The site of angiotensin generation: focus on the heart

De plaats van angiotensine vorming,
met nadruk op het hart.

Proefschrift

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Chapter 1

General introduction

The renin-angiotensin system (RAS) has traditionally been viewed as a circulating system. Kidney-derived renin cleaves liver-derived angiotensinogen in the circulation, to form angiotensin I (AngI). Subsequently, AngII is generated from AngI by angiotensin-converting enzyme (ACE) located at the luminal side of the endothelium or by ACE in blood plasma. According to the classical concept the RAS is a hormonal system, designed to deliver the effector peptide AngII to different tissue sites, where it exerts its effects via stimulation of AngII receptors. Two types of AngII receptors have been described so far, AT_1 and AT_2[1].

AngII plays an important role in the cardiovascular homeostasis. Not only is it a potent vasoconstrictor, it also stimulates renal sodium reabsorption and adrenal aldosterone production and -release. Furthermore, it is believed to act as a growth factor[2]. All these effects are mediated via the AT_1 receptor. The role of the AT_2 receptor is less clear; it may oppose some of the AT_1 receptor-mediated effects[3-5]. The proportion of AT_1 and AT_2 receptors changes during development. In fetal tissues AT_2 receptor expression is dominant, but this abundance is followed by a rapid decline immediately after birth[6].

In light of the vasoconstrictor and growth stimulating effects of AngII it is not surprising that RAS blockade is beneficial in cardiovascular diseases. Renin inhibitors, although at present not further developed due to their poor oral bioavailability, reduce blood pressure effectively[7-9]. ACE inhibitors, the class of RAS inhibitors that was developed first, are effective blood pressure lowering agents, and cause regression of cardiac hypertrophy and postinfarction remodeling in subjects with heart failure[10,11]. This also appears to apply to AT_1-receptor antagonists, the most recently developed class of RAS inhibitors[12-16].

The effects of ACE inhibitors on cardiac hypertrophy were shown to be independent, at least partly, from the blood pressure lowering effect of these drugs[10,17]. This may indicate that ACE inhibitors have local effects in the heart, in addition to their blood pressure lowering effects. Indeed, the existence of a so-called local RAS, as opposed to the circulating RAS, has been proposed in the heart[18]. In fact, such local renin-angiotensin systems may exist in many organs. RAS components and their messenger RNAs have now been identified in kidney, adrenal, brain, ovary, testis, eye and heart[18-26]. Many investigators have speculated on the role and regulation of these local renin-angiotensin systems. So far it has been difficult to clearly separate the circulating- and the local RAS. It is even possible that the circulating RAS serves to deliver renin and angiotensinogen to tissue sites, where local angiotensin production may then occur.

The cardiac RAS is believed to play an important role in cardiac growth and remodeling[18]. A better understanding of how and where angiotensins are produced in the heart may shed further light on the mechanism by which RAS inhibitors exert their effects in the heart.

1.1 Circulating renin-angiotensin system

Renin

The kidney releases both renin and its inactive precursor, prorenin, into the circulation. Renal renin release is influenced by blood pressure, the sodium load of the organism, the activity of the sympathetic nervous system, and a number of humoral or locally generated factors. AngII inhibits renin release directly via AT_1-receptors and indirectly via a rise in blood pressure and an increase of the sodium chloride load.

At present, the kidney is the only organ that is known to convert prorenin into renin. Normally, approximately 90% of plasma total renin (renin +

prorenin) is present as prorenin. Following a bilateral nephrectomy, plasma renin, but not plasma prorenin, decreases to very low levels[27,28]. This suggests that the kidney is the main or only source of plasma renin, whereas prorenin may be synthesized extrarenally as well. Indeed, prorenin release into the circulation has been demonstrated for several extrarenal organs, e.g. the ovary, testis and adrenal[29,30].

The function of prorenin remains unclear. Recently, renin receptors have been described[31,32], which bind renin and prorenin equally well[31]. Renin binding at tissue sites may be involved in the cascade leading to local angiotensin generation. Possibly, prorenin competitively prevents renin from binding to these receptors, thereby blocking the local formation of AngII[31] (Fig. 1). Alternatively, prorenin might be converted to renin at tissue sites following receptor binding. This activation may occur either on the cell surface or intracellularly (Fig. 1)

Renin appears to be metabolized mainly by the liver[33]. The half-life of circulating renin is approximately 30 min[28]. Renal clearance of renin is very low[34]. Renin has been detected immunohistochemically in the proximal tubule, and low levels can be measured in urine.

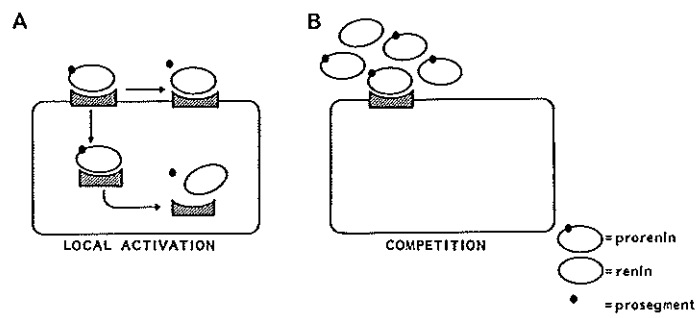

Figure 1. Prorenin binding to (pro)renin binding receptors may lead to prorenin activation (A), either extra- or intracellularly, thereby stimulating local angiotensin generation, or, when activation does not occur (B), it may competitively prevent renin from binding, thereby inhibiting local angiotensin generation.

Angiotensinogen

The liver is the main source of circulating angiotensinogen. Although angiotensinogen mRNA has been demonstrated in other organs (e.g. kidney, brain and heart)[35] it remains uncertain whether extrahepatically produced angiotensinogen contributes to the plasma levels of angiotensinogen.

Angiotensinogen production in the liver is stimulated by estrogens, glucocorticoids and AngII[36,37]. Plasma angiotensinogen is cleared mainly by the kidneys via receptor-mediated endocytosis, its elimination half-life being approximately 10 hours[38].

ACE

ACE has been detected on many cell types, but the conversion of circulating AngI is probably mainly mediated by endothelial ACE. Plasma ACE appears to be of minor importance[39]. Its levels are partly determined by the so-called insertion/deletion ACE gene polymorphism[40]. This may also apply to tissue (i.e., endothelial) ACE[41].

ACE expression is subject to negative feedback by AngII[42].

Angiotensin I and II

Both AngI and II are rapidly degraded by angiotensinases located in the vessel wall, their half-lives in the circulation being less than one minute. In early studies in sheep[43], AngI and II clearance across the pulmonary and combined systemic vascular beds was calculated from the arteriovenous differences measured across these beds during infusion of high doses of AngI or II into the pulmonary artery. These studies already indicated that the reaction of circulating renin with circulating angiotensinogen ("plasma renin activity", PRA) was insufficient to make up for the rapid degradation of both angiotensins.

More recently, the regional clearance and metabolism of AngI and AngII have been quantified, both in humans[44,45] and in pigs[39,46], by giving constant infusions of radiolabeled ^{125}I-AngI or ^{125}I-AngII. In humans the infusions were

given through an antecubital vein, and in pigs into the left cardiac ventricle. Blood was obtained from various arterial and venous sampling sites for measurements of radiolabeled AngI and II. Additional measurements of the levels of endogenous AngI and II, and measurements of PRA at physiological pH made it possible to estimate how much of the venous AngI could be attributed to arterial delivery, how much to de novo synthesis, and what proportion of de novo synthesized AngI depends on the action of circulating renin on circulating angiotensinogen. It was also possible to calculate how much of the venous angiotensin II originated from arterial delivery and how much originated from conversion of arterially delivered AngI.

It was found that, in all vascular beds studied[44, 46] (heart, kidney, limbs, liver, head, skin), a major proportion of venous AngI originated from de novo production and that PRA contributed little to this production. One can therefore conclude that AngI is produced at tissue sites and that part of it is released into the circulation. While most of venous AngI appears not to be generated by the action of circulating renin on circulating angiotensinogen, the level of venous AngI produced at tissue sites correlated strongly with the level of PRA[46]. This suggests that it is kidney-derived renin that is responsible for the production of AngI at tissue sites.

For AngII, the situation is different. Most, if not all, venous AngII appears to originate from AngII delivered by the artery and from that generated by conversion of arterially delivered angiotensin I[45]. Thus, angiotensin I produced at tissue sites and released into the circulation may have escaped conversion to AngII. It is possible that AngI produced in the tissue enters the blood at a level distal to the site where arterially delivered AngI is converted to AngII by the vascular endothelium, so that this conversion site is bypassed. AngI formed at tissue sites may enter the circulation at the level of the capillaries or venules, whereas conversion of AngI to AngII occurs at the level of the arterioles. Alternatively, AngII is produced in the tissue and may remain there.

Effect of RAS inhibitors on circulating RAS components

Inhibition of the RAS always results in the onset of feed-back processes (Table 1). Remikiren inhibits plasma renin activity, thereby leading to reduced plasma AngI and AngII concentrations[7]. Consequently, renin release from the kidney will increase, resulting in elevated immunoreactive renin levels[7,9]. However, due to the presence of the renin inhibitor, this renin is enzymatically inactive. ACE inhibitors also cause a rise in plasma renin, and as a result of that, a rise in plasma AngI. Plasma AngII is reduced initially, but it may rise to levels above normal during chronic treatment, as a result of the increased renin and AngI concentrations[47]. AT_1-receptor antagonists will not only increase plasma renin and AngI, but plasma AngII as well[13,14].

1.2 Cardiac renin-angiotensin system

Renin

Renin mRNA concentrations in normal hearts are very low or undetectable[48,49], suggesting that under normal conditions cardiac renin synthesis is unlikely to occur. It is possible, however, that during foetal development[50] or under pathological conditions[51], the renin gene is expressed in the heart. Cultured cardiac cells (cardiomyocytes, fibroblasts, vascular smooth muscle cells and endothelial cells) have all been reported to synthesize renin[50,52,53]. However, the relevance of such findings for the *in vivo* situation may be questioned. Moreover, since appropriate control measurements with specific renin inhibitors were usually not performed, it is possible that renin-like enzymes such as cathepsin D[54], and not renin itself, were detected. Renin has been measured in the heart of normal and nephrectomized pigs[55]. AngI-generating activity of cardiac tissue was identified as renin by its inhibition by a specific active site-directed renin inhibitor. The levels of renin in cardiac tissue (expressed per g

wet weight) were found to be similar to those in plasma. The tissue levels of renin, therefore, could not be accounted for by trapped plasma, suggesting that renin may have been sequestered actively by the heart. Both in cardiac tissue and in plasma, renin fell to undetectable levels after nephrectomy, suggesting that under normal circumstances most, if not all, renin present in the heart originates from the kidney.

Not much is known about cardiac renin sequestration. Renin, like ACE, was found to be enriched in a purified cardiac membrane fraction prepared from left ventricular tissue, suggesting that uptake of kidney-derived renin in the heart may occur through binding to cell membranes[55]. This would fit with the recently described renin receptors[31,32]. Binding to cell membranes is also suggested by chemical cross-linking studies using membrane fractions prepared from various tissues, including heart and blood vessels[56]. The possibility that prorenin, following its binding to these receptors, is locally activated cannot be excluded. However, such local activation has never been demonstrated.

Angiotensinogen

Angiotensinogen mRNA can be detected in the heart[22,57]. Its levels are approximately 1% of the angiotensinogen mRNA levels in the liver[22]. An increase in cardiac angiotensinogen mRNA has been reported following myocardial infarction[57], but this could not be confirmed by others[51]. Cultured neonatal rat cardiomyocytes and fibroblasts appeared to release angiotensinogen into the medium under serum-free conditions[50]. Other cells in the heart that have been reported to synthesize angiotensinogen are vascular smooth muscle cells[58] and endothelial cells[59].

