Conversion and degradation of [^{125}I] labelled angiotensin I in isolated perfused porcine coronary and carotid arteries

A H Jan Danser, Suchandana Chowdury, Larissa M de Lannoy, Willem J van der Giessen, Pramod R Saxena, and Maarten A D H Schalekamp

Objective: The aims were (1) to quantitate angiotensin I to II conversion on the endothelial surface and at deeper sites in isolated arteries, (2) to assess whether the angiotensin II that is formed at deeper sites is released into the vascular lumen, and (3) to examine whether enzymes other than angiotensin converting enzyme (ACE) are involved in vascular angiotensin I to II conversion. **Methods:** Metabolism of [^{125}I]-angiotensin I was studied in isolated perfused porcine coronary and carotid arteries after luminal administration of the labelled peptide (in the perfusion fluid) and after adventitial administration (in the organ bath). Measurements were made both in the presence and in the absence of captopril. **Results:** [^{125}I]-angiotensin II was a major metabolite and its formation was virtually completely blocked by captopril, after both luminal and adventitial administration of [^{125}I]-angiotensin I. In coronary arteries (n = 8), the [^{125}I]-angiotensin I to II conversion rate after adventitial administration was about half that after luminal administration. In coronary arteries (n = 6) the conversion rate after adventitial administration was 10-20 times lower than after luminal administration. Degradation of [^{125}I]-angiotensin I into peptides other than [^{125}I]-angiotensin II was also observed, with both luminal and adventitial administration. No [^{125}I]-angiotensin I or II was released into the organ bath after luminal administration of [^{125}I]-angiotensin I, and very little [^{125}I]-angiotensin I and II entered the lumen after adventitial administration of [^{125}I]-angiotensin I. **Conclusions:** (1) Vascular angiotensin I to II conversion is not limited to the endothelial surface. (2) ACE is the most important, if not the only, enzyme responsible for vascular angiotensin I to II conversion. (3) If angiotensin I and II are formed in the adventitia or media, little of these peptides will enter the vascular lumen.
Cardiovascular Research 1995;**29**:789-795

Downloaded from by guest on December 9, 2014

The classical view of angiotensin II, the most important biologically active product of the renin-angiotensin system, as a circulating hormone has been challenged in recent years. Previously it was thought that angiotensin II was derived solely from the conversion of circulating angiotensin I by membrane bound angiotensin converting enzyme (ACE) of the vascular endothelium. This enzyme is identical with kininase II. However, there is now evidence that many tissues, including heart and blood vessels, contain a complete renin-angiotensin system[1-3] and that angiotensin I and angiotensin II may also be generated locally, outside the circulating plasma.[4-7] There is even good evidence that in a number of vascular beds angiotensin I production at tissue sites may contribute to the circulating levels of angiotensin I and II.[5 7] Angiotensin II produced in vascular tissue from in situ synthesised angiotensin I or from angiotensin I taken up from the circulation may serve functions other than the regulation of vascular tone. It may for instance contribute to the development of vascular hypertrophy.[8]

The formation of angiotensin II in the vessel wall may not depend on endothelial ACE only. Endothelium denuded rat aortic rings contain ACE activity, as determined with a synthetic tripeptide substrate.[9] ACE has also been demonstrated in endothelium denuded porcine coronary arteries by using [^{125}I]-labelled angiotensin I as substrate.[10] In addition, angiotensin I has been found to contract endothelium denuded rat aortic rings.[11] In support of these findings, ACE activity has been shown in cultured rat aortic smooth muscle cells.[12]

Several enzymes other than ACE have been described which can convert angiotensin I to angiotensin II in vitro.[13-16] A chymostatin sensitive angiotensin II generating enzyme (CAGE), capable of catalysing angiotensin I to II conversion, has been isolated from aortic tissue of dog, monkey, and man.[17 18] This enzyme is localised mainly in the adventitia,[18] and is thought to be identical with an angiotensin II forming chymase which has recently been discovered in human heart tissue.[19 20] Human heart chymase is a serine proteinase with high specificity for angiotensin I. Chymase mRNA is expressed in endothelial cells and in mast cells[21] and may be induced by vascular injury.[22]

We decided to compare angiotensin I metabolism after luminal and adventitial administration of [^{125}I]-labelled angiotensin I to isolated perfused porcine arteries. The aim of the present study was (1) to quantitate angiotensin I to II conversion both on the endothelial surface and at deeper sites in isolated arteries, (2) to assess whether the angiotensin II

Cardiovasculair Onderzoeksinstituut Erasmus University Rotterdam (COEUR), Erasmus University Rotterdam, Dr Molewaterplein 50, 3015 GE Rotterdam, The Netherlands – Department of Pharmacology: A H J Danser, S Chowdury, L M de Lannoy, P R Saxena; Department of Cardiology: W J van der Giessen; Department of Internal Medicine: M A D H Schalekamp. Correspondence to Dr Danser.

that is formed at deeper sites is released into the vascular lumen, and (3) to examine whether angiotensin converting enzymes other than ACE are involved in vascular angiotensin I to II conversion.

Methods

Blood vessel preparation

Carotid and left anterior descending coronary arteries were obtained from 20 pigs (weight 16-24 kg, crossbred Yorkshire × Landrace) during anaesthesia with 160 mg·kg^{-1} α chloralose, which was given into the superior caval vein, followed by a continuous intravenous infusion of low dose sodium pentobarbitone (5 mg·kg^{-1}·h^{-1}). The pigs had been used for acute pharmacological experiments involving intravenous saline or calcitonin gene related peptide infusions. All experiments were performed in accordance with the *Guiding principles in the care and use of animals* as approved by the American Physiological Society and under the regulations of the animal care committee of the Erasmus University, Rotterdam, The Netherlands.

