Investigations of Components of the Renin-Angiotensin System in Rat Vascular Tissue

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SUMMARY  Investigations were performed on components of the renin-angiotensin system (RAS) in homogenate extracts of vascular tissue and aortic smooth muscle cells cultivated in vitro. Determinations of isoelectric points and pH optima indicated the existence in aortic homogenate extracts of two local angiotensin I (AI)-forming enzymes (AIFE) that were different from those of plasma, renal cortex, veins, and aortic smooth muscle cells. The pH optima for AI-converting enzyme (ACE) from vascular tissues, aortic smooth muscle cells, and plasma were in the same range (pH 8.0–8.5), and in agreement with those measured previously in other tissues. In contrast, in vitro studies with the ACE inhibitors MK-421 and MK-422 and measurement of isoelectric points suggested that aortic ACE was different from the plasma enzyme. AIFE and ACE activities were found to be elevated in spontaneously hypertensive rats (SHR). The biochemical characteristics of the enzymes investigated in the vascular tissue of SHR were not different from those of the normotensive controls. AI- and AII-degrading enzymes were found both in aortic tissue and in aortic smooth muscle cells. One potent AI-degrading enzyme different from ACE was observed in aortic tissue. A high ratio of AI/AII immunoreactivities in arterial walls suggests the availability of renin substrate, and that AI-degrading enzymes are the rate-limiting enzymes for AII formation. The results further support the concept of an intrinsic vascular RAS. (Hypertension 6: 383–390, 1984)

KEY WORDS  • renin • angiotensin-I-converting enzyme • angiotensinases • rat aorta • rat vena cava

The present investigation continues our previous studies in this field and was undertaken to recognize, quantify, and partly characterize those components of the vascular RAS likely to be involved in local angiotensin II (AII) formation. We have also studied venous tissue, mainly to find out if there exists an additional intrinsic venous RAS, which may be important for the hemodynamics of capacitance vessels. Furthermore, it was our intention to investigate the intracellular RAS of aortic smooth muscle cells cultivated in vitro, since intracellular AII formation in juxtaglomerular cells has already been postulated. To demonstrate a physiological or pathophysiological role of the vascular RAS, the occurrence and behavior of AII activity at the receptor site of vascular smooth muscle cells should be investigated under varying conditions. However, at this point, such quantitative measurements are practically impossible; therefore, we studied the enzymatic activities and angiotensin I (AI) and AII immunoreactivities involved in local AII formation.

Methods

Preparation of Aortic and Other Homogenate Extracts

Male and female Wistar rats and spontaneously hypertensive rats (SHR) of the Kyoto strain that weighed 190 to 220 g were anesthetized with Inactin (Byk-Gulden, Konstanz, West Germany, 110 mg/kg b.w. i.p.). The aortas were removed, immediately cut into 0.5 mm pieces, and transferred into 0.9% saline.
Homogenization was performed in two steps with a Potter-Elvehjem homogenizer (Braun, Melsungen, West Germany) (3 × 1 minutes with 1-minute intervals) and an MSE ultrasonic disintegrator 100 W (Measuring and Scientific Equipment Ltd., Crawley, Sussex, England) (9 × 2 seconds with 1-second intervals). The homogenate was centrifuged for 3 minutes in an Eppendorf centrifuge 3200 (Hamburg, West Germany) (25,000 g), and the supernatant used. All steps were performed at below 4° C. These conditions were found to be optimal in previously performed systematic studies. Homogenates of the vena cava were prepared in the same way. Those of other structures (kidney cortex, pulmonary, and testicular tissues) were submitted to a similar treatment but with an ultrasonic disintegration of 15 × 2 seconds and 1-second intervals.

Cell Culture Technique
The isolation and growth of aortic smooth muscle cells (5th to 6th generation) in vitro have been described in detail previously. Cultures with 60 × 10^6 to 80 × 10^6 cells per ml 0.9% saline were ultrasonically disintegrated 3 × 2 seconds and 1-second intervals.