The angiotensinogen concentration in porcine cardiac tissue are 10-25% of the levels in plasma, which is compatible with its diffusion from plasma into the interstitium[55]. Angiotensinogen is generally believed to be distributed equally across the extracellular fluid[60]. It appears therefore that cardiac angiotensinogen is largely derived from the circulation. The contribution of locally synthesized

angiotensinogen, if present at all, is probably small. In support of this assumption, the angiotensinogen concentrations in the coronary effluent of the isolated perfused rat Langendorff heart are < 0.1% of the levels normally found in rat blood plasma[61].

ACE

ACE has been demonstrated in the rat heart by autoradiography, using a radiolabeled ACE inhibitor[62], as well as by measurements of its activity in cardiac homogenates[63]. ACE mRNA is readily detectable in cardiac tissue[63]. According to some investigators, cardiac ACE is normally limited to the coronary vascular endothelial cells and the endocardium[64]. Cardiac ACE protein and mRNA are increased following myocardial infarction[64-66] as well as during pressure overload induced left ventricular hypertrophy[63]. Under these conditions the localization of ACE may no longer be limited to the endothelium. In humans, following myocardial infarction, ACE can be detected in the remaining viable cardiomyocytes near the infarct scar of the aneurysmal left ventricle, as well as in fibroblasts, vascular smooth muscle cells and macrophages in the scar area itself[65]. In rats, following coronary occlusion, ACE was demonstrated in fibroblasts in the healthy hypertrophying part of the heart[66].

Angiotensin I and II

The concentration of AngI in cardiac tissue (expressed per g wet weight) is similar to the level in plasma, whereas the cardiac tissue concentration of AngII is two to three times the level in plasma[55,67]. The cardiac angiotensin levels, therefore, are far too high to be explained by trapped blood or by simple diffusion of angiotensins from plasma into the interstitial fluid. However, such relatively high concentrations cannot be taken as unequivocal evidence that AngI and II are generated in cardiac tissue independently of the circulating renin-angiotensin system. For example, AngII may have been sequestered from the circulation by a receptor-dependent process.

As mentioned above, measurements of circulating angiotensins during ^{125}I-AngI infusion into the left cardiac ventricle provided evidence for the release of AngI from cardiac tissue sites. These measurements of circulating angiotensins provided no evidence for the release of tissue AngII into the circulation. Thus, taken together, the evidence available so far suggests that AngI and possibly AngII are generated in cardiac tissue, and that of these locally generated angiotensins , only AngI is released into the circulation. AngI and II may be formed in different tissue compartments which are not all capable of exchanging freely with the circulation. Levels of AngI and II were undetectably low in both plasma and cardiac tissue following a bilateral nephrectomy[55]. This is a strong indication that cardiac angiotensin generation *in vivo* depends on renin from the kidney.

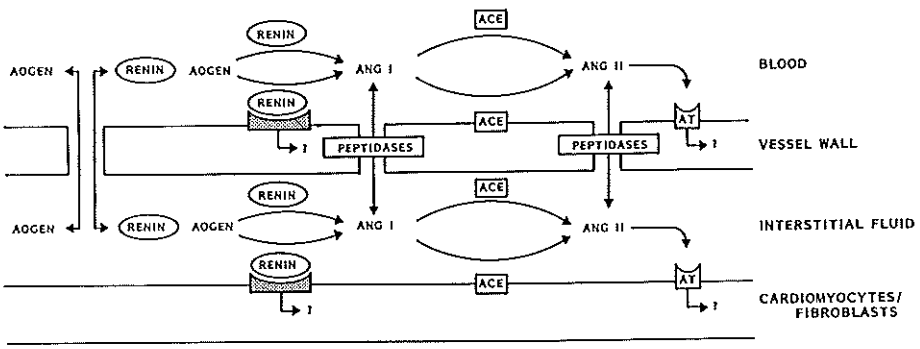

Figure 2. Origin of cardiac angiotensin I and II. See text (under Cardiac renin-angiotensin system, Angiotensin I and II) for a detailed description. Not addressed in the Figure are 1) intracellular angiotensin generation by locally synthesized renin or renin taken up from the circulation (depicted by a question mark) and 2) how AT_1 receptor-mediated endocytosis (also depicted by a question mark) might affect the intracellular AngII levels.

A scheme depicting the possible angiotensin production sites is shown in Fig. 2. Angiotensin production may either occur in extracellular fluid (blood plasma and/or interstitial fluid) or at the tissue-fluid interphase (i.e., on the cell membrane). A third possibility, not shown in the Figure, is intracellular production of angiotensin by renin that is locally synthesized or taken up from the circulation. Future investigation will have to address 1) what proportion of angiotensinogen cleavage occurs by intracellular renin, membrane-associated renin or renin in the fluid phase and 2) the exact localization of AngI and II in cardiac tissue (intracellular, extracellular or membrane-bound). Extracellularly formed AngII may, via receptor-mediated endocytosis, reach the intracellular compartment.

Effect of RAS inhibitors on cardiac RAS levels

At present, not much is known about changes in cardiac RAS component levels during treatment with RAS inhibitors (Table 1). Renin is elevated in cardiac tissue of both humans[68] and pigs[69] treated with ACE inhibitors, whereas cardiac angiotensinogen is decreased under these conditions[68,69]. ACE inhibition with perindopril led to a 2-4 fold increase in cardiac AngI[70]. Cardiac AngII did not change unless very high doses of quinalapril were applied. The AT_1 receptor antagonist losartan increased cardiac AngI and II approximately 7- and 2-fold, respectively[71]. As a consequence of these non-parallel changes in cardiac AngI and II, the cardiac AngII/I ratio decreased both with quinalapril and losartan. The decrease in cardiac AngII/I ratio during quinalapril treatment most likely illustrates the degree of ACE inhibition obtained in cardiac tissue. The decrease in cardiac AngII/I ratio during losartan treatment is more difficult to explain, especially since cardiac ACE activity was unchanged following losartan treatment[71]. The authors speculated that a proportion of the measured tissue level of AngII may have been receptor-bound and protected from metabolism and that the displacement of receptor-bound AngII by losartan may have accelerated local tissue metabolism of AngII, with a consequent decrease in the AngII/I ratio.

Table 1. Effect of renin inhibition, ACE inhibition and AT_1 receptor blockade on renin-angiotensin system component levels in blood plasma and heart.

	Immuno-reactive renin	Enzym-atically active renin	AngI	AngII	AngII/I ratio
Plasma					
renin inhibition	↑	↓	↓	↓	N.D.
ACE inhibition	↑	↑	↑	↓	↓
AT_1 receptor blockade	↑	↑	↑	↑	=
Heart					
renin inhibition	N.D.	N.D.	N.D.	N.D.	N.D.
ACE inhibition	↑	↑	↑	= ↓	↓
AT_1 receptor blockade	N.D.	N.D.	↑	↑	↓

↑, increase; ↓, decrease; =, no change; N.D., not done. Data are taken from references 7, 9, 13, 14, 68, 69, 70 and 71.

1.3 Aim of the thesis

In order to obtain detailed information about angiotensin production at tissue sites in the heart we have studied:

1. The cardiac uptake and clearance of arterially delivered renin, angiotensinogen, angiotensin I and angiotensin II.

2. The localization and clearance of locally generated angiotensin I and II in the heart.

We made use of a modified version of the isolated perfused rat Langendorff heart, which allowed the separate collection of coronary effluent (CE) and interstitial transudate (IST). RAS components were infused in the perfusion system and their presence in CE, IST and tissue was investigated under steady-state conditions and following discontinuation of their infusion (chapters 2-4). Infusion of AngI resulted in local generation of AngII, whereas during combined infusion of renin and angiotensinogen both AngI and II were locally generated (chapters 2 and 4). Infusions were performed under control conditions and in the presence of an AT_1 receptor antagonist in order to investigate the binding of AngII to cardiac AT_1 receptors (chapter 4). AT_1 receptor-mediated uptake of arterially delivered AngII in the heart and other organs, as well as the in vivo tissue half life of plasma AngII, was studied in pigs (chapter 5). Finally, AngI-to-II conversion on the endothelial surface and at deeper sites in coronary arteries was investigated using and in vitro model allowing luminal and adventitial administration of AngI (chapter 6).

1.4 References

1. de Gasparo M, Husain A, Alexander W, Catt KJ, Chiu AT, Drew M, Goodfriend Th, Harding JW, Inagami T, Timmermans PBMWM: Proposed update of angiotensin receptor nomenclature. *Hypertension.* 1995; 25: 924-927.
2. Schelling P, Fischer H, Ganten D: Angiotensin and cell growth: a link to cardiovascular hypertrophy? *J Hypertens.* 1991; 9: 3-15.
3. Stoll M, Steckelings M, Paul M, Bottari SP, Metzger R, Unger T: The angiotensin AT_2-receptor mediates inhibition of cell proliferation in coronary endothelial cells. *J Clin Invest.* 1995; 95: 651-657.
4. Ichiki T, Labosky PA, Shiota C, Okuyama S, Imagawa Y, Fogo A, Niimura F, Ichikawa I, Hogan BL, Inagami T: Effects on blood pressure and exploratory behaviour of mice lacking angiotensin II type-2 receptor. *Nature.* 1995; 377: 748-750.
5. Hein L, Barsh GS, Pratt RE, Dzau VJ, Kobilka BK: Behavioural and cardiovascular effects of disrupting the angiotensin II type-2 receptor in mice. *Nature.* 1995; 377: 744-747.
6. Grady EF, Sechi LA, Griffin CA, Schambelan M, Kalinyak JE: Expression of AT_2-receptors in the developing rat fetus. *J Clin Invest.* 1991; 88: 921-933.
7. Camenzind E, Nussberger J, Juillerat L, Munafo A, Fischli W, Coassolo P, van Brummelen P, Kleinbloesem CH, Waeber B, Brunner HR: Effect of the renin response during renin inhibition: oral Ro 42-5892 in normal humans. *J Cardiovasc Pharmacol.* 1991; 18: 299-307.
8. Ryan MJ, Hicks GW, Batley BL, Rapundalo ST, Patt WC, Taylor DG, Keiser JA: Effect of an orally active renin inhibitor CI-992 on blood pressure in normotensive and hypertensive monkeys. *J Pharmacol Exp Ther.* 1994; 268: 372-379.
9. van den Meiracker AH, Admiraal PJJ, Man in 't Veld AJ, Derkx FHM, Ritsema van Eck HJ, Mulder P, van Brummelen P, Schalekamp MADH: Prolonged blood pressure reduction by orally active renin inhibitor RO 42-5892 in essential hypertension. *Br Med J.* 1990; 301: 205-210.
10. Dahlöf B: Regression of left ventricular hypertrophy - are there differences between antihypertensive agents? *Cardiology.* 1992; 81: 307-315.
11. Latini R, Maggioni AP, Flather M, Sleight P, Tognoni G: ACE inhibitor use in patients with myocardial infarction. Summary of evidence from clinical trials. *Circulation.* 1995; 92: 3132-3137.
12. Goa KL, Wagstaff AJ: Losartan potassium: A review of its pharmacology, clinical efficacy and tolerability in the managment of hypertension. *Drugs.* 1996; 51: 820-845.
13. Azizi M, Chatellier G, Guyene TT, Murieta-Goeffroy D, Menard J: Additive effects of combined angiotensin-converting enzyme inhibition and angiotensin II antagonism on blood pressure and renin release in sodium-depleted normotensives. *Circulation.* 1995; 92: 825-834.
14. van den Meiracker AH, Admiraal PJJ, Janssen JA, Kroodsma JM, de Ronde WAM, Boomsma F, Sissmann J, Blankestijn PJ, Mulder PGM, Man in 't Veld AJ, Schalekamp MADH: Hemodynamic and biochemical effects of the AT_1 receptor antagonist irbesartan in hypertension. *Hypertension.* 1995; 25: 22-29.
15. Ruzicka M, Yuan B, Leenen FHM: Effects of enalapril versus losartan on regression of volume overload-induced cardiac hypertrophy in rats. *Circulation.* 1994; 90: 484-491.
16. Schieffer B, Wirger A, Meybrunn M, Seitz S, Holtz J, Riede UN, Drexler H: Comparative effects of chronic angiotensin-converting enzyme inhibition and angiotensin II type 1 receptor blockade on cardiac remodeling after myocardial infarction in the rat. *Circulation.* 1994; 89: 2273-2282