The vessels (diameter 2-3 mm) were dissected free from surrounding tissue, and 1-2 cm sections without side branches were mounted horizontally in a double jacketed 4 ml organ bath containing Krebs buffer (composition in mmol·litre^{-1}: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, and glucose 8.3), which was continuously gassed with 95% O_2 and 5% CO_2 at 37°C, as described by Hulsmann *et al.*[22] The vessels were perfused with carbogenated Krebs buffer from a 6 ml reservoir kept at 37°C in a water bath, using a roller pump. The flow was kept at 1 ml·min^{-1}. The perfusate was collected in the reservoir and reperfused through the vessel, thus creating a closed perfusion circuit (fig 1). This set up allowed us to obtain samples from both the reservoir ("perfusate") and the organ bath ("bath fluid") after the addition of [^{125}I]-angiotensin I to either the perfusate reservoir or the organ bath.

Preparation of radiolabelled angiotensins

Monoiodinated [^{125}I]-angiotensin I was prepared with the chloramine-T method and purified as described previously.[5] The specific radioactivity of the [^{125}I]-angiotensin I preparation was 3.6 × 10^6 counts·min^{-1} (cpm) pmol^{-1} (74 kBq·pmol^{-1}). [^{125}I]-labelled preparations of angiotensin II, angiotensin III, angiotensin-(3-8), angiotensin-(4-8), angiotensin-(2-10), angiotensin-(1-7), and tyrosine were also made.[5]

Separation and measurement of radiolabelled angiotensins

[^{125}I]-angiotensin I and its metabolites were extracted from the samples obtained during the experiment (see below) by reversible adsorption to octadecylsilyl silica (SepPak C18, Waters). The SepPak cartridges were conditioned with 4 ml methanol and equilibrated with 2 × 4 ml of cold water. Samples were passed through the cartridge at 4°C, followed by a wash with 2 × 4 ml of cold water. Adsorbed angiotensins were eluted with 3 ml methanol into polypropylene tubes and the methanol was evaporated under vacuum rotation at 4°C, using a Savant Speed Vac concentrator.

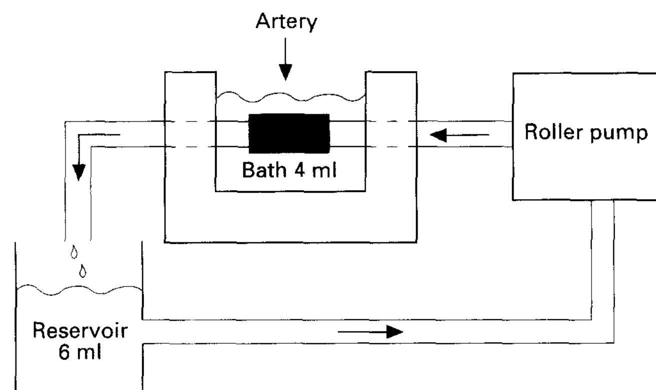


Figure 1 *Schematic presentation of the experimental set up. A 1-2 cm vessel segment is mounted in a double jacketed organ bath that contains Krebs buffer at 37°C. The buffer is continuously gassed with 95% O_2 and 5% CO_2. The vessel lumen is constantly perfused with gassed Krebs buffer from a reservoir kept in a water bath at 37°C, using a roller pump. Volume of the bath was 4 ml. Total volume of the reservoir including pump and connecting cannulas was 6 ml. [^{125}I]-labelled angiotensin I was added to the reservoir ("luminal administration") or the organ bath ("adventitial administration").*

[^{125}I]-labelled angiotensins were separated by reversed phase high pressure liquid chromatography (HPLC), according to the method of Nussberger *et al*,[23] using a Nucleosil C18 steel column of 250 × 4.6 mm and 10 μm particle size. Mobile phase A was 0.085% orthophosphoric acid containing 0.02% sodium azide. Mobile phase B was methanol. The flow was 1.5 ml·min^{-1} and the working temperature was 45°C. SepPak methanol extracts were dissolved in 100 μl of HPLC solvent (mobile phase A) and injected. Elution was performed as follows: 65% A/35% B from 0 to 9 min, followed by a linear gradient to 45% A/55% B until 18 min. The eluate was collected in 20 s fractions into polypropylene tubes, and the concentrations of [^{125}I]-angiotensin I and its radiolabelled metabolites in the HPLC fractions were measured in the gamma counter.

There was virtually complete recovery of radioactivity after SepPak extraction. Moreover, more than 95% of [^{125}I]-angiotensin I or II that had been added to SepPak extracts was recovered after HPLC separation. Results of [^{125}I]-angiotensin I and II measurements were therefore not corrected for the small losses during extraction and HPLC separation.

Experimental protocol

The blood vessel preparations were allowed to equilibrate for 30 min. In each carotid and coronary artery two experiments were performed. In one experiment approximately 10^6 cpm [^{125}I]-angiotensin I were added to the perfusate reservoir ("luminal administration"). Samples of 100 μl were taken from both organ bath and reservoir at t = 0, 1, 2, 5, 10, 20, and 40 min after the addition of [^{125}I]-angiotensin I, and were immediately mixed with 10 μl inhibitor solution containing 125 mM disodium EDTA and 25 mM 1,10-phenanthroline, to inhibit ACE and angiotensinases, respectively. The samples were stored at −70°C and assayed within one week. In the second experiment [^{125}I]-angiotensin I (approximately 10^6 cmp) was added to the organ bath ("adventitial administration"), after which 100 μl samples were taken from both organ bath and reservoir at t = 0, 5, 10, 20, 30, and 40 min. The samples were immediately mixed with inhibitor solution as described above, and stored at −70°C. The two experiments were performed in random order, and between the two experiments the Krebs buffer in the organ bath and reservoir was refreshed several times in order to wash out all radioactivity.