Isoelectric Focusing
To determine isoelectric points (pI), a LKB 8100-1 column (LKB Produkter AB, Bromma, Sweden), LKB Ampholine and glycerol gradients, in which the tissue extracts were evenly distributed, were used according to the instructions of the manufacturer. Columns were focused for at least 15 hours at 400 V. Fractions of 2 to 3 ml were eluted at a flow rate of 1 ml/min. Following determinations of pH, all fractions were dialyzed against cold distilled water for 30 to 33 hours, lyophilized, and redissolved in 0.7 ml and 0.9% saline.

Estimation of Molecular Weights
Sephadex gels G-100 or G-200, 0.9% saline as solvent, and high or low molecular weight calibration kits (provided by Pharmacia Fine Chemicals, Uppsala, Sweden) were applied.

Angiotensin I Radioimmunoassay (RIA)
The samples to be assayed were pipetted into 0.15 M phosphate buffer, pH 7.4, containing 0.1% bovine gamma-globulin (RIA buffer), so that the total volume was 0.5 ml in all cases. Immunoreactivities were determined according to the method described previously to all samples were added 0.05 ml of labeled AI (NEN, Boston, Massachusetts, specific activity about 1800 μCi/μg) and All antibodies (raised in our laboratory) were used.

Measurement of Angiotensin I and Angiotensin II in Tissue Samples
Between 220 and 260 mg of aortic tissue was homogenized in 2.2 to 2.6 ml 100% ethanol containing an inhibitor solution 1:10 (composition of the inhibitor solution: 0.25 M EDTA, 0.05 M o-phenanthroline, 0.378 M phenylmethane-sulfonyl fluoride [PMSF], and 0.2% neomycin sulfate) according to the homogenization procedure described above. Two homogenate samples of 1.0 ml each were transferred into 6 ml 100% ethanol, and the peptides were extracted as described previously. One sample was used for the measurement of AI, the other for that of All. The peptide concentrations were determined by radioimmunoassay. In some cases, thin-layer chromatography (silica gel plates 60 F^254, Merck, Darmstadt, West Germany) was performed following the alcoholic extraction in order to determine the amounts of angiotensin within the immunoreactive material. The AI antibodies used did not cross-react either with 10,000 ng angiotensinogen or with 1000 pg All, heptapeptide, hexapeptide, pentapeptide, or tetrapeptide. The All antibodies did not cross-react with 10,000 ng angiotensinogen or with 1000 pg AI, pentapeptide, or tetrapeptide. Cross-reaction was observed with 1000 pg of the All degradation products heptapeptide and hexapeptide. Therefore, the angiotensin values, in particular those of All, were expressed as "angiotensin immunoreactivities."

Determination of Angiotensin-I-Forming Enzyme (AIFE) Activity
Rat renin substrate of (24-hour) bilaterally nephrectomized rats was purified by means of fractionated ammonium sulfate precipitation, followed by Sephadex gel filtration with gel G-75. The renin substrate preparation was dissolved in 0.15 M phosphate buffer of the desired pH, with the components NaH_2PO_4 and NaH_2PO_4 for the pH range between 4.5 and 9.0, or NaH_2PO_4 and phosphoric acid for lower pH values. The substrate concentration (60,000 ng/ml) was in the saturation range for the small enzymatic activities measured.

In 1.5 ml test tubes (Eppendorf, Hamburg, West Germany), 0.05 ml sample was added to 0.05 ml renin substrate solution and 0.01 ml inhibitor solution (79.87 mM 2,3-dimercaptoopropanol-1, 53.73 mM EDTA and 169.93 mM 8-oxyquinoline sulfate). After adjustment of pH, the mixture was incubated for 20 minutes at 37° C. In some cases, reaction kinetic determinations were performed, and linearity of AI formation demonstrated. The incubation was stopped by chilling the test tubes in ice; 0.05 ml was transferred into 0.45 ml RIA buffer, and the activities were measured by RIA.
Determination of Angiotensin-I- and Angiotensin-II-Degrading Enzyme Activities