17. Linz W, Schaper J, Wiemer G, Albus U, Schölkens BA: Ramipril prevents left ventricular hypertrophy with myocardial fibrosis without blood pressure reduction: a one year study in rats. *Br J Pharmacol.* 1992; 107: 970-975.
18. Lindpaintner K, Ganten D: The cardiac renin-angiotensin system. An appraisal of present experimental and clinical evidence. *Circ Res.* 1991; 68: 905-921.
19. Campbell DJ, Lawrence AC, Towrie A, Kladis A, Valentijn AJ: Differential regulation of angiotensin peptide levels in plasma and kidney of the rat. *Hypertension.* 1991; 18: 763-773.
20. Kim S, Tokuyama M, Hosoi M, Yamamoto K: Adrenal and circulating renin-angiotensin system in stroke-prone hypertensive rats. *Hypertension.* 1992; 20: 280-291.
21. Lightman A, Tarlatzis BC, Rzasa PJ, Culler MD, Caride VJ: The ovarian renin-angiotensin system: renin-like activity and angiotensin II/III immunoreactivity in gonadotropin-stimulated and unstimulated human follicular fluid. *Am J Obstet Gynecol.* 1987; 156: 808-816.
22. Dzau VJ, Ellison KE, Brody T, Ingelfinger J, Pratt RE: A comparative study of the distributions of renin and angiotensinogen messenger ribonucleic acids in rat and mouse tissues. *Endocrinology.* 1987; 120: 2334-2338.
23. Inagami T: Renin in the brain and neuroblastoma cells: an endogenous and intracellular system. *Neuroendocrinology.* 1982; 34: 475-483.
24. Phillips MI, Stenstrom B: Angiotensin II in the brain comigrates with authentic angiotensin II in high pressure liquid chromatography. *Circ Res.* 1985; 72: 406-412.
25. Deinum J, Derkx FHM, Danser AHJ, Schalekamp MADH: Identification and quantification of renin and prorenin in the bovine eye. *Endocrinology.* 1990; 126: 1673-1682.
26. Danser AHJ, Derkx FHM, Admiraal PJJ, Deinum J, de Jong PTVM, Schalekamp MADH: Angiotensin levels in the eye. *Invest Ophthalmol Vis Sci.* 1994; 35: 1008-1018.
27. Sealey JE, White RP, Laragh JH, Rubin AL: Plasma prorenin and renin in anephric patients. *Circ Res.* 1977; 41 (suppl II): 17-21.
28. Derkx FHM, Wenting GJ, Man in 't Veld AJ, Verhoeven RP, Schalekamp MADH: Control of enzymatically inactive renin in man under various pathological conditions: implications for the interpretation of renin measurements in peripheral and renal venous plasma. *Clin Sci Mol Med.* 1978; 54: 529-538.
29. Lenz T, Sealey JE: Tissue renin systems as a possible factor in hypertension. In: Laragh JH, Brenner BM, eds. Hypertension: Pathophysiology, Diagnosis, and Management. 1990 New York: Raven Press Ltd. pp. 1319-1328.
30. Sealey JE, Goldstein M, Pitarresi T, Kudlak TT, Glorioso N, Fiamengo SA, Laragh JH: Prorenin secretion from human testis: no evidence for secretion of active renin or angiotensinogen. *J Clin Endocrinol Metab.* 1988; 66: 974-978.
31. Sealey JE, Catanzaro DF, Lavin TN, Gahnem F, Pitarresi T, Hu L-f, Laragh JH: Specific prorenin/renin binding (ProBP). Identification and characterization of a novel membrane site. *Am J Hypertens.* 1996; 9: 491-502.
32. Nguyen G, Delarue F, Berrou J, Rondeau E, Sraer J-D: Specific receptor binding of renin on human mesangial cells in culture increases plasminogen activator inhibitor-1 antigen. *Kidney Int.* 1996; 50:1897-1903.
33. Marks DL, Kost LJ, Kuntz SM, Romero JC, Larusso NF: Hepatic processing of recombinant human renin: mechanisms of uptake and degradation. *Am J Physiol.* 1991; 261: G349-G358.
34. Sealey JE, Laragh JH: The renin-angiotensin-aldosterone system for normal regulation of blood pressure and sodium and potassium homeostasis. In: Laragh JH, Brenner BM, eds. Hypertension: Pathophysiology, Diagnosis, and Management. 1990 New York: Raven Press Ltd. pp. 1287-1317.
35. Campbell DJ, Habener JF: Angiotensinogen gene is expressed and differentially regulated in multiple tissues of the rat. *J Clin Invest.* 1986; 78: 31-39.

36. Klett C, Hellmann W, Hackenthal E, Ganten D: Modulation of tissue angiotensinogen gene expression by glucocorticoids, estrogens, and androgens in SHR and WKY rats. *Clin Exp Hypertens.* 1993; 45: 683-708.
37. Herrmann HC, Morris BJ, Reid IA: Effect of angiotensin II and sodium depletion on angiotensinogen production. *Am J Physiol.* 1980; 238: E145-149.
38. Yayama K, Yoshiya M, Takahashi K, Matsui T, Takano M, Okamoto H: Role of the kidney in the plasma clearance of angiotensinogen in the rat: plasma clearance and tissue distribution of ^{125}I-angiotensinogen. *Life Sci.* 1995; 57: 1791-1801.
39. Danser AHJ, Koning MMG, Admiraal PJJ, Derkx FHM, Verdouw PP, Schalekamp MADH: Metabolism of angiotensin I by different tissues in the intact animal. *Am J Physiol.* 1992; 263: H418-H428.
40. Rigat B, Hubert C, Alhenc-Gelas F, Cambien F, Corvol P, Soubrier F: An insertion/deletion polymorphism in the angiotensin I converting enzyme gene accounting for half the variance of serum enzyme levels. *J Clin Invest.* 1990; 86: 1343-1346.
41. Danser AHJ, Schalekamp MADH, Bax WA, Maassen van den Brink A, Saxena PR, Riegger GAJ, Schunkert H: Angiotensin-converting enzyme in the human heart. Effect of the deletion/insertion polymorphism. *Circulation.* 1995; 92: 1387-1388.
42. Schunkert H, Ingelfinger JR, Hirsch AT, Pinto Y, Remme WJ, Jacob H, Dzau VJ: Feedback regulation of angiotensin converting enzyme activity and mRNA levels by angiotensin II. *Circ Res.* 1993; 72: 312-318.
43. Fei DT, Scoggins BA, Tregear GW, Coghlan JP: Angiotensin I, II and III in sheep. A model of angiotensin production and metabolism. *Hypertension.* 1981; 3: 730-737.
44. 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.
45. 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: 173-184.
46. Danser AHJ, Koning MMG, Admiraal PJJ, Sassen LMA, Derkx FHM, Verdouw PD, Schalekamp MADH: Production of angiotensins I and II at tissue sites in the intact pig. *Am J Physiol.* 1992; 263: H429-H437.
47. Mooser V, Nussberger J, Juillerat L, Burnier M, Waeber B, Bidiville J, Pauly N, Brunner HR: Reactive hyperreninemia is a major determinant of plasma angiotensin II during ACE inhibition. *J Cardiovasc Pharmacol.* 1990; 15: 276-282.
48. Ekker M, Tronik D, Rougeon F: Extra-renal transcription of the renin genes in multiple tissues of mice and rats. *Proc Natl Acad Sci USA.* 1989; 86: 5155-5158.
49. Iwai N, Inagami T: Quantitative analysis of renin gene expression in extrarenal tissues by polymerase chain reaction method. *J Hypertens.* 1992; 10: 717-24.
50. Dostal DE, Rothblum KN, Chernin MI, Cooper GR, Baker KM: Intracardiac detection of angiotensinogen and renin: a localized renin-angiotensin system in neonatal rat heart. *Am J Physiol.* 1992; 263: C838-C850.
51. Passier RCJJ, Smits JFM, Verluyten MJA, Daemen MJAP: Expression and localization of renin and angiotensinogen in rat heart after myocardial infarction. *Am J Phys.* 1996; 271: H1040-H1048.
52. Re R, Fallon JT, Dzau VJ, Quay SC, Haber E: Renin synthesis by canine aortic smooth muscle cells in culture. *Life Sci.* 1982; 30: 99-106.
53. Lilly LS, Pratt RE, Alexander RW, Larson DM, Ellison KE, Gimbrone MA, Dzau VJ: Renin expression by vascular endothelial cells in culture. *Circ Res.* 1985; 57: 312-318.

54. Hackenthal E, Hackenthal R, Hilgenfeldt U: Isorenin, pseudorenin, cathepsin D and renin. A comparative enzymatic study of angiotensin-forming enzymes. *Biochim Biophys Acta.* 1978; 522: 574-588.
55. Danser AHJ, van Kats JP, Admiraal PJJ, Derkx FHM, Lamers JMJ, Verdouw PD, Saxena PR, Schalekamp MADH: Cardiac renin and angiotensins. Uptake from plasma versus in situ synthesis. *Hypertension.* 1994; 24: 37-48
56. Campbell DJ, Valentijn AJ: Identification of vascular renin-binding proteins by chemical cross-linking: inhibition of binding of renin by renin inhibitors. *J Hypertens.* 1994; 12: 879-890.
57. Lindpaintner K, Lu W, Niedermaier N, Schieffer B, Just H, Ganten D, Drexler H: Selective activation of cardiac angiotensinogen gene expression in post-infarction ventricular remodeling in the rat. *J Mol Cell Cardiol.* 1993; 23: 133-143.
58. Naftilan AJ, Zuo WM, Ingelfinger J, Ryan TJ, Pratt RE, Dzau VJ: Localization and differential regulation of angiotensinogen mRNA expression in the vessel wall. *J Clin Invest.* 1991; 87: 1300-1311.
59. Kifor I, Dzau VJ: Endothelial renin-angiotensin pathway: Evidence for intracellular synthesis and secretion of angiotensins. *Circ Res.* 1987; 60: 422-428.
60. Lynch KR, Peach MJ: Molecular biology of angiotensinogen. *Hypertension.* 1991; 17: 263-269.
61. Lindpaintner K, Jin M, Niedermaier N, Wilhelm MJ, Ganten D. Cardiac angiotensinogen and its local activation in the isolated perfused beating heart. *Circ Res.* 1990; 67: 564-573.
62. Yamada H, Fabris B, Allen AM, Jackson B, Johnston CI, Mendelsohn FAO: Localization of angiotensin converting enzyme in rat heart. *Circ Res.* 1991; 68: 141-149.
63. Schunkert H, Dzau VJ, Tang SS, Hirsch AT, Apstein CS, Lorell BH: Increased rat cardiac angiotensin converting enzyme activity and mRNA expression in pressure overload left ventricular hypertrophy. Effects on coronary resistance, contractility, and relaxation. *J Clin Invest.* 1990; 86: 1913-1920.
64. Falkenhahn M, Franke F, Bohle RM, Zhu YC, Stauss HM, Bachmann S, Danilov S, Unger T: Cellular distribution of angiotensin-converting enzyme after myocardial infarction. *Hypertension.* 1995; 25: 219-226.
65. Hokimoto S, Yasue H, Fujimoto K, Yamamoto H, Nakao K, Kaikita K, Sakata R, Miyamoto E: Expression of angiotensin-converting enzyme in remaining viable myocytes of human ventricles after myocardial infarction. *Circulation.* 1996; 94: 1513-1518.
66. Sun Y, Cleutjens JPM, Diaz-Arias AA, Weber KT: Cardiac angiotensin converting enzyme and myocardial fibrosis in the rat. *Cardiovasc Res.* 1994; 28: 1423-1432.
67. Campbell DJ, Kladis A, Duncan AM: Nephrectomy, converting enzyme inhibition and angiotensin peptides. *Hypertension.* 1993; 22: 513-522.
68. Danser AHJ, van Kesteren CAM, Bax WA, Tavenier M, Derkx FHM, Saxena PR, Schalekamp MADH: Prorenin, renin, angiotensinogen and angiotensin-converting enzyme in normal and failing human hearts. Evidence for renin-binding. *Circulation.* 1997; in press.
69. van Kats JP, Sassen LMA, Danser AHJ, Admiraal PJJ, Verdouw PD, Schalekamp MADH: ACE-inhibition in the heart is overcome by increased cardiac renin uptake. *J Hypertens.* 1994; 12 (suppl 3): S95.
70. Campbell DJ, Kladis A, Duncan AM: Effects of converting enzyme inhibitors on angiotensin and bradykinin peptides. *Hypertension.* 1994; 23: 439-449.
71. Campbell DJ, Kladis A, Valentijn AJ: Effects of losartan on angiotensin and bradykinin peptides and angiotensin-converting enzyme. *J Cardiovasc Pharmacol.* 1995; 26: 233-240.