In a second series of experiments, captopril was added in a concentration of 0.4 mM before the addition of [^{125}I]-angiotensin I. This concentration of captopril had previously been shown to inhibit ACE but not other enzymes involved in the metabolism of angiotensin I.[24]

After completion of the experiments the length of the vessel segments was measured. The vessels were then blotted on dry paper and weighed. The wet weight of the coronary artery segments (n = 11) did not differ from that of the carotid artery segments (n = 9): 70(SEM 7) mg and 99(11) mg, respectively, and the lengths were likewise similar: 1.5(0.1) and 1.6(0.2) cm.

Reagents

[Ile5]-angiotensin-(1-10) decapeptide (angiotensin I), [Ile5]-angiotensin-(1-8) octapeptide (angiotensin II), and [Ile5]-angiotensin-(2-8) heptapeptide (angiotensin III) were from Bachem. [Ile5]-angiotensin-(2-10) nonapeptide (angiotensin-(2-10)) was from Senn Chemicals. [Ile5]-angiotensin-(3-8) hexapeptide (angiotensin-(3-8)), [Ile5]-angiotensin-(4-8) pentapeptide (angiotensin-(4-8)), [Ile5]-angiotensin-(1-7) heptapeptide (angiotensin-(1-7)), and tyrosine were from Peninsula Laboratories. Captopril was obtained from Bristol-Myers Squibb. Methanol, ortho-phosphoric acid (both analytical grade), and 1,10-phenantroline were purchased from Merck. Water for HPLC was prepared with a Milli-Q system from Waters.

Calculations

[^{125}I]-angiotensin I is eliminated either by conversion to [^{125}I]-angiotensin II by ACE or other angiotensin converting enzymes, or by breakdown to small biologically inactive peptides by various other enzymes. We refer to the latter process as "degradation" of [^{125}I]-angiotensin I.[24] The first order rate constants for degradation and conversion of [^{125}I]-angiotensin I are denoted as k_1 and k_2, respectively. The rate constant for [^{125}I]-angiotensin I elimination (k_{el}) is taken to be equal to the sum of the first order rate constants for conversion and degradation.

The metabolism of [^{125}I]-angiotensin I in a closed perfusion circuit can be described by the equation for a first order process, so that k_{el} can be calculated as follows:

$$k_{el} = -1/t \cdot \ln([[^{125}\text{I}]\text{-angiotensin I}]_t/[[^{125}\text{I}]\text{-angiotensin I}]_0) \quad (1)$$

in which $[[^{125}\text{I}]\text{-angiotensin I}]_t$ is the concentration of [^{125}I]-angiotensin I at time t and $[[^{125}\text{I}]\text{-angiotensin I}]_0$ is the concentration of [^{125}I]-angiotensin I at t = 0 (immediately after the addition of [^{125}I]-angiotensin I).

The elimination of [^{125}I]-angiotensin II involves degradation only. Experiments in pigs, in which [^{125}I]-angiotensin I was infused into the left cardiac ventricle,[24] and experiments in humans, in which

Downloaded from by guest on December 9, 2014

[125I]-angiotensin I and [125I]-angiotensin II were infused in a peripheral vein,[25] did not show a difference between the rate constants for degradation of infused [125I]-angiotensin I and infused [125I]-angiotensin II. Results of these experiments were also compatible with the assumption that the rate constants of infused [125I]-angiotensin I and [125I]-angiotensin II generated from infused [125I]-angiotensin I are not different. Applying these assumptions to the present experiments, k_1 can be calculated as follows:

$$k_1 = -1/t \cdot \ln(([[^{125}I]\text{-angiotensin II}]_t + [[^{125}I]\text{-angiotensin I}]_t)/[[^{125}I]\text{-angiotensin I}]_0) \quad (2)$$

in which$[[^{125}I]\text{-angiotensin II}]_t$ is the concentration of [125I]-angiotensin II at time t.

Finally, k_2 can be obtained by subtracting k_1 (calculated with formula 2) from k_{el} (calculated with formula 1).

[125I]-angiotensin I metabolic clearance rates due to degradation plus conversion or due to degradation or conversion separately were calculated by multiplying $k_1 + k_2$, k_1, or k_2 respectively with the volume of distribution (6 ml for luminal administration, 4 ml for adventitial administration). No corrections were made for the small volume changes that occurred as a consequence of fluid sampling for angiotensin measurements.

The percentage of [125I]-angiotensin I metabolism due to conversion is defined as follows:

$$\text{Contribution of conversion to metabolism (\%)} = [k_2/(k_1 + k_2)] \cdot 100. \quad (3)$$

Statistics

All data are reported as mean(SEM). Differences between carotid and coronary arteries and differences between experiments with and without captopril were evaluated for statistical significance ($P < 0.05$) by using the Mann-Whitney U test for unpaired observations. Differences in metabolism between luminal and adventitial administration of [125I]-angiotensin I were evaluated for statistical significance by using the Mann-Whitney U test for paired observations.

Results

Vascular metabolites of [125I]-angiotensin I during luminal or adventitial administration of [125I]-angiotensin I

With luminal administration of [125I]-angiotensin I, radioactivity (that is, [125I]-angiotensin I and its radiolabelled metabolites) was limited to the perfusate, both in carotid and coronary arteries. With adventitial administration, however, radioactivity was not limited to the bath fluid. Some radioactivity reached the perfusate in coronary and carotid arteries during the course of the experiment. The amount of radioactivity in the perfusate tended to plateau after 20-30 min at approximately 0.5-1.0% (carotid arteries) or 10-15% (coronary arteries) of total radioactivity. With both luminal and adventitial administration, radioactivity in vascular tissue, as measured after termination of the experiment, was marginal (approximately 1% of total radioactivity). Because of these low levels we did not attempt to measure intact [125I]-angiotensin I or II in the vessel wall.