To 0.4 ml RIA buffer containing 1000 pg/ml AI or All (both from Beckman, Geneva, Switzerland), 0.1 ml of sample was added. For the blanks, 0.1 ml saline was taken instead. After mixing, all samples were incubated for 20 to 40 minutes at 37°C. In some cases, reaction kinetic determinations were performed. The incubation was stopped by chilling in ice, and the activities were measured by RIA. For the determination of pH optima, 0.15 M phosphate buffers were prepared as described above. To 0.5 ml of each buffer, 0.05 ml AI (All) (10 ng/ml saline) was added, and then 0.1 ml of sample (or saline for the blanks) was added. After incubation, 0.05 ml of the mixture was transferred into another test tube with 0.45 ml RIA buffer. All samples were measured by RIA.

The activities were plotted semilogarithmically against incubation time, and the period of time necessary to reduce the angiotensin concentration to 50% of its initial value (t 1/2) was determined. For each sample the reciprocal of t 1/2 was calculated, which is a measure of the enzymatic activity.

Determination of Angiotensin-Converting-Enzyme Activity

Angiotensin-converting-enzyme (ACE) activity was measured either with a modification of the method of Piquilloud et al., described previously, or with a modification of the method of Cushman and Cheung: 0.1 ml of sample was incubated with 0.1 ml ACE substrate (Hippuryl-His-Leu, Serva Feinbiochemika, Heidelberg, West Germany; 8.4 mM in 0.5 M phosphate buffer, pH 8.3, containing 0.75 M sodium chloride) at 37°C for 60 minutes. The incubation was stopped with 0.2 ml 1 N HCl; 1.5 ml ethyl acetate was added, and the samples were mixed for 10 seconds on a vortex mixer. After centrifugation for 10 minutes at 750 g, 1 ml of the upper layer was transferred into glass test tubes and dried at 120°C for 35 minutes. The residues were redissolved in 2.0 ml 1 M sodium chloride and the hippuric acid concentration measured 15 to 25 minutes later (Zeiss Spectrophotometer PM 4, Aikerkochen, West Germany, 228 nm). The effects of the ACE inhibitors MK-421 (ethyl ester maleate salt of N-[S]-1-[ethoxycarbonyl]-3-phenyl-propyl-Ala-L-Pro, 11 nmol/ml) and MK-422 (diacid form of MK-421, 11 nmol/ml; both from Merck, Sharp and Dohme, West Point, Pennsylvania) were investigated in order to demonstrate the specificity of the data obtained. For this study, the Cushman method was used.

Results

Angiotensin-I-Forming Enzymes

The isoelectric focusing behavior of aortic AIFE is depicted in Figure 1. The highest maximum was found at pH 5.6, another one at pH 1.0. The pi values varied within the ranges of 5.45 to 5.75 and 1.0 to 1.8, respectively (four experiments). For renin from renal cortex, pi 4.8 was determined.

The effect of pH on aortic AIFE activity is shown in Figure 2. For the experiments in the upper panel, extracts from total aortic homogenate were applied, which is the starting material for the isoelectric focusing procedure. In the lower panel, the pH behavior of the fractions containing AIFE with pi 1.0 or 5.6, separated by isoelectric focusing, is demonstrated. The AIFE with pi 5.6 had a pH optimum between 7.5 and 8.0, which was significantly different from that of the enzyme with pi 1.0 (pH optimum below 4.5). An
Figure 3. Column isoelectric focusing of angiotensin I-forming enzymes from aortic homogenate extracts of SHR, Wistar Kyoto strain (---) and of normotensive rats of the same strain (......). pH gradient: 3.5 to 10. Starting material: 190 mg wet tissue for SHR; 605 mg for normotensive rats.

Additional pH optimum at 6.5 was only determined in extracts of total aortic homogenate.