Chapter 2

Renin-angiotensin system components in the interstitial fluid of the isolated perfused rat heart. Local production of angiotensin I.

2.1 Abstract

A modification of the isolated perfused rat heart, in which coronary effluent (CE) and interstitial transudate (IST) were separately collected, to investigate the uptake and clearance of exogenous renin, angiotensinogen and angiotensin I (AngI) as well as the cardiac production of AngI. The levels of these compounds in IST were considered to be representative of the levels in the cardiac interstitial fluid. During perfusion with renin or angiotensinogen, the steady-state levels (mean ± s.d.) in IST were 64 ± 34% ($p<0.05$ for difference from the arterial level, n=8) and 108 ± 42% (n=6) of the arterial level, respectively, and the levels in CE were not significantly different from those in IST. AngI was not detectable in IST during perfusion with Tyrode's buffer or angiotensinogen. It was very low in IST during perfusion with renin, and rose to much higher levels during combined renin/angiotensinogen perfusion. The total production rate of AngI present in interstitial fluid could be largely explained by the renin-angiotensinogen reaction in the fluid phase of the interstitial compartment. In contrast, the total production rate of AngI present in CE and the net ejection rate of AngI via CE were, respectively, 4.6 ± 2.2 and 2.8 ± 1.3 ($p<0.01$ and $p<0.05$ for difference from 1.0, n=6) times higher than could be explained by AngI formation in the fluid phase of the intravascular compartment. AngI from the interstitial fluid contributed little to the AngI in the intravascular fluid, and *vice versa*. These data reveal two tissue sites of AngI production, i.e. the interstitial fluid and a site closer to the blood compartment, possibly vascular surface-bound renin. There was no evidence that the release of locally produced AngI into CE and IST occurred independently of blood-derived renin or angiotensinogen.

2.2 Introduction

A local renin-angiotensin system (RAS) in the heart may contribute to the pathogenesis of congestive heart failure, cardiac hypertrophy and remodeling, and reperfusion arrhythmias[1-4]. The RAS components, renin, angiotensinogen, angiotensin-converting enzyme (ACE), and angiotensin (Ang) I and II, which are all present in circulating blood, have also been identified in cardiac tissue[5,6]. In addition, the tissue concentrations of AngI and II are too high to be explained simply by passive diffusion out of the blood and distribution into the interstitial fluid[6].

Perfusion of the isolated rat Langendorff heart with AngI leads to the appearance of AngII in the coronary effluent[7,8], and perfusion with renin leads to the appearance of both AngI and II[7]. Part of the AngI in the coronary venous blood appears to originate from local production at cardiac tissue sites[9], but it is not known how much of this local production depends on renin that is synthesized by the heart and how much on renin from the kidney. It has been reported that, in pigs, the release of locally produced AngI into the coronary circulation is directly proportional to the level of renin activity in circulating plasma[9], and that the cardiac tissue levels of renin, AngI and AngII are undetectably low 30 hours after bilateral nephrectomy, when plasma renin activity was practically zero[6]. In the rat, the cardiac tissue levels of AngI and II were also lowered by nephrectomy, although small quantities of AngII are still detectable both in cardiac tissue and in blood[5]. These observations indicate that the presence of AngI and II in cardiac tissue depends, at least in part, on kidney-derived renin.

Little is known about the cardiac uptake of blood-derived RAS components. The sites of cardiac AngI and II production are also unknown. A unique model to address these issues is a modified version of the Langendorff heart[10-12], which enables the investigator to separately collect the coronary effluent and the transudate derived from the interstitial fluid compartment. We report here on the use of this model to study the transport and distribution of blood-derived renin and angiotensinogen and to investigate the local intracardiac production of AngI.

2.3 Materials and methods

2.3.1 Chemicals

[Ile^5]-Ang-(1-10) decapeptide (AngI) was obtained from Bachem, Bubendorf, Switzerland. ^{51}Cr-EDTA and ^{125}I-labeled human serum albumin (HSA) were obtained from Amersham, 's Hertogenbosch, The Netherlands. Bovine serum albumin (BSA) was from Sigma, St. Louis, MO, USA. 1,10-phenanthroline was from Merck, Darmstadt, Germany. Sodium pentobarbital was obtained from Apharma, Arnhem, The Netherlands. The AT_1 receptor antagonist, losartan, was a kind gift of Dr. R.D. Smith, Du Pont Merck, Wilmington, Del., USA. The renin inhibitor, remikiren, was a kind gift of Dr. P. van Brummelen, Hoffmann-La Roche, Basel, Switzerland. All other reagents were of standard laboratory grade.

2.3.2 Preparation of renin and angiotensinogen

Renin was prepared from rat or porcine kidneys. Both rat renin and porcine renin were used to perfuse the rat Langendorff hearts. Most of these perfusions were carried out with porcine renin, because of the limited availability of sufficient quantities of rat renin.

Angiotensinogen was prepared from plasma of nephrectomized rats, pigs or sheep. Rat angiotensinogen was used as a substrate for rat renin measurements, and porcine or sheep angiotensinogen was used for porcine renin measurements. Under the conditions of our experiments, sheep angiotensinogen yielded higher quantities of AngI than porcine angiotensinogen when incubated with porcine renin. The angiotensinogens were also used to perfuse the Langendorff heart. Most of these perfusions were carried out with porcine or sheep angiotensinogen, because of the limited availability of sufficient quantities of rat angiotensinogen.

For renin preparation, kidney tissue was homogenized (1/1, weight/volume) with a Polytron PT10/35 (Kinematica, Luzern, Switzerland) in 0.01 mol/L phosphate buffer, pH 7.4, containing 0.15 mol/L NaCl. The homogenate was dialyzed for 48 h at 4 °C against 0.05 mol/L glycine buffer, pH 3.3, containing 0.001 mol/L disodium EDTA and 0.095 mol/L NaCl[6]. This was followed by dialysis for 24 h against 0.1 mol/L phosphate buffer, pH 7.4, containing 0.001 mol/L disodium EDTA and 0.075 mol/L NaCl. The content of the dialysis bag was then collected, and denatured protein was removed by centrifugation at 20,000*g* for 20 min at 4 °C. The supernatant ('semipurified renin') was stored at -80 °C. The renin concentration was 125 pmol AngI/min per mL in the rat renin preparation, and 600 pmol AngI/min per mL in the porcine renin preparation, as assessed by incubation with rat and porcine angiotensinogen, respectively[6].

Angiotensinogen was prepared as described before[13]. The semipurified preparations of rat, porcine and sheep angiotensinogen were stored at -80 °C. The angiotensinogen concentrations in these preparations were 2500, 500 and 300 pmol/mL respectively.

2.3.3 Preparation of the modified Langendorff heart

All experiments were performed under the regulations of the Animal Care Committee of the Erasmus University, Rotterdam, The Netherlands, in accordance with the 'Guiding principles in the care and use of animals' as approved by the American Physiological Society.

Male Wistar rats (Harlan, Zeist, The Netherlands; 280-400 g) were anesthetized with pentobarbital (60 mg/kg, IP), and heparinized (5,000 units/kg, IV). The hearts (1.0-1.4 g) were rapidly excised, cooled in ice-cold Tyrode's buffer (125 mmol/L NaCl, 4.7 mmol/L KCl, 1.4 mmol/L $CaCl_2$, 20 mmol/L $NaHCO_3$, 0.4 mmol/L NaH_2PO_4, 1.0 mmol/L $MgCl_2$, and 10 mmol/L D-glucose, pH 7.4) until contractions stopped, and prepared for Langendorff perfusion. Continuously carbogen-gassed

(95% O_2/5% CO_2) Tyrode's buffer at 37 °C was perfused immediately after cannulation of the aorta, at a constant perfusion pressure of 80 mm Hg. Coronary flow was between 4 and 8 mL/min. Subsequently the pulmonary artery was cannulated and the caval and pulmonary veins were carefully ligated (Fig 1). After the ligation procedure, which took 30-45 minutes, the hearts were stabilized for 30 min.

With this modified Langendorff heart preparation it is possible to collect separately coronary effluent and interstitial transudate[10-12]. Coronary effluent (CE), ejected by the right ventricle, was collected via the cannulated pulmonary artery. Dead space of the pulmonary cannula was 0.1 mL. Interstitial transudate (IST), which keeps dripping from the heart, was collected at the apex. IST flow was 0.03-0.16 mL/min corresponding with 0.7-2% of the coronary flow. An IST flow > 2% of the coronary flow was considered to be an indication of leakage, e.g. from veins that were not properly ligated. Hearts with such a high IST flow were therefore not used.

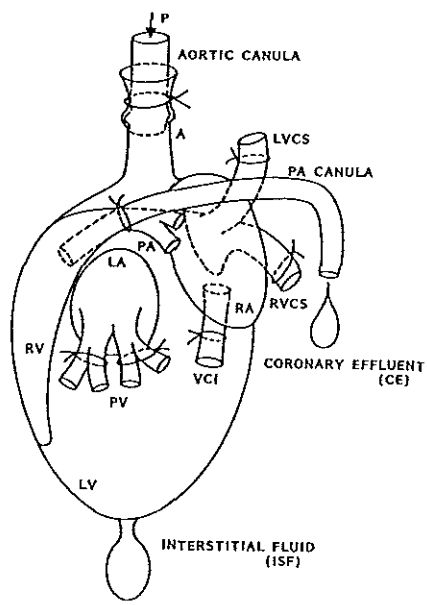

Figure 1. Modified Langendorff rat heart preparation. P, perfusate; A, aorta; PA, pulmonary artery; LVCS, left vena cava superior; LA, left atrium; RVCS, right vena cava superior; RA, right atrium; VCI, vena cava inferior; RV, right ventricle; PV, pulmonary veins; LV, left ventricle.

2.3.4 Checking for leakage of perfusate into the interstitial transudate (IST)

The hearts were perfused with red blood cells to check for leakage of perfusate into IST. The blood cells were isolated from heparinized (30 units/mL) rat blood and washed two times with carbogen-gassed Tyrode's buffer, containing heparin (30 units/mL). The cells were diluted in this buffer to a concentration of $5x10^9$ cells/mL, and infused via a T-connection, into the cannulated aorta by using a Harvard 22 pump (South Natick, MA, USA) at a speed of 0.1 mL/min for either 10 or 20 min. The cells were counted in CE and IST samples collected from the moment the infusion was started to the end of infusion.

2.3.5 Measurements of intravascular and interstitial fluid volumes

The hearts were perfused with ^{125}I-HSA or ^{51}Cr-EDTA. The radiolabeled markers were diluted to a concentration of 0.2 μCi/mL with Tyrode's buffer, and infused, via a T-connection, into the cannulated aorta at a speed of 0.1 mL/min. ^{125}I-HSA was infused for 1 min, and ^{51}Cr-EDTA for 10 min.