With both means of [125I]-angiotensin I administration, [125I]-angiotensin II appeared to be a major metabolite. Peaks with retention times corresponding to those of [125I]-tyrosine, [125I]-angiotensin-(4-8) and [125I]-angiotensin-(2-10) could also be identified (fig 2). After adventitial [125I]-angiotensin I administration, most of the radioactivity present in the perfusate was found in the peak corresponding with [125I]-tyrosine (fig 2).

Release of [125I]-angiotensin I and II into the lumen after adventitial administration of [125I]-angiotensin I

With adventitial administration of [125I]-angiotensin I to coronary arteries, small quantities of [125I]-angiotensin I and [125I]-angiotensin II could be detected in the perfusate 20 min after [125I]-angiotensin I had been added to the bath fluid. At the end of the experiment, the levels of [125I]-angiotensin I and II in the perfusate were less than 10% of the levels in the bath (fig 2).

No intact [125I]-angiotensin I or [125I]-angiotensin II could be detected in the perfusate during adventitial administration of [125I]-angiotensin I to carotid arteries. Likewise, with luminal administration, both to coronary and carotid arteries, none of these radiolabelled peptides could be detected in the bath fluid (fig 2).

Vascular [125I]-angiotensin I metabolic clearance rates during luminal or adventitial administration of [125I]-angiotensin I

The decrease in [125I]-angiotensin I and the increase in [125I]-angiotensin II in the perfusion fluid after luminal [125I]-angiotensin I administration are shown in fig 3. The changes in [125I]-angiotensin I and II in the bath fluid after adventitial [125I]-angiotensin I administration are shown in fig 4. No major differences were detected between coronary and carotid arteries. [125I]-angiotensin I levels followed first order kinetics. The changes in [125I]-angiotensin I and II levels were analysed as described under "Calculations", and the best fitting curves are shown in figs 3 and 4.

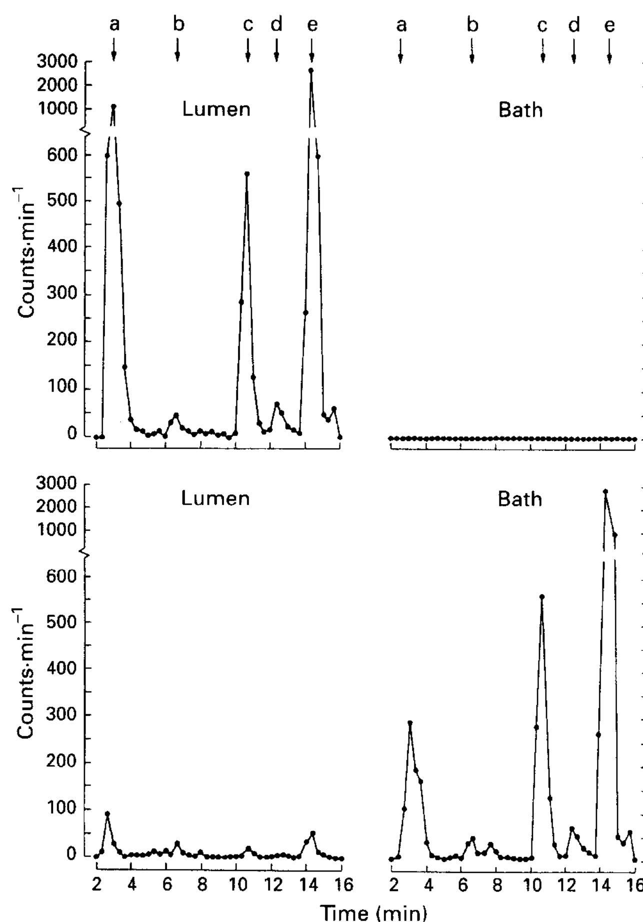


Figure 2 *Examples of high pressure liquid chromatography elution profiles of [125I]-labelled angiotensins. Top panels: Perfusate ("lumen") and bath fluid ("bath") taken from a carotid artery 20 min after the addition of [125I]-angiotensin I to the reservoir ("luminal administration"). Bottom panels: Perfusate and bath fluid taken from a coronary artery 40 min after the addition of [125I]-angiotensin I to the bath ("adventitial administration"). a = [125I]-tyrosine; b = [125I]-angiotensin-(4-8); c = [125I]-angiotensin II; d = [125I]-angiotensin-(2-10); e = [125I]-angiotensin I.*

Downloaded from by guest on December 9, 2014

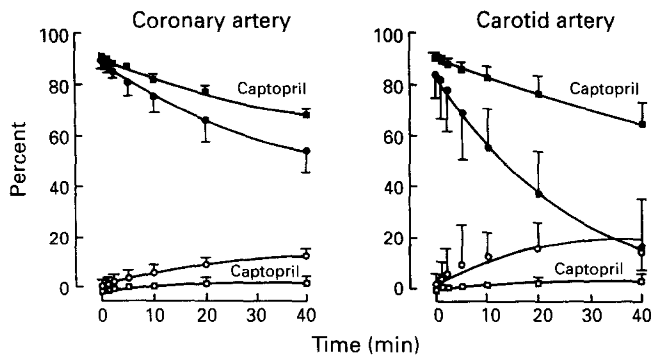


Figure 3 *Decrease in [^{125}I]-angiotensin I (filled symbols) and increase in [^{125}I]-angiotensin II (empty symbols) in the perfusate of coronary (left panel) and carotid arteries (right panel) after luminal administration of [^{125}I]-angiotensin I in the presence (n = 8) or absence (n = 3) of captopril. Error bars = SEM; where no SEM is given it was smaller than the symbol. The solid lines are the best fitting curves describing the decrease in [^{125}I]-angiotensin I and the increase in [^{125}I]-angiotensin II.*

The metabolic clearance rate (MCR) of [^{125}I]-angiotensin I was higher with luminal [^{125}I]-angiotensin I administration than with adventitial administration, and this was true both for the coronary (fig 5 and table) and carotid arteries (fig 6 and table). In the coronary arteries the MCRs of [^{125}I]-angiotensin I due to degradation and conversion were both significantly lower with adventitial [^{125}I]-angiotensin I administration than with luminal administration (fig 5 and table). In the carotid arteries the MCR of [^{125}I]-angiotensin I due to degradation during luminal [^{125}I]-angiotensin I administration tended to be higher than the MCR during adventitial administration, but the difference was not significant. The MCR due to conversion was higher with luminal administration than with adventitial administration in the carotid arteries (fig 6 and table).