Figure 3 compares the results of the isoelectric focusing of aortic AIFE from SHR with those of normotensive controls. In both cases, identical pI values (5.6) were obtained. However, significantly increased enzymatic activities were observed for the aortas of the hypertensive rats.

In venous tissue, the main isoelectric point for AIFE was determined in the range of 4.25 to 4.35 (n = 2). For AIFE from disintegrated aortic smooth muscle cells, the main pI was found at 6.5 (Figure 4, upper panel); there were also two smaller maxima at pH 2.0 and 5.0. By measuring the effect of pH on AIFE activity from disintegrated aortic smooth muscle cells, two optima were found (Figure 4, lower panel): the main maximum at pH 5.0, and a smaller one in the range between 6.5 and 7.0. When estimating the absolute AIFE activity in aortic smooth muscle cells at pH 5.0, we found values in the range between 100 and 650 ng AI/hr g protein.

Angiotensin-I-Converting Enzyme

The isoelectric point of ACE from aortic homogenate extracts was determined at 4.8. The pI values of four experiments ranged between 4.6 and 5.0 and were similar to those of lung and epididymis. The pI of ACE in extracts from testicular homogenate was measured at 5.4. For ACE from disintegrated aortic smooth muscle cells, isoelectric points were determined between 6.5 and 7.5; a smaller peak was observed at 3.5. For plasma ACE, the isoelectric point was 4.45, close to that of the veins.

The effect of pH on ACE activity in extracts from aortic tissue and aortic smooth muscle cells is illustra-
For plasma ACE, the pH optimum was found in the range between 8.0 and 8.5.

Investigating the effect of MK-422 concentration on aortic ACE activity, we found that 0.3 nmol of ACE blocker per ml incubation volume was required to inhibit 50% of the initial aortic ACE activity (IC_{50}). The same MK-422 concentration blocked 87.5% of the plasma ACE. With MK-421, the IC_{50} values for ACE from lung and aorta were in the same range (IC_{50} = 2 nmol/ml) and differed from renal cortex (IC_{50} = 0.03 nmol/ml) and plasma (IC_{50} = 0.05 nmol/ml).

Angiotensin-I-Degrading Enzymes

Isoelectric focusing of Al-degrading enzymes from aortic tissue revealed two isoelectric points: one between 3.6 and 3.9, the other between 4.2 and 4.7 (Figure 6). The highest enzymatic activity was measured for the enzyme with a pI between 3.6 and 3.9. In further studies, the influence of pH on Al-degrading enzyme activity from aortic tissue was evaluated, using Al as substrate; this was repeated in the presence of the inhibitor MK-421. The pH optimum was found in the range of 7.4 to 7.9. The only measurable inhibition by MK-421 observed was between pH 8.1 and 8.8 (Figure 7).

Angiotensin-II-Degrading Enzymes

The main isoelectric point of aortic AlII-degrading enzymes was determined between pH 4.5 and 5.0; another point was determined between pH 8.0 and 8.25. A pH optimum at 8.1 was observed for the AlII-degrading enzyme from aortic tissue homogenate. The results of an isoelectric focusing experiment of AlII-degrading enzymes from aortic smooth muscle cells are shown in Figure 8, upper panel; the effect of pH on the enzymatic activity is shown in the lower panel.
Earlier results have shown increased amounts of AIFE activity in the aortic tissue of SHR, which are supported by recent data but are at variance with those of other investigators. It was not our intention to further investigate the issue of elevated arterial AIFE in SHR, but to examine whether aortic AIFE of SHR is identical with that of normotensive controls. The isoelectric points of aortic AIFE from both groups of animals were found to be identical, that is, 5.6. These investigations yielded a further noteworthy finding: the difference of AIFE activity with pl 5.6, when comparing SHR with their controls, was markedly greater than that reported earlier when activities were measured in crude aortic homogenate extracts without prior separation by isoelectric focusing. This may be explained by the assumption that it is mainly the activity of the enzyme with pl 5.6 that is elevated in SHR. Another possibility is that enzymatic effectors (inhibitors) present in the aortic wall are separated by the isoelectric focusing procedure.