One-min CE samples and individual IST drops (approximately 50 μL) were collected during the infusion period, and the hearts were removed immediately after the perfusion had been switched off. Radioactivity levels (counts per minute or cpm) of CE, IST and the whole heart were measured with a Minaxi 5000 multiple channel gamma-counter (Packard Instruments, IL, USA). Intravascular and extracellular fluid volumes (mL/g heart wet weight) were considered to be equal to the distribution volumes of ^{125}I-HSA and ^{51}Cr-EDTA, respectively, in the heart, and were calculated as the ratio between the radioactivity of the heart (cpm/g) and the radioactivity of CE (cpm/mL). The intravascular compartment contains the fluid present in the coronary vascular bed and right ventricle. The interstitial fluid volume (mL/g) was calculated by subtracting the intravascular fluid volume from the extracellular fluid volume.

2.3.6 Perfusions with renin-angiotensin system components and collection of coronary effluent (CE) and interstitial transudate (IST)

The Langendorff hearts were perfused with Tyrode's buffer via the cannulated aorta. The buffer contained the AT_1 receptor antagonist, losartan, in a concentration of 10^{-6} mol/L. This concentration is sufficient to prevent AngII-mediated vasoconstriction[14]. After a 30-min stabilization period, the RAS components were infused, via a T-connection, into the cannulated aorta at a speed of 0.1 mL/min.

CE and IST were collected during and after the infusions. One-min (4-8 mL) or 4- to 5-min (16-40 mL) samples of CE were collected into BSA-coated 10- or 50-mL polystyrene tubes, and 1-min (approximately 50 µL) or 9- to 10-min (approximately 450-500 µL) samples of IST were collected into BSA-coated 1.5-mL Eppendorf cups. The Eppendorf cups and polystyrene tubes used to collect samples for AngI measurement contained a mixture of inhibitors, 5 or 25 µL in the Eppendorf cups (for the 1-min and 9- to 10-min IST samples) and 250 or 2500 µL (for the 1-min and 9- to 10-min CE samples) in the polystyrene tubes, in order to prevent *ex vivo* formation of AngI, conversion of AngI to AngII and the degradation of AngI. The mixture consisted of 0.2 mmol/L of the renin inhibitor remikiren, 125 mmol/L disodium EDTA and 25 mmol/L 1,10-phenanthroline[6]. Remikiren is an inhibitor of human renin (IC_{50} 7 x 10^{-10} mol/L). It also inhibits porcine renin (IC_{50} 5 x 10^{-8} mol/L)[6]. The Eppendorf cups and polystyrene tubes were kept on ice during the perfusions, so that the samples were rapidly cooled during their collection and remained cold (0-4 °C) during the experiment. After the experiment was finished, the samples for AngI measurement were frozen at -80 °C. Samples for the measurement of renin and angiotensinogen were frozen at -20 °C.

2.3.7 Control perfusion with Tyrode's buffer to study the release of endogenous renin, angiotensinogen and angiotensin I

Langendorff hearts were perfused with Tyrode's buffer for a period of 40 min. The

30-min stabilization period after heart preparation (see above) was omitted here, because we assumed that after such a long period the endogenous RAS components would have been washed away from the CE and IST. Nine-min collections of CE and IST were used for measurement of AngI. Each 9-min collection was followed by a 1-min collection for measurement of renin and angiotensinogen.

2.3.8 Perfusion with renin, angiotensinogen or angiotensin I to study the uptake and washout of exogenous renin-angiotensin system components

Langendorff hearts were perfused either with porcine renin, diluted 1/8 with Tyrode's buffer, with undiluted porcine angiotensinogen, or with AngI, diluted to a concentration of 400 pmol/mL with Tyrode's buffer. The RAS components were infused into the perfusion system for 60 min (renin and angiotensinogen) or 15 min (AngI). After the infusion had been switched off, the hearts were either rapidly frozen in liquid nitrogen, or subjected to a 10-min washout period.

One-min samples of CE and individual drops of IST were collected during the infusion and washout periods for measurement of renin, angiotensinogen or AngI. The frozen hearts were used for measurement of the steady-state tissue levels of these RAS components. Hearts frozen after 60 min of perfusion with Tyrode's buffer served as controls.

2.3.9 Perfusion with renin or angiotensinogen or renin combined with angiotensinogen to study the cardiac production of angiotensin I

Langendorff hearts were perfused with rat renin or angiotensinogen to study AngI production from endogenous (rat) angiotensinogen and renin respectively. Other Langendorff hearts were perfused with porcine or sheep angiotensinogen to investigate whether these hearts were capable of producing AngI, in the absence of exogenous

renin, possibly by the action of a renin-like enzyme in the heart. Renin, diluted 1/4 with Tyrode's buffer or undiluted angiotensinogen were infused into the perfusion system for 40 min, followed by 10 min washout. Nine-min collections of CE and IST were used for measurement of AngI. Each 9-min collection was followed by a 1-min collection for measurement of renin or angiotensinogen.

Finally, a number of Langendorff hearts were perfused with porcine renin combined with porcine or sheep angiotensinogen to study the cardiac production of AngI from both exogenous renin and exogenous angiotensinogen. Renin, diluted 1/4 with Tyrode's buffer, and undiluted angiotensinogen were infused into the perfusion system for 60 min, followed by a 30 min washout. The renin and angiotensinogen solutions were kept at 0-4 °C with ice until they reached the cannulated aorta. Ten-min collections of CE and IST were used for measurement of AngI. Each 10-min collection period was followed by a 5-min collection for measurement of renin and angiotensinogen.

2.3.10 Biochemical measurements

Renin

The concentration of renin in CE, IST or cardiac tissue homogenate was determined by measuring the rate of AngI generation during incubation, at pH 7.4 and 37 °C, with known amounts of angiotensinogen, in the presence of a mixture of ACE-, angiotensinase-, and serine protease-inhibitors[6]. Renin concentration was defined as the maximal AngI generation rate (V_{max}) at saturating concentrations of angiotensinogen.

For measurement of renin in cardiac tissue, the hearts were rapidly frozen in liquid nitrogen. The frozen hearts were then minced and homogenized (1/3, weight/volume) in 0.01 mol/L phosphate buffer, pH 7.4, containing 0.15 mol/L NaCl, with a Polytron PT10/35. Homogenate used for the measurement of renin was treated as follows. One mL of homogenate was dialyzed for 48 hrs at 4 °C against 0.05

mol/L glycine buffer, pH 3.3, containing 0.095 mol/L NaCl[6,15]. This was followed by dialysis at 4 °C for 24 hrs against 0.1 mol/L phosphate buffer, pH 7.4, containing 0.075 mol/L NaCl. The content of the dialysis bags was then collected, denatured protein was removed by centrifugation at 20,000*g* for 20 min at 4 °C, and volume was adjusted to 1 mL with phosphate buffer. Experiments, in which 0.1 mL rat or porcine renin was added to 1 g frozen tissue before homogenization, showed that the recovery of renin was better than 90%.

In the experiments, in which the hearts were perfused with Tyrode's buffer (control perfusion) or with rat renin, the renin concentration was determined by incubation with rat angiotensinogen. In the experiments, in which the hearts were perfused with porcine renin, the renin concentration was determined with the use of porcine angiotensinogen. In the experiments, in which the hearts were perfused with porcine renin combined with porcine angiotensinogen or sheep angiotensinogen, the renin concentration was determined by using porcine or sheep angiotensinogen, respectively.

The incubation mixture of the renin assay consisted of (1) 100 µL undiluted CE or 100 µL IST diluted 1/3 in 0.01 mol/L phosphate buffer, pH 7.4, containing 0.15 mol/L NaCl, or 100 µL acid-pretreated tissue homogenate, (2) 100 µL of 0.01 mol/L phosphate buffer, pH 7.4, containing 0.15 mol/L NaCl, (3) 200 µL angiotensinogen, and (4) 14 µL of an inhibitor mixture containing phenylmethylsulfonyl fluoride (0.07 mol/L), disodium EDTA (0.14 mol/L), 8-hydroxyquinoline sulfate (0.10 mol/L), and aprotinin (2000 kallikrein-inhibiting units per mL).

Incubation time was 1 or 2 hours, and AngI generation was linear during this period. AngI was measured with a sensitive radioimmunoassay[16]. V_{max} was calculated according to the equation: $V_{max} = V \times (K_m + [S])/[S]$, in which V is the measured AngI generation rate, K_m is the Michaelis-Menten constant and [S] is the angiotensinogen concentration in the incubate. K_m was determined by 5-, 10- and 20-min incubations of renin with serial dilutions of angiotensinogen and by constructing Lineweaver-Burk plots of the measured AngI generation rates. K_m for the reaction of rat renin with rat angiotensinogen was 2400 pmol/mL, which agrees with the values

reported in the literature[17,18]. K_m was 420 pmol/mL for the reaction of porcine renin with porcine angiotensinogen and 110 pmol/mL for the reaction of porcine renin with sheep angiotensinogen.

The lowest level that could be measured for rat renin (incubated with rat angiotensinogen) was approximately 10 fmol AngI/min per mL in CE, 40 fmol/min per mL in IST and 40 fmol AngI/min per g in cardiac tissue. For porcine renin (incubated with either porcine or sheep angiotensinogen), it was approximately 5 fmol AngI/min per mL in CE, 25 fmol AngI/min per mL in IST and 25 fmol AngI/min per g in cardiac tissue.

Angiotensinogen

The concentration of angiotensinogen in CE, IST or cardiac tissue homogenate was measured as the maximum quantity of AngI generated during incubation, at pH 7.4 and 37 °C, with porcine kidney renin, in the presence of a mixture of ACE-, angiotensinase- and serine protease-inhibitors[6]. For measurement of angiotensinogen in cardiac tissue, the frozen hearts were rapidly minced and homogenized as described above under 'Renin', but the dialysis step that was used for renin measurement, was omitted here. Experiments, in which 0.1 mL rat, porcine or sheep angiotensinogen was added to 1 g frozen tissue before homogenization, showed that the recovery of angiotensinogen was better than 90%.

The incubation mixture of the angiotensinogen assay consisted of (1) 100 µL undiluted CE, 100 µL IST diluted 1/3 in 0.01 mol/L phosphate buffer, pH 7.4, containing 0.15 mol/L NaCl, or 100 µL tissue homogenate, (2) 150 µL porcine renin diluted 1/50 in 0.01 mol/L phosphate buffer, pH 7.4, containing 0.15 mol/L NaCl, and (3) 14 µL inhibitor mixture (see above). Incubation time was 1 hour, and the conditions of the assay were chosen in such a way that AngI formation was completed within 1 hour.

The lowest level of angiotensinogen that could be measured were 0.1 pmol/mL in CE, 0.4 pmol/mL in IST, and 0.4 pmol/g in cardiac tissue.

Angiotensin I

The AngI concentration of CE and IST, collected during AngI perfusion, was measured directly with a sensitive AngI radioimmunoassay[16]. Measurements were made in 50 µL undiluted CE and 50 µL IST diluted 1/1.5 in 0.25 mol/L phosphate buffer, pH 7.4, containing 0.15 mol/L NaCl. Recovery of AngI added to CE or IST was better than 95%. The lowest level of AngI that could be measured with the direct radioimmunoassay were 15 fmol/mL in CE and 40 fmol/mL in IST.