The percentage of [^{125}I]-angiotensin I metabolism that could be attributed to [^{125}I]-angiotensin I to II conversion amounted to 45(SEM 4)% (luminal administration, n = 8) and 37(3)% (adventitial administration, n = 8) in coronary arteries, and to 62(5)% (luminal administration, n = 6) and 47(8)% (adventitial administration, n = 6) in carotid arteries.

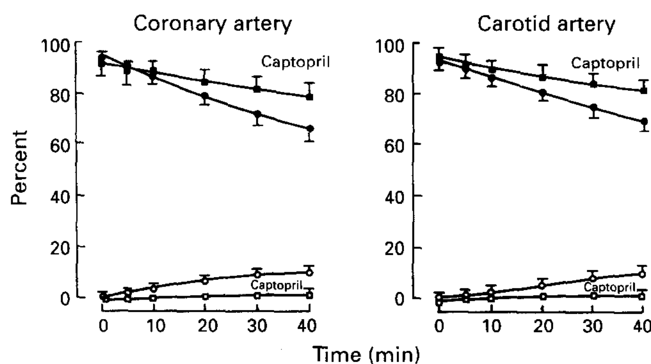


Figure 4 *Decrease in [^{125}I]-angiotensin I (filled symbols) and increase in [^{125}I]-angiotensin II (empty symbols) in the bath fluid of coronary (left panel) and carotid arteries (right panel) after adventitial administration of [^{125}I]-angiotensin I in the presence (n = 6) or absence (n = 3) of captopril. Error bars = SEM; where no SEM is given it was smaller than the symbol. The solid lines are the best fitting curves describing the decrease in [^{125}I]-angiotensin I and the increase in [^{125}I]-angiotensin II.*

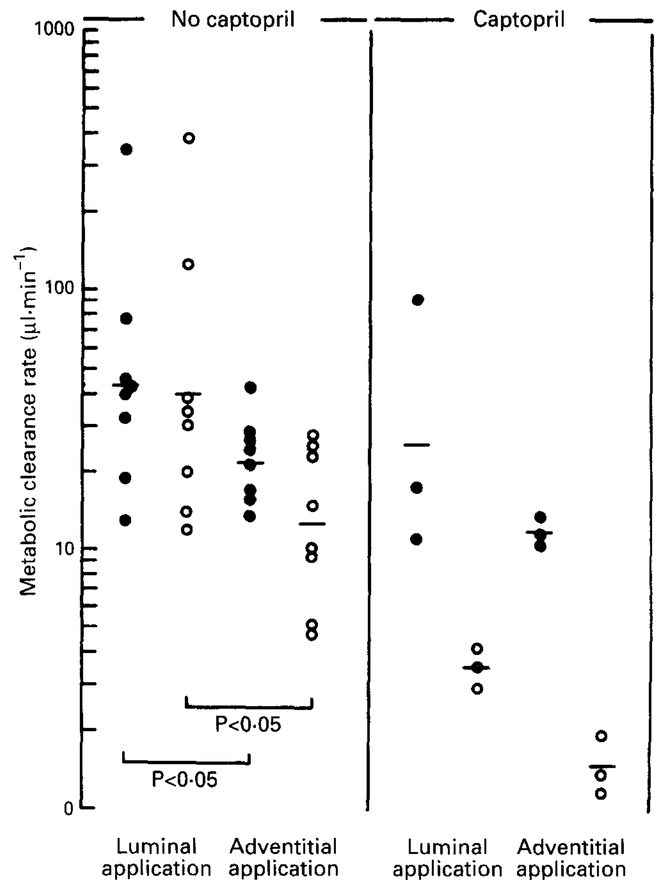


Figure 5 *Metabolic clearance rate of [^{125}I]-angiotensin I due to degradation (filled symbols) and conversion (empty symbols) in coronary arteries after luminal or adventitial [^{125}I]-angiotensin I administration in the absence (left panel) and presence (right panel) of captopril. Geometric means are shown with horizontal bars.*

Effect of captopril on vascular [^{125}I]-angiotensin I metabolic clearance rates during luminal or adventitial administration of [^{125}I]-angiotensin I

Captopril reduced the [^{125}I]-angiotensin II peak in the HPLC fractions to values close to the background (figs 3 and 4). The peak corresponding with [^{125}I]-angiotensin-(4-8) became undetectable. In the presence of captopril, the peaks corresponding with [^{125}I]-tyrosine and [^{125}I]-angiotensin-(2-10) were the main metabolites of [^{125}I]-angiotensin I, both with luminal and adventitial [^{125}I]-angiotensin I administration. Consequently, the MCR of [^{125}I]-angiotensin I due to conversion fell to very low levels after captopril, with both luminal and adventitial [^{125}I]-angiotensin I administration (figs 5 and 6). Captopril had little effect on [^{125}I]-angiotensin I degradation.

Ratio between the vascular metabolic clearance rate (MCR) of [^{125}I]-angiotensin I during adventitial administration and during luminal administration. Values are means(SEM).