In venous tissue, an AIFE with a different pl value (4.35) was found. This suggests that AIFE of venous tissues is not identical with that of the arteries. Further studies are required to clarify if the venous RAS should be considered an intrinsic one. Recent investigations have shown that venous AIFE is not elevated in SHR in comparison to that of arteries. This indicates that the influence of the RAS on the low and high pressure systems, for example, following therapeutic application of ACE inhibitors in congestive heart failure, may not affect pre- and afterload to the same extent.

ACE was demonstrable in most of the organs and tissues that we investigated. The reproducibility of the pl and other values, as well as the fact that approximately 65% of ACE activity remained soluble following a 1-hour ultracentrifugation at 100,000 g, indicates that the main activities of the enzyme are not associated with membrane fractions (unpublished data from recent experiments). The highest specific ACE activities of the rat were found in testis and epididymis, followed by the lung. Up to now, neither the pathophysiologic role of this enzyme nor the origin of plasma ACE has been clarified yet. ACE is present in endothelial cells and, therefore, also in the vascular walls. The question remains as to whether this vascular wall enzyme is identical to those of other origins, and under what conditions its activities can be modulated.

Recently, it was reported that vascular ACE is elevated in SHR. This observation is supported by our results. The pl value measured for aortic tissue was 4.8, in the same range as the values found for lung and epididymis. The isoelectric points of ACE in plasma and veins were slightly lower. On the other hand, the value for testis (pl = 5.4) differed clearly. A further characterization was attempted by determining the IC50 values with the ACE inhibitors MK-421 and MK-422. The significantly different results with MK-422 indicated the existence of an intrinsic vascular enzyme, which appeared to be different from that of plasma. It is likely that MK-421 has been activated by the tissue extract, since the inhibitory effect of MK-422 was only

### Table 1. Angiotensin I and II Immunoreactivities in Rat Aortic Tissue Homogenate

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1 ml of homogenate contained about 100 mg tissue (wet weight).

Angiotensin Levels

The angiotensin immunoreactivities measured in the arterial walls are summarized in Table 1. For measurements of the octapeptide, thin-layer chromatography indicated that the main compound of the immunoreactivity was All.

Discussion

A major problem in the field of vascular AIFE is the issue of local All formation. This includes the question of whether the enzymes involved are present in loco, and if and to what extent they are synthesized and/or stored there. Hitherto, the experimental results available have suggested either the existence or absence of an intrinsic vascular renin system. In contrast, there is no doubt of the occurrence of ACE synthesis in arterial walls. Our intention was primarily to recognize and investigate those enzymes within the vascular walls that are involved in All formation. This is a prerequisite for further studies of their possible physiological and pathophysiological role.

In aortic homogenate extracts, we found one AIFE with a pl of 5.6 and a pH optimum between 7.5 and 8.0, which is in agreement with the findings of Barrett et al. We assume that this enzyme may be synthesized in the arteries, since we measured different pl values for plasma and kidney renin (pl = 4.8). The other AIFE with the extreme pl value of 1.0 had possibly been altered by the isoelectric focusing procedure. For this enzyme, however, we determined a significantly different pH optimum (below pH 4.5); with aortic homogenate extracts, the pH optimum was 4.8 (Figure 2). A similar pH optimum was demonstrated by Barrett et al. for aortic homogenate and for plasma as well. We estimated the molecular weight of this enzyme at between 17,000 and 20,000 (preliminary results). Therefore, we assume that this is a second AIFE present in aortic tissue. Its isoelectric properties have possibly been changed by the separation procedure, but with preservation of the enzymatic activity, which was still well measurable even after 2 to 4 weeks of storage. The AIFE's molecular weight suggests that it is a fragment of the original renin molecule. Comparable fractionation has been described by Galen et al., who reported a renin of another origin with a similar molecular weight. The extreme acid pl value could be caused by oxidation of the -SH groups, which is enhanced at the anode of the isoelectric focusing column.

