

[2] MPA, MPB, MPC, MPD, and MPE refer to puppies.

Table 3 shows the organ uptake of [^{125}I]-big renin in the newborn puppy. The liver, kidneys, and lungs, have the greatest % of the total counts injected. There was no chromatographic evidence of conversion of high-molecular-weight renin to lower-molecular-weight renin in any of the organ extracts.

DISCUSSION

Evidence suggests that plasma inactive renin is a mixture of renin zymogen and active renin of similar weight (8). In Fig. 2, trypsin activated [^{125}I]-inactive renin eluted in a directly adjacent fraction that was partially overlapping on chromatography.

The clearance curve (Fig. 3) in the whole blood of newborn puppies, 21–28 days of age, may represent the disappearance of an (inactive-active) mixture of plasma [^{125}I]-high-molecular-weight renin. Whether the mixture was predominantly inactive or active cannot be ascertained. Plasma has 40–70% of its renin activity in the form of inactive renin (3, 11) whereas the proportion of inactive to active renin in hog, rat, and human kidneys (14), is less than 1%. The disappearance curve of [^{125}I]-big renin (Fig. 3) is represented as the sum of two exponentials, suggesting that the whole blood consists of at least two compartments, a plasma and a non-plasma compartment. The initial fast component had a half-life of 10 ± 1.5 min and the slow component had a half-life of 40 ± 4.6 min. The rapid component probably represents big renin distribution from plasma into a non-plasma compartment, whereas the slow component represents the metabolic degradation of big renin (6). The disappearance of endogenous active renin in nephrectomized newborn piglets (16) was 17 ± 3 at 1–5 days of age, and 12.9 ± 0.9 min at 45–50 days of age (statistically similar).

Table 3 shows that the highest % of [^{125}I]-big renin was bound in the liver, kidneys, and lungs at the termination of the 120-min study. Whether these organs are the major sites of inactive renin activation and metabolism, or conversion to angiotensin 11 remains speculation.

To date, there has been no published evidence that high-molecular-weight plasma renin is converted to low-molecular-weight renin; or low-molecular-weight plasma renin to high-molecular-weight renin *in vivo*, in the fetus, newborn, or adult. In this study (Figs. 2 and 4), there was no evidence of *in vivo* conversion of exogenous plasma big renin, 56,000 MW, to low-molecular-weight renin systemically or in renal and non-renal tissue extracts in the newborn canine puppy.

The reasons for the higher % of inactive renin/active renin in the plasma needs further study. Inactive renin, in association or dissociation with active renin, reacts to falls in blood pressure (1), adrenergic system regulation (1, 4), angiotensin 11, and sodium balance (1, 18), in the adult. Both inactive and active renin levels are much higher in the fetal and newborn lamb and human (19, 20) than in the adult. Under basal conditions there is a negative angiotensin 11-inactive renin, and not an angiotensin 11-active renin reflex arc in the fetal lamb (20); and inactive renin shows a greater response to angiotensin 11 (20).

Bailie (2) showed that the release of inactive renin from the newborn pig kidney parallels the release of active renin and found no evidence that inactive renin was converted to active renin *in vivo*. Our laboratory has evidence for activation of inactive plasma renin *in vivo* (unpublished) after infusion of exogenous inactive renin in the newborn canine puppy; and Rumpf *et al.* (17) showed that the % of active renin in human plasma was correlated with the level of plasma kallikrein.

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