	MCR adventitial/MCR luminal		
	MCR due to degradation and conversion	*MCR due to degradation*	*MCR due to conversion*
Coronary artery (n = 8)	0.51(0.09)*	0.62(0.11)*	0.44(0.14)*
Carotid artery (n = 6)	0.13(0.05)*	0.64(0.26)	0.06(0.01)*

*P < 0.05 for difference from 1.00 (Mann-Whitney U test for paired observations).

Downloaded from by guest on December 9, 2014

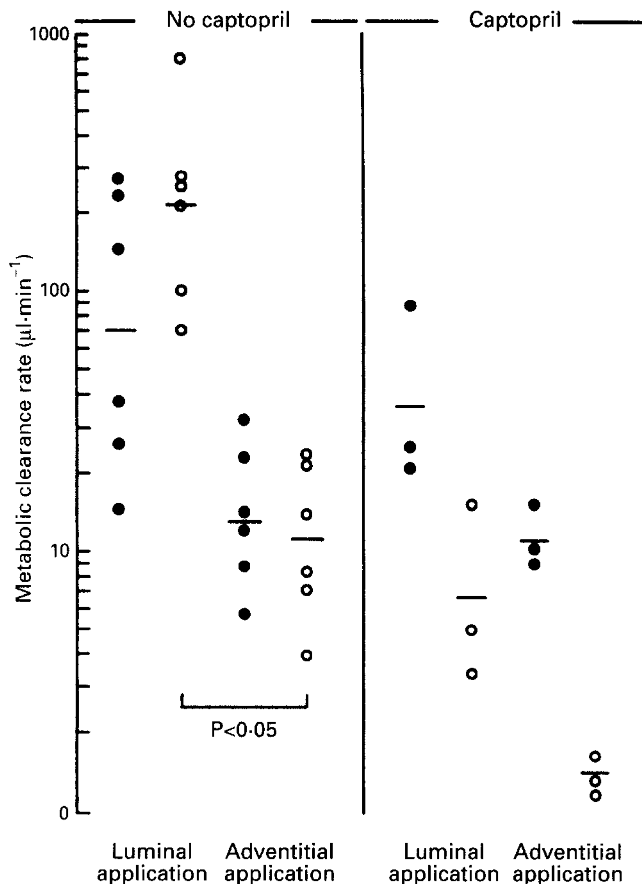


Figure 6 *Metabolic clearance rate of [^{125}I]-angiotensin I due to degradation (filled symbols) and conversion (empty symbols) in carotid arteries after luminal or adventitial [^{125}I]-angiotensin I administration in the absence (left panel) and presence (right panel) of captopril. Geometric means are shown with horizontal bars.*

With captopril, the percentage of [^{125}I]-angiotensin I metabolism that could be attributed to [^{125}I]-angiotensin I to II conversion amounted to 16(7)% (luminal administration, n = 3; P < 0.05 for the difference with the experiments in the absence of captopril) and 9(4)% (adventitial administration; P < 0.05) in coronary arteries, and to 15(1)% (luminal administration, n = 3; P < 0.05) and 10(1)% (adventitial administration; P < 0.05) in carotid arteries. Thus after captopril more than 85% of [^{125}I]-angiotensin I metabolism in the coronary and carotid arteries was due to degradation.

Discussion

Previous studies have shown that the vessel wall contains multiple enzymes, for example tissue plasminogen activator, tonin, cathepsin A, cathepsin G, CAGE, and chymase, which are capable of converting angiotensin I to angiotensin II in vitro and which cannot be blocked by ACE inhibitors.[13–19 26 27] However, in most of these studies the conversion of angiotensin I to angiotensin II was studied using vascular tissue homogenates and it is not known to what extent homogenisation of the tissues may cause the release of several cellular enzymes that are not normally involved in the metabolism of angiotensins. We have observed that angiotensin I and II are much more rapidly degraded in cardiac tissue homogenates than in the intact tissue.[28] In published studies in which intact vessels were used angiotensin II formation was either not quantitated directly[26] or measured after adding highly unphysiological amounts (50 µg per vessel) of angiotensin I.[27]

In the present study we investigated angiotensin I metabolism under more physiological conditions, using intact segments of porcine coronary and carotid arteries, perfused with carbogenated Krebs buffer at 37°C. This model allowed us to administer angiotensin I to the blood vessel in the perfusion fluid (luminal application) or in the surrounding bath fluid (adventitial application). With luminal application, angiotensin I is primarily exposed to the vascular endothelium. Adventitially administered angiotensin I – which most probably corresponds to the angiotensin I that is formed in the vessel wall in the in vivo situation – may also reach the endothelium, but is primarily exposed to enzymes present in the media or adventitia. With both ways of administration, one would expect that endothelially produced angiotensin I metabolites would appear in the perfusate.

Measurements of [^{125}I]-angiotensin I and II in the perfusion and bath fluids showed that [^{125}I]-angiotensin I to II conversion occurred not only after luminal [^{125}I]-angiotensin I administration but also after adventitial administration, and that very little of the [^{125}I]-angiotensin II formed after adventitial [^{125}I]-angiotensin I administration was released into the perfusate. Moreover, the [^{125}I]-angiotensin II formed after luminal [^{125}I]-angiotensin I administration was released only in the perfusate.

These findings lend support to the concept that vascular conversion of angiotensin I is not limited to the luminal endothelial surface. Our present observation that little angiotensin I and II passed through the wall of the carotid and coronary arteries from the lumen to the bath and vice versa, together with our previous work[7] indicating rapid exchange between the angiotensins in circulating blood and the tissue interstitial fluid, is compatible with the idea that the exchange occurs mainly at the capillary level. There seems to be little accumulation of angiotensins in the present experiments because only low levels of radioactivity were found in the vessel segments at the end of the perfusion.