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1 ml of homogenate contained about 100 mg tissue (wet weight).
approximately 10-fold higher. In the lung, ACE is mainly present in vascular walls, so we may consider the lung enzyme a vascular one. It is generally assumed that plasma ACE derives from the lung. However, our results do not support this contention.

An additional question is whether ACE is the only enzyme consuming the decapeptide AI as substrate, or if other peptidases are involved as well. We therefore studied AI degradation and observed that there is at least one potent AI-degrading enzyme that differs from aortic ACE. The pI value of this enzyme ranges between 3.6 and 3.9, with a pH optimum at 7.8. This enzyme can neither be activated by sodium chloride nor be affected by the ACE inhibitors MK-421 and MK-422 in vitro; it can be partially blocked by EDTA (2.5 mmol/ml, final concentration; unpublished data). The isoelectric focusing study indicated the existence of a second AI-degrading enzyme with a pI between 4.2 and 4.7. This enzyme is likely to be ACE, as described above. Upon studying the effect of pH on AI-degrading activity in the presence of MK-421, we found a pronounced inhibition of enzymatic activity in the range of the pH optimum of ACE (pH 8.0–8.4). The activity of the AI-degrading enzyme with pH optimum at 7.8 was two to three times higher than that of ACE, as shown in Figure 7. Therefore, this enzyme has to be taken into consideration when discussing the local AI formation. Initial experiments with SHR showed that the activity of AI-degrading enzymes is lower, whereas that of ACE is higher. This should result in a marked alteration of the local AI steady state and should lead to enhanced AI formation.

Peptidases responsible for angiotensin degradation (angiotensinas) have been described in blood and in various other organs. Basically, one peptidase may react with both AI and All, even though its affinity for these peptides may be different. The angiotensin-degrading enzymes are also involved in the regulation of All, and possibly of AII, formation. The pI value (5.0) of the vascular All-degrading enzyme was in the same range as that determined for one AI-degrading enzyme. Only one pH optimum at 8.1 has been measured for All-degrading enzymes so far, but preliminary determinations of the molecular weight indicate the existence of several enzymes.

In aortic smooth muscle cells, AI-degrading enzyme activity was readily measurable. The pI optimum was found at 8.0, which is in the same range as that determined in total aortic homogenate. However, the pI value (4.3) was different from that of aortic tissue (5.0). The AI-degrading enzymes in aortic smooth muscle cells had pI values (5.0 and 6.6) different from those in total aortic homogenate. Two pH optima, 6.0 and 8.0, were determined. We assume that one of the pI values of the AI-degrading enzymes (i.e., 6.6) belongs to ACE, since the isoelectric point of ACE in smooth muscle cells was found to be in the same range. The pI value for ACE in smooth muscle cells differs from that of total aortic homogenate (pI 4.8). This also applies to the other AI-degrading enzyme, since the pH optimum of this enzyme was 6.0 in the cells and 7.8 in the aortic tissue. Therefore, one can hypothesize that there may be an additional intrinsic RAS in aortic smooth muscle cells. The biochemical characteristics of this system are not reflected by those from total aortic homogenate. At this point, the relevance of this system is conjectural; the detection of an enzyme in a given structure does not necessarily imply a biological role.

In conclusion, a number of enzymes of the RAS could be detected in vascular tissues. Additional studies are required to further identify them, since differences of pI values only, or of other single biochemical characteristics, may not definitely prove enzyme proteins to be different. Conclusive evidence of an intrinsic RAS of the vascular walls necessitates the measurement of local peptide activities. These investigations are being performed, and first results point to the presence of relatively high values for AI and All immunoactivities (Table 1). It appears that the ratio of AI to All values is higher in aortic tissue than in plasma. From this, we conclude a local AI formation, which implies that angiotensinogen has access to the vessel wall or is synthesized there. Moreover, the relatively high AI values indicate that aortic AI-degrading enzymes, for example ACE, should be rate-limiting for vascular All formation.

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