The AngI concentration of CE, IST or cardiac tissue, during perfusion with Tyrode's buffer, renin, angiotensinogen or renin combined with angiotensinogen, was measured by radioimmunoassay, after SepPak extraction and reversed phase high performance liquid chromatography (HPLC) separation[6,9,16]. For measurement of AngI, the frozen hearts were minced and homogenized (1/10, weight/volume) in an iced solution of 0.1 mol/L HCl/80% ethanol. Homogenates were centrifuged at 20,000*g* for 25 min at 4 °C. Ethanol in the supernatant was evaporated under constant air flow. The remainder of the supernatant was diluted in 20 mL of 1% ortho-phosphoric acid and centrifuged again at 20,000*g*. The supernatant was diluted with 1% ortho-phosphoric acid 1:1 (vol/vol). CE, IST or tissue homogenate supernatants were concentrated over the SepPak columns (SepPak C18, Waters, Millford, MA, USA), and the concentrated extracts were subjected to HPLC followed by radioimmunoassay. ^{125}I-labeled AngI had been added to the samples before SepPak extraction (CE and IST samples) or before cardiac tissue homogenization (tissue homogenate samples), as an internal standard. Recovery was better than 70%, and the AngI results were corrected for incomplete recovery. The lowest level of AngI that could be measured with the AngI radioimmunoassay after HPLC separation were 0.05 fmol/mL in CE, 2.5 fmol/mL in IST and 2.0 fmol/g in cardiac tissue.

2.3.11 Calculations

In our calculations, IST that is dripping from the heart is distinguished from the interstitial fluid (ISF) that is present in cardiac tissue. A distinction is also made

between *exogenous* arterially delivered AngI and *endogenous* AngI that is formed in the Langendorff preparation.

The production of *endogenous* AngI present in ISF (fmol/min) was calculated as follows:

$$AngI_{ISF} \text{ production} = [AngI_{IST}] \times \text{clearance of } AngI_{ISF} \quad (1)$$

in which $[AngI_{IST}]$ is the steady state concentration of *endogenous* AngI (fmol/mL) in IST.

The clearance of $AngI_{ISF}$ was calculated according to the equation:

$$\text{Clearance of } AngI_{ISF} = \text{ISF volume} \times \ln 2/t_{1/2} \quad (2)$$

in which the cardiac ISF volume (mL) is the difference between the distribution volumes of ^{51}Cr-EDTA and ^{125}I-HSA, and $t_{1/2}$ is the half-life (min) of *exogenous* $AngI_{IST}$, as measured in the AngI perfusion experiments.

The production rate of $AngI_{ISF}$ (fmol/min,) predicted on the basis of the renin-angiotensinogen reaction in the fluid phase of the interstitial compartment, was derived from the following equation:

$$AngI_{ISF} \text{ production in the fluid phase} = AGA_{IST} \times \text{interstitial fluid volume} \quad (3)$$

in which AGA_{IST} is the AngI-generating activity (fmol AngI/min per mL) of IST caused by the renin-angiotensinogen reaction in the fluid phase of the interstitial compartment. AGA_{IST} was derived from the levels of renin and angiotensinogen in IST and from the Michaelis-Menten constant according to the equation:

$$AGA_{IST} = [R_{IST}] \times [Aog_{IST}]/(K_m + [Aog_{IST}]) \quad (4)$$

in which $[R_{IST}]$ is the concentration of renin (fmol AngI/min per mL) measured in

IST, [Aog_{IST}] is the concentration of angiotensinogen (pmol/mL) in IST, and K_m (pmol/mL) is the Michaelis-Menten constant (see 'Biochemical Measurements' above').

$AngI_{ISF}$ production calculated according to equation (1) is referred to as 'measured' $AngI_{ISF}$ production, because the independent variables in this equation were measured experimentally. $AngI_{ISF}$ production, predicted by equation (3), is referred to as 'predicted' Ang_{ISF} production.

The production rate of *endogenous* $AngI_{CE}$ (fmol/min), was calculated as follows:

$$AngI_{CE} \text{ production} = [AngI_{CE}] \times Q/(1\text{-}ER) \quad (5)$$

in which [$AngI_{CE}$] is the steady state concentration of *endogenous* AngI (fmol/mL) in CE, Q is the perfusate flow (mL/min), and ER is the extraction ratio of AngI. ER was calculated as the fraction of *exogenous* AngI that is extracted from the perfusion fluid during its passage from the arterial to the venous side of the coronary vascular bed, as measured in the AngI perfusion experiments. This equation may overestimate $AngI_{CE}$ production, because it accounts for the extraction of arterially delivered AngI, whereas *endogenous* AngI is added to the perfusion fluid during its passage through the coronary vascular bed.

The net ejection rate of *endogenous* AngI (fmol/min) via CE was calculated as follows:

$$AngI_{CE} \text{ ejection} = [AngI_{CE}] \times Q \quad (6)$$

The production rate of $AngI_{CE}$ (fmol/min), predicted on the basis of the renin-angiotensinogen reaction in the fluid phase of the intravascular compartment, was derived from the following equation:

$AngI_{CE}$ production in the fluid phase = AGA_{CE} x (intravascular fluid volume + 0.1

mL) (7)

in which AGA_{CE} is the AngI-generating activity (fmol AngI/min per mL) of the intravascular compartment caused by the renin-angiotensinogen reaction in the fluid phase. The intravascular fluid volume (mL) is the distribution volume of ^{125}I-HSA, and 0.1 mL is the dead space of the pulmonary artery cannula. AGA_{CE} was derived from the levels of renin and angiotensinogen in CE and from the Michaelis-Menten constant according to the equation:

$$AGA_{CE} = [R_{CE}] \times [Aog_{CE}]/(K_m + [Aog_{CE}]) \quad (8)$$

in which $[R_{CE}]$ is the concentration of renin (fmol AngI/min per mL) measured in CE, $[Aog_{CE}]$ is the concentration of angiotensinogen (pmol/mL) in CE, and K_m (pmol/mL) is the Michaelis-Menten constant (see 'Biochemical Measurements' above).

$AngI_{CE}$ production calculated according to equation (5) is referred to as 'measured' $AngI_{CE}$ production, because the independent variables in this equation were measured experimentally. $AngI_{CE}$ production, predicted by equation (7), is referred to as 'predicted' $AngI_{CE}$ production.

Our analysis is based on the following assumptions: 1) the disappearance of *exogenous* AngI from IST and from the cardiac interstitial fluid follows first-order kinetics, and the half-life of *exogenous* AngI is not different between the two fluid compartments, 2) the steady-state concentrations of *exogenous* AngI in IST and interstitial fluid are not different, and 3) these assumptions also apply to *endogenous* AngI.

In assumptions (1) and (2) above, the interstitial fluid is considered to represent a single compartment and the only source of IST. This is supported by kinetic studies of the transport of inert low- and high-molecular weight substances from the perfusion fluid into IST[19] and by studies of the release of cardiac enzymes and metabolites into IST[11,12,19,20] as well as by studies of the cardiac glucose uptake, in which glucose levels in CE and IST were compared in the same type of Langedorff model as we used

here.[10] For assumptions (1) and (2) to be valid, it is important to exclude leakage of perfusate directly into IST, caused by damage to the coronary vessels or inadequate ligation of the veins. Perfusions with red blood cells showed that such leakage only marginally contributed to the formation of IST (see under 'Results')

Assumption (3) implies that in the interstitial fluid the half-life of *endogenous* AngI is the same as the half-life of *exogenous* AngI. The half-life of AngI in interstitial fluid is determined by its metabolism by peptidases, by its back-diffusion into the intravascular compartment, and by its loss via IST. It seems logical to assume that in the interstitial fluid these mechanisms act on *endogenous* AngI in the same way as on *exogenous* AngI.

Is the half-life of *endogenous* AngI in the IST drop also the same as the half-life of *endogenous* AngI in the interstitial fluid? As described in 'Results', the half-life of *exogenous* AngI in IST in the collection tube was much longer than the half-life in the IST drop while it was still on the cardiac surface. There may be little back-diffusion of AngI from the IST drop into the interstitial fluid, and AngI in the IST drop may be less exposed to peptidases than AngI in the interstitial fluid. This would result in a longer half-life in the IST drop on the cardiac surface than in the interstitial fluid in cardiac tissue, so that the level of *endogenous* AngI in the IST might be higher than the level of *endogenous* AngI in the interstitial fluid. Equation (1) would then lead to an overestimation of the true production of the AngI that is present in the interstitial fluid. However, the AngI production calculated according to equation (1) was close to the value predicted by equation (3) (see under 'Results'), and, since equation (3) gives the lowest possible value, the level of *endogenous* AngI does not appear to be higher in IST than in the interstitial fluid.

2.3.12 Statistical analysis

Data are expressed as means ± s.d., except when indicated otherwise. In the uptake and washout experiments, the concentrations in CE, IST and cardiac tissue are expressed as a percentage of the arterial concentration. In the AngI generation

experiments the concentrations in CE and IST are given as absolute values. Intraindividual differences were evaluated for statistical significance by Student's paired t-test. Differences were considered to be significant for values of $p < 0.05$.

2.4 Results

2.4.1 Leakage of perfusate into the interstitial transudate (IST)

During 10-min and 20-min perfusions with red blood cells, the cell counts in IST were approximately 1% of the counts in simultaneously collected CE (Table 1). Leakage of perfusate into IST may occur when the coronary vessels are damaged or after the veins have been improperly ligated. Our results show that such leakage only marginally contributed to the formation of IST.

Table 1. Red blood cell counts in coronary effluent and interstitial fluid during perfusion of the modified Langendorff heart with red blood cells.

Cardiac fluid	10 min perfusion n=4	20 min perfusion n=4
Coronary effluent (CE) (red cells, 10^{12}/L)	0.11 ± 0.5	0.17 ± 0.08
Interstitial transudate (IST) (red cells, 10^{12}/L)	0.001 ± 0.001	0.002 ± 0.004
[IST/CE]x100%	0.9 ± 0.8	1.1 ± 1.6

Values are means ± s.d..

2.4.2 Intravascular and interstitial fluid volumes

^{125}I-HSA, when infused into the rat Langendorff heart, entered the IST and reached levels comparable to those in CE after approximately 10 minutes. After 1-min perfusion with ^{125}I-HSA the level in IST was approximately 10% of the level in CE. We used the ^{125}I-HSA level in CE, collected during the first minute of infusion and the level in cardiac tissue after 1 min of infusion, to calculate the intravascular fluid volume, which consists of the fluid in the coronary vascular bed and the right ventricle. It was 38% (mean, n=4) of the cardiac wet weight (Table 2), which is in agreement with previous studies in isolated hearts[21].

^{51}Cr-EDTA, when infused into the perfusion system, reached levels in IST comparable to those in CE within 5 minutes of infusion. No further increase was observed when the infusion was prolonged. We used ^{51}Cr-EDTA levels in CE and cardiac tissue after 10 min of infusion, to calculate the extracellular fluid volume. It was 61% (mean, n=6) of the cardiac wet weight (Table 2). Since 38% of the cardiac weight consisted of intravascular fluid, the interstitial fluid volume is estimated to be 23% of the cardiac wet weight.

2.4.3 Release of endogenous renin, angiotensinogen and angiotensin I

During 40-min perfusion with Tyrode's buffer immediately after preparation of the Langendorff hearts (n=4), renin and AngI were below the detection limit of the assay in both CE and IST. Angiotensinogen was undetectable in CE but not in IST. Angiotensinogen in IST was 4 ± 1 pmol/mL after 10 min of perfusion with Tyrode's buffer, and decreased to just above the detection limit (0.4 pmol/mL) after 40 min.

Table 2. Intravascular and extracellular fluid volumes of the modified Langendorff heart determined with ^{125}I-human serum albumin and ^{51}Cr-EDTA perfusions.

Cardiac fluid or cardiac tissue	^{125}I-HSA n=4	^{51}Cr-EDTA n=6
Coronary effluent (cpm/mL)	14600 ± 5490	7650 ± 1790
Interstitial transudate (cpm/mL)	1790 ± 898	6720 ± 2360
Cardiac tissue (cpm/g)	5500 ± 2150	5090 ± 1110
Volume of distribution (mL/g cardiac wet weight)	0.38 ± 0.08	0.61 ± 0.06

Values are means ± s.d.. ^{125}I-HSA was infused into the perfusion system for 1 min, and ^{51}Cr-EDTA for 10 min. The intravascular space consists of the volume of fluid in the coronary vascular bed and the right ventricle. HSA, human serum albumin. cpm, counts per minute.