The conversion that occurs on the endothelial surface as well as at deeper sites appears to be mediated by ACE, because the ACE inhibitor captopril caused nearly complete inhibition of angiotensin II formation at these sites. Therefore, in the pig, enzymes other than ACE do not seem to play a major role in the conversion of circulating angiotensin I.

The evidence provided by our observations on perfused arteries that ACE dependent angiotensin I to II conversion is not limited to the endothelium extends previous studies in which the formation of [^{125}I]-angiotensin II was followed during incubation of tissue pieces from endothelium denuded porcine coronary arteries with [^{125}I]-angiotensin I.[10] From these studies it was calculated that approximately 25% of the vascular ACE was extraendothelial. Similar findings have been reported for the rat aorta; de-endothelialisation of this vessel resulted in a 30% decrease in ACE activity of aortic tissue homogenates, suggesting that 70% of rat aortic ACE is situated outside the endothelium.[9] The higher contribution of extraendothelial sites to angiotensin I to II conversion in the rat aorta might be related to the localisation of ACE in the smooth muscle layer.[12] Radioligand binding studies have shown that ACE in the porcine carotid artery and in the

Downloaded from by guest on December 9, 2014

human internal mammary artery is found mainly in the endothelial layer and in the adventitia.[29 30] These species differences in ACE localisation may partially explain the conflicting data concerning the effects of ACE inhibitors on myointimal proliferation after vascular injury. In the rat, the myointimal proliferative response to endothelial damage of the carotid artery was prevented by ACE inhibitor treatment.[31] This could not be confirmed in the pig[32] and likewise in humans the use of ACE inhibitors for the prevention of restenosis after angioplasty proved to be ineffective.[33 34] It should be noted, however, that the doses used in the rat experiments were up to 70 times higher than those used in man.

The possibility that our results might be explained by endothelial ACE activity of the adventitial vasa vasorum[29] must be addressed. Our observations that, with adventitial administration of [^{125}I]-angiotensin I, little of the peptide entered into the vascular perfusate and that [^{125}I]-angiotensin II formed in the vascular lumen did not enter into the surrounding bath fluid make it unlikely that conversion at the luminal surface of the vasa vasorum is a major source of the [^{125}I]-angiotensin II found in the bath fluid.

The [^{125}I]-angiotensin I to II conversion rates we measured after adventitial administration of [^{125}I]-angiotensin I may not be representative of the in vivo conversion rates. In the normal in vivo situation angiotensin I within the vascular tissue may have been taken up from the circulating blood or may have been formed locally in the tissue at extracellular and intracellular sites. Blood derived angiotensin I and locally formed angiotensin I are most probably metabolised differently, since blood derived angiotensin I has to pass the endothelial barrier which is rich in converting and degrading enzymes.

Our study clearly shows that ACE present in the adventitia or media is functionally active. One characteristic component of the adventitia is the noradrenergic nerve endings which are located at the adventitial-medial border. Angiotensin II produced by ACE in the vicinity of the nerve endings could act presynaptically to facilitate noradrenaline release. Angiotensin II mediated facilitation of neurotransmitter release has been shown in a number of vascular preparations.[35 36] It is further possible that angiotensin II formed locally within the vascular media is involved in the induction of medial hypertrophy.[8]

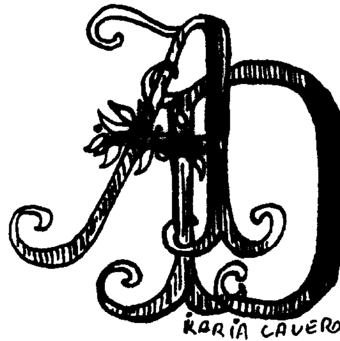
Key terms: angiotensin; angiotensin converting enzyme; captopril; endothelium; renin; blood vessel wall.

Received 27 September 1994; accepted 19 January 1995. Time for primary review 41 days.