2.4.4 Uptake and washout of exogenous renin, angiotensinogen and angiotensin I

Renin

During perfusion with porcine renin (n=8), the steady-state renin level in CE was 78% of the arterial level (mean value, $p < 0.05$ for difference from the arterial level; see Table 3). Thus, some of the arterially delivered renin was removed from the perfusate by the heart. A steady-state level in IST was reached within 20 min (Fig. 2), and it was not significantly different from that in CE (Table 3, Fig. 2). After discontinuation of the renin perfusion, renin disappeared from CE in a biphasic pattern (Fig. 3). The rapid first phase had a $t_{1/2}$ of 0.42 ± 0.03 min, and the slow second phase had a $t_{1/2}$

of 3.3 ± 0.8 min. Renin disappeared from IST in a monophasic way with a $t_{1/2}$ of 3.9 ± 1.4 min.

The cardiac tissue level of renin (per g tissue), immediately after the renin perfusion had been switched off, was 56 ± 9% (n=5) of the arterial level (per mL perfusate). Since the renin level in CE was 78% of the arterial level and not different from the level in IST, and since in a different series of experiments the cardiac extracellular space was found to be 0.61 mL/g heart weight (mean value, n=6; see Table 2), the cardiac tissue level of renin is expected to be 0.61 x 78 = 48% (mean) of the arterial level, assuming that renin in the heart is located in the extracellular fluid compartment and that 1 g of tissue corresponds with a volume of 1 mL[6,22]. This calculated value is close to the renin level that was actually measured in cardiac tissue (56 % of the arterial level). Thus, the cardiac tissue level of renin is consistent with its location in the extracellular fluid at a concentration similar to that of CE. Renin was not detectable in control hearts perfused with Tyrode's buffer (n=4).

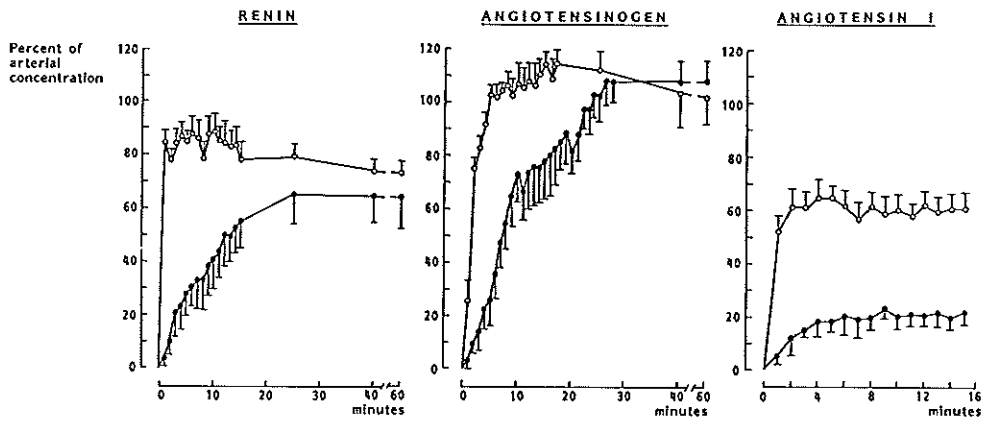

Figure 2. Levels of porcine renin (left panel; n=8), porcine angiotensinogen (middle panel; n=6) and angiotensin I (right panel; n=5) in coronary effluent (open circles) and interstitial transudate (closed circles) during perfusion of the modified Langendorff heart with these renin-angiotensin system components. Values (means and s.e.m.) are expressed as percentage of the arterial level.

Angiotensinogen

During perfusion with porcine angiotensinogen (n=6), the steady state angiotensinogen level in CE was not significantly different from the arterial level (Table 3). Thus, in contrast with renin, removal of angiotensinogen from the perfusate by the heart could not be demonstrated. Angiotensinogen did reach the IST, and in IST a steady-state level comparable to that in CE was reached after approximately 30 min (Fig. 2). After discontinuation of the angiotensinogen perfusion, angiotensinogen disappeared rapidly ($t_{1/2}$ 0.54 ± 0.32 min) from CE in a monophasic way (Fig. 3), and there was no evidence for a slow second phase, as there was during renin washout. Angiotensinogen disappeared from IST, also in a monophasic way, with a $t_{1/2}$ of 2.9 ± 1.0 min.

Table 3. Steady-state levels of renin, angiotensinogen and angiotensin I in coronary effluent and interstitial transudate during perfusion of the modified Langendorff heart with renin, angiotensinogen or angiotensin I, respectively.

Cardiac fluid	Renin n=8	Angiotensinogen n=6	Angiotensin I n=5
Coronary effluent	78.3 ± 14.6	102.9 ± 17.3	59.9 ± 10.3
Interstitial transudate	63.9 ± 34.4	107.8 ± 41.7	16.8 ± 4.6

Values are means ± s.d.. Renin, angiotensinogen and angiotensin I (AngI) levels in coronary effluent (CE) and interstitial transudate (IST) are expressed as a percentage of the arterial level, which was calculated as the rate of renin, angiotensinogen and angiotensin I infusion into the perfusion system divided by the perfusate flow. Arterial renin, angiotensinogen and AngI were 1.1 ± 0.2 pmol AngI/min per mL, 11.4 ± 5.3 pmol/mL and 6.4 ± 2.6 pmol/mL, respectively. Steady-state levels of renin and angiotensinogen were averages of the levels after 25 min, 40 min and 60 min of renin or angiotensinogen perfusion (see Fig. 1). Steady-state levels of AngI were averages of the levels after 13, 14 and 15 min of AngI perfusion (see Fig. 1). The renin and angiotensinogen levels were not significantly different between IST and CE. AngI in IST was lower than in CE ($p<0.01$). The level of angiotensinogen in CE was not significantly different from the arterial level. The renin and angiotensin I levels in CE were lower than the arterial level ($p<0.05$ and $p<0.01$, respectively).

The cardiac tissue level of angiotensinogen (per g tissue), immediately after the angiotensinogen perfusion had been switched off, was 53 ± 18% (n=5) of the arterial level (per mL perfusate). Since the angiotensinogen levels in CE and IST were not different from the arterial level, and since in a different series of experiments the cardiac extracellular fluid space was found to be 0.61 mL/g (mean, n=6; see Table 2), the cardiac tissue level of angiotensinogen is expected to be 0.61 x 100 = 61% (mean) of the arterial level, assuming that angiotensinogen is located in the extracellular fluid compartment and that 1 g tissue corresponds with a volume of 1 mL[6,22]. This calculated value is similar to the angiotensinogen level that was actually measured in cardiac tissue (53% of the arterial level). Thus, as with renin, the cardiac tissue level of angiotensinogen is consistent with its location in the extracellular fluid at a concentration similar to that of CE. Angiotensinogen was not detectable in control hearts perfused with Tyrode's buffer (n=4).

Angiotensin I

During perfusion with AngI (n=5), the steady-state AngI level in CE was 60% of the arterial level (mean, $p < 0.01$ for difference from the arterial level; see Table 3). Thus, 40% of the arterially delivered AngI was removed from the perfusate by the heart. A steady-state level of AngI in IST was reached within 5 min (Fig. 2). It was 17% of the arterial level, which was significantly lower than the level in CE ($p < 0.01$). After discontinuation of the AngI perfusion, AngI disappeared rapidly from CE in a monophasic way (Fig. 3), with a $t_{1/2} < 0.5$ min. AngI disappeared from IST, also in a monophasic way, with a $t_{1/2}$ of 0.9 ± 0.6 min. AngI that was added to samples of CE and IST after they had been collected from control hearts perfused with Tyrode's buffer (n=2) had a half life > 40 min at 37 °C (in the absence of the mixture of ACE- and angiotensinase-inhibitors that was routinely used during CE and IST collection). Thus, the rapid disappearance of AngI from IST that was observed in the AngI perfusion experiments, was not caused by the presence of peptidases in IST, but by the rapid removal of AngI from the cardiac interstitial fluid.

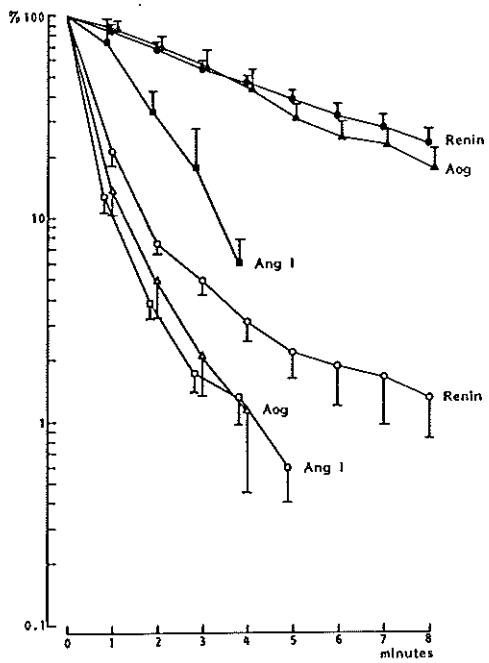

Figure 3. Washout of porcine renin (n=8), porcine angiotensinogen (Aog, n=6) and angiotensin I (n=5) from coronary effluent (open circles) and interstitial transudate (closed circles) of the modified Langendorff heart after its perfusion with these renin-angiotensin system components. Values (means and s.e.m.) are expressed as percentage of the level immediately before discontinuation of infusion of the renin-angiotensin system components into the perfusion system.

The cardiac tissue level of AngI (per g tissue), immediately after the AngI perfusion had been switched off, was less than 5% (n=3) of the arterial level (per mL perfusate). The AngI levels in CE and IST were 60% and 17% of the arterial level, whereas in a different series of experiments the intravascular and interstitial fluid spaces were 0.38 and 0.23 mL/g, respectively (see Table 2). Thus, the cardiac tissue level of AngI is expected to be 0.38 x 60 + 0.23 x 17 = 27% of the arterial level, assuming that AngI is located in the extracellular fluid compartment and that 1 g

tissue corresponds with a volume of 1 mL[6,22]. The difference from the measured result might be related to the rapid degradation of AngI in the vascular compartment. It is possible that the short period between the moment the AngI perfusion was stopped and the moment the tissue was transferred into liquid nitrogen, was long enough for the endothelial peptidases to cause a loss of most of the AngI. AngI was not detectable in control hearts perfused with Tyrode's buffer (n=3).

2.4.5 Cardiac production of angiotensin I

Results obtained during perfusion with rat renin (n=4), in the absence of exogenous angiotensinogen, are shown in Fig. 4. AngI was not detectable in CE and IST samples collected before renin was infused into the perfusion system. In samples collected during renin perfusion, AngI remained undetectable in CE, whereas in IST AngI rose to levels above the detection limit of the assay. After an initial increase, AngI in IST decreased, despite the continuous perfusion with renin. This may be due to the washout of endogenous (rat) angiotensinogen during the course of the experiment.

AngI was not detectable in CE and IST samples collected during perfusion with rat, porcine or sheep angiotensinogen (n=3). Thus, there was no evidence for cardiac AngI production by endogenous (rat) renin or renin-like enzymes (cathepsins).

AngI was easily detectable in samples of CE and IST collected during perfusion with porcine renin combined with porcine or sheep angiotensinogen (Fig. 5 and Tables 4 and 5). During combined renin/angiotensinogen perfusion, the level of AngI in CE had reached its steady state maximum in the 30-40-min and 45-55-min samples, but not in the 0-10-min and 15-25-min samples. This contrasts with the levels of renin and angiotensinogen, which had reached their steady-state maximum in CE within less than 5 min (see Fig. 2). The slow increase of AngI in CE, as compared with the rapid increase of renin and angiotensinogen is an indication that the renin-angiotensinogen reaction in the fluid-phase of the intravascular compartment was not the only source of AngI in CE. Also in IST, AngI had reached its steady-state

maximum in the 30-40-min and 45-55-min samples, but not in the 0-10-min and 15-25-min samples; this corresponds well with the time required for renin and angiotensinogen to reach their steady state maximum in IST (see Fig. 2).