1 Lindpaintner K, Wilhelm MJ, Jin M, *et al.* Tissue renin-angiotensin systems: focus on the heart. *J Hypertens* 1987; **5**(suppl 2):S33–8.
2 Campbell DJ, Lawrence AC, Towrie A, Kladis A, Valentijn A. Differential regulation of angiotensin peptides in plasma and kidney of the rat. *Hypertension* 1991;**18:**763–73.
3 Kim S, Tokuyama M, Hosoi M, Yamamoto K. Adrenal and circulating renin-angiotensin system in stroke-prone hypertensive rats. *Hypertension* 1992;**20:**280–91.
4 Hilgers KF, Kuczera M, Wilhelm MJ, *et al.* Angiotensin formation in the isolated rat hindlimb. *J Hypertens* 1989;**7:**789–98.
5 Admiraal PJJ, Derkx FHM, Danser AHJ, Pieterman H, Schalekamp MADH. Metabolism and production of angiotensin I in different vascular beds in subjects with hypertension. *Hypertension* 1990;**15:**44–55.
6 Taddei S, Favilla S, Duranti P, Simonini N, Salvetti A. Vascular renin-angiotensin system and neurotransmission in hypertensive persons. *Hypertension* 1991;**18:**266–77.
7 Danser AHJ, Koning MMG, Admiraal PJJ, *et al.* Production of angiotensin I and II at tissue sites in intact pigs. *Am J Physiol* 1992;**263:**H429–37.
8 Griffin SA, Brown WCB, MacPherson F, *et al.* Angiotensin II causes vascular hypertrophy in part by a non-pressor mechanism. *Hypertension* 1991;**17:**626–35.
9 Pipili E, Manolopoulos VG, Catravas JD, Maragoudakis ME. Angiotensin converting enzyme activity is present in the endothelium-denuded aorta. *Br J Pharmacol* 1989;**98:**333–5.
10 Van der Giessen WJ, Danser AHJ, van Beusekom HMM, *et al.* Local angiotensin II production in porcine coronary arteries: reduction of release during neointimal hyperplasia induced by stent implantation. *Coronary Artery Dis* 1992;**3:**730–7.
11 Egleme C, Cressier F, Wood JM. Local formation of angiotensin II in the rat aorta: effect of endothelium. *Br J Pharmacol* 1990;**100:**237–40.
12 Andre P, Schott C, Nehlig H, Stoclet JC. Aortic smooth muscle cells are able to convert angiotensin I to angiotensin II. *Biochem Biophys Res Commun* 1990;**173:**1137–42.
13 Klickstein LB, Kaempfer CE, Wintroub BU. The granulocyte angiotensin system: angiotensin I converting enzyme activity of cathepsin G. *J Biol Chem* 1982;**257:**1504–6.
14 Wintroub BU, Schechter NB, Lazarus GS, Kaempfer CE, Schwartz LB. Angiotensin I conversion by human and rat chymotryptic proteinases. *J Invest Dermatol* 1984;**83:**336–9.
15 Miller JJ, Changaris DG, Levy RS. Conversion of angiotensin I to angiotensin II by cathepsin A isoenzymes of porcine kidney. *Biochem Biophys Res Commun* 1988;**154:**1122–9.
16 Tang SS, Loscalzo J, Dzau VJ. Tissue plasminogen activator activates renin angiotensin *in vitro*. *J Vasc Med Biol* 1989; **1:**67–74.
17 Okunishi H, Miyazaki M, Toda N. Evidence for a putative new angiotensin II-generating enzyme in the vascular wall. *J Hypertens* 1984;**2:**277–84.
18 Okunishi H, Miyazaki M, Okamura T, Toda N. Differential distribution of two types of angiotensin II-generating enzymes in the aortic wall. *Biochem Biophys Res Commun* 1987;**149:** 1186–92.
19 Urata H, Kinoshita A, Misono KS, Bumpus FM, Husain A. Identification of a highly specific chymase as the major angiotensin II-forming enzyme in the human heart. *J Biol Chem* 1990;**36:**22348–57.
20 Shiota N, Okunishi H, Fukamizu A, *et al.* Activation of two angiotensin-generating enzymes in the balloon-injured artery. *FEBS Lett* 1993;**323:**239–42.
21 Urata H, Boehm KD, Philip A, *et al.* Cellular localization and regional distribution of an angiotensin II-forming chymase in the heart. *J Clin Invest* 1993;**91:**1269–81.
22 Hulsmann AR, Raatgreep HR, Bonta IL, Stijene T, Kerrebijn KF, de Jongste JC. The perfused human bronchial tube. Characteristics of a new model. *J Pharmacol Methods* 1992; **28:**29–34.
23 Nussberger J, Brunner DB, Waeber B, Brunner HR. Specific measurement of angiotensin metabolites and in vitro generation of angiotensin II in plasma. *Hypertension* 1986;**8:**476–82.
24 Danser AHJ, Koning MMG, Admiraal PJJ, Derkx FHM, Verdouw PD, Schalekamp MADH. Metabolism of angiotensin I by different tissues in the intact animal. *Am J Physiol* 1992; **263:**H418–28.
25 Admiraal PJJ, Danser AHJ, Jong MS, Pieterman H, Derkx FHM, Schalekamp MADH. Regional angiotensin II production in essential hypertension and renal artery stenosis. *Hypertension* 1993;**21:**173–84.
26 Cornish KG, Joyner WL, Gilmore JP. Direct evidence for the presence of a different converting enzyme in the hamster cheek pouch. *Circ Res* 1979;**44:**540–4.
27 Okunishi H, Miyazaki M, Toda N. Evidence for a putatively new angiotensin II generating enzyme in the vascular wall. *J Hypertens* 1984;**2:**277–84.
28 Danser AHJ, van Kats JP, Admiraal PJJ, *et al.* Cardiac renin and angiotensins. Uptake from plasma versus in situ synthesis. *Hypertension* 1994;**24:**37–48.
29 Rogerson FM, Chai SY, Schlawe I, Murray WK, Marley PD, Mendelsohn FAO. Presence of angiotensin converting enzyme in the adventitia of large blood vessels. *J Hypertens* 1992;**10:** 615–20.
30 Johnston CI. Renin-angiotensin system: a dual tissue and hormonal system for cardiovascular control. *J Hypertens* 1992; **10**(suppl 7):S13–26.
31 Powell JS, Clozel JP, Muller RKM, *et al.* Inhibitors of angiotensin-converting enzyme prevent myointimal proliferation after vascular injury. *Science* 1989;**245:**186–8.
32 Lam JYT, Lacoste L, Bourassa MG. Cilazapril and early atherosclerotic changes after balloon injury of porcine carotid arteries. *Circulation* 1992;**85:**1542–7.

Downloaded from by guest on December 9, 2014

33 MERCATOR Study Group. Does the new angiotensin converting enzyme inhibitor cilazapril prevent restenosis after percutaneous transluminal coronary angioplasty? Results of the MERCATOR study: a multicenter, randomized, double-blind placebo-controlled trial. *Circulation* 1992;**86:**100–10.
34 Desmet W, Vrolix M, de Scheerder I, van Lierde J, Willems J, Piessens J. Angiotensin-converting enzyme inhibition with fosinopril sodium in the prevention of restenosis after coronary angioplasty. *Circulation* 1994;**89:**385–92.
35 Starke K. Regulation of noradrenaline release by presynaptic receptor systems. *Rev Physiol Biochem Pharmacol* 1977;**77:** 1–124.
36 Westfall TC. Local regulation of adrenergic neurotransmission. *Physiol Rev* 1977;**57:**659–728.



Downloaded from by guest on December 9, 2014