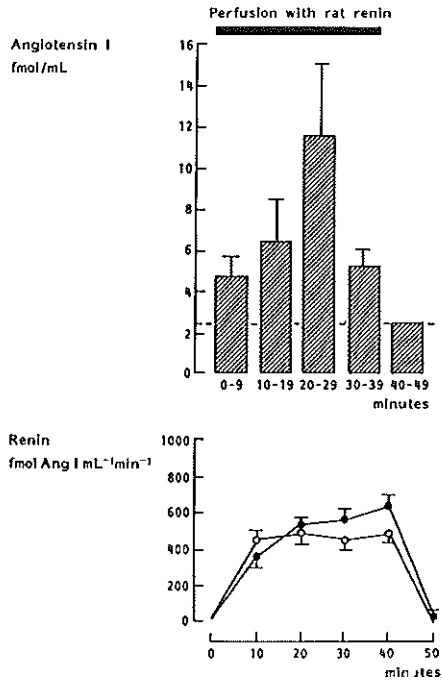

Figure 4. *Top panel.* Angiotensin I levels in interstitial transudate during 40-min perfusion of the modified Langendorff heart with rat renin followed by a 10-min washout (n=4). Samples were collected over 9 minutes. Angiotensin I in simultaneously collected samples of coronary effluent was below the detection limit of the assay. Values are means and s.e.m..

Bottom panel. Renin levels in coronary effluent (open circles) and interstitial transudate (closed circles) during and after 40 minute perfusion of the modified Langendorff heart with rat renin (n=4). One-min samples were collected every 10 minutes. Values are means and s.e.m..

Table 4. Levels of renin, angiotensinogen and angiotensin I in coronary effluent and interstitial transudate during perfusion of the modified Langendorff heart with porcine renin and porcine angiotensinogen.

RAS component		Sample collection period 30-40 min n=6	45-55 min n=6
Renin (fmol AngI/ min per mL)	CE	2430 ± 418	2400 ± 433
	IST	1620 ± 275	1670 ± 280
	IST/CE	0.70 ± 0.22	0.73 ± 0.21
Angiotensinogen (pmol/mL)	CE	10.4 ± 2.2	10.5 ± 2.1
	IST	8.2 ± 4.3	8.5 ± 4.3
	IST/CE	0.82 ± 0.43	0.81 ± 0.35
Angiotensin I (fmol/mL)	CE	35.8 ± 13.9	29.5 ± 10.2
	IST	62.2 ± 25.0	63.5 ± 29.1
	IST/CE	2.51 ± 1.14*	2.53 ± 0.83*

Values are means ± s.d.. RAS, renin-angiotensin system. CE, coronary effluent. IST, interstitial transudate.
* Significantly different from 1.00 ($p<0.05$).

Figs. 6 and 7 give the measured $AngI_{ISF}$ and $AngI_{CE}$ production rates as derived from equations (1) and (5) (see 'Calculations' above), for the 30-40-min and 45-55-min samples. The measured production rates are compared with the production rates predicted by equations (3) and (7), which are based on the assumption that the renin-angiotensinogen reaction in the intravascular and interstitial compartments is occurring in the fluid phase only. The renin and angiotensinogen levels that were entered into these calculations are shown in Tables 4 and 5.

As shown in Figs. 6 and 7, the production of $AngI_{ISF}$ could be accounted for by the renin-angiotensinogen reaction in the fluid phase of the interstitial compartment. The production of $AngI_{CE}$, however, was too high to be accounted for

by the renin-angiotensinogen reaction in the fluid phase of the intravascular compartment. In the porcine renin/porcine angiotensinogen perfusion experiments, the measured $AngI_{CE}$ production rate was 4.6 ± 2.2 times (average of the two samples collected at 30-40 min and 45-55-min of perfusion, $p<0.01$ for difference from 1.0, n=6) the rate predicted on the basis of the renin-angiotensinogen reaction in the fluid phase. In the porcine renin/sheep angiotensinogen perfusion experiments, the discrepancy was even greater, the measured production rate being 7.1(4.1-10.1) times (mean value and range, n=3) the predicted rate.

Table 5. Levels of renin, angiotensinogen and angiotensin I in coronary effluent and interstitial transudate during perfusion of the modified Langendorff heart with porcine renin and sheep angiotensinogen.

RAS component		Sample collection period	
		30-40 min n=3	45-55 min n=3
Renin (fmol AngI/ min per mL)	CE IST IST/CE	1875 (1441-2444) 2225 (1032-4151) 1.11 (0.59-1.70)	1948 (1618-2251) 2075 (1096-3512) 1.04 (0.59-1.56)
Angiotensinogen (pmol/mL)	CE IST IST/CE	7.0 (5.7-9.3) 6.1 (5.7-6.8) 0.91 (0.73-1.00)	6.5 (5.9-7.7) 6.5 (5.9-7.5) 0.99 (0.97-1.00)
Angiotensin I (fmol/mL)	CE IST IST/CE	90 (38-150) 186 (77-370) 1.96 (1.38-2.45)*	100 (38-160) 236 (126-380) 2.56 (2.00-3.29)*

Values are means and ranges. RAS, renin-angiotensin system. CE, coronary effluent. IST, interstitial transudate. Significantly different from 1.00 ($p<0.05$)

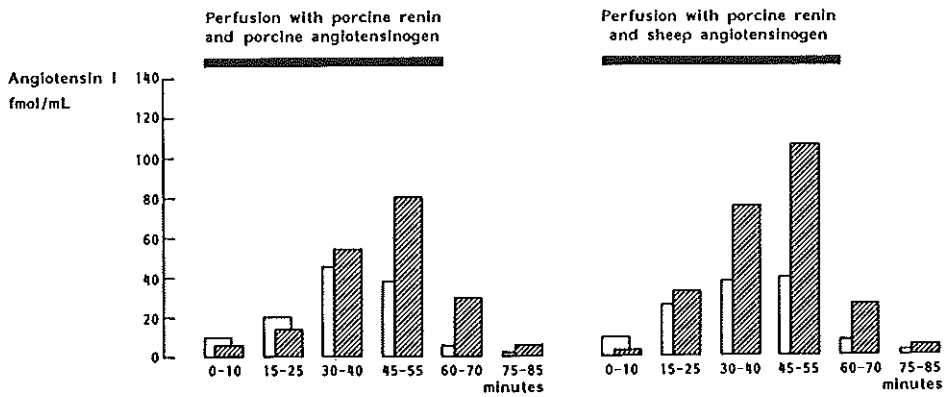

Figure 5. Angiotensin I levels in coronary effluent (open bars) and interstitial transudate (hatched bars) during 60-min perfusion of the modified Langendorff heart with porcine renin, combined with porcine angiotensinogen (left panel) or sheep angiotensinogen (right panel), followed by a 30-min washout. Results of two individual experiments are shown.

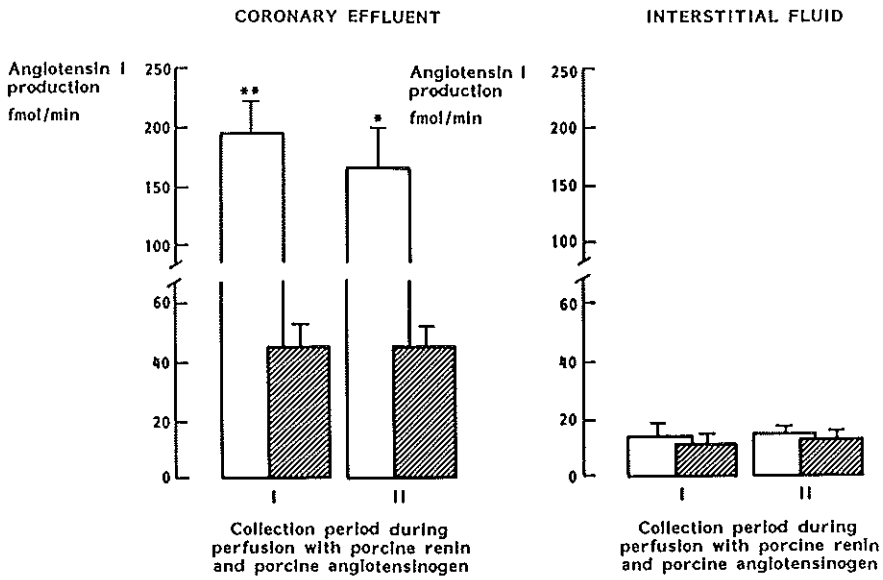

Figure 6. *Left panel.* Measured total production rate (open bars) vs. predicted production rate (hatched bars) of angiotensin I present in coronary effluent during perfusion of the modified Langendorff heart with porcine renin combined with porcine angiotensinogen (means and s.e.m., n=6). Predicted production rate was calculated on the basis of the renin-angiotensinogen reaction in the fluid phase of the intravascular compartment. Collection periods I and II were at 30-40 min and 45-55 min of perfusion, respectively. Measured production rate in each of the two collection periods was significantly higher than predicted (*, $p<0.05$; **, $p<0.01$).
Right panel. Measured total production rate (open bars) vs. predicted production rate (hatched bars) of angiotensin I in the interstitial fluid during perfusion of the modified Langendorff heart with porcine renin combined with porcine angiotensinogen (means and s.e.m., n=6). Predicted production rate was calculated on the basis of the renin-angiotensinogen reaction in the fluid phase of the interstitial compartment. Measured and predicted production rates were not different.

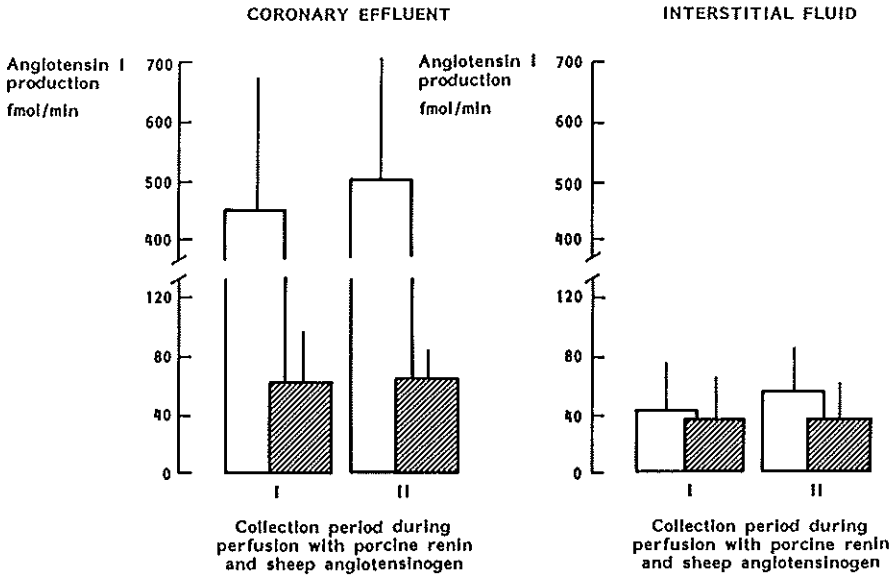

Figure 7. *Left panel.* Measured total production rate (open bars) vs. predicted production rate (hatched bars) of angiotensin I present in coronary effluent during perfusion of the modified Langendorff heart with porcine renin combined with sheep angiotensinogen (means + half range, n=3). Predicted production rate was calculated on the basis of the renin-angiotensinogen reaction in the fluid phase of the intravascular compartment. Collection periods I and II were at 30-40 min and 45-55 min of perfusion, respectively. Measured production rate in each of the two collection periods was higher than predicted in all three experiments.
Right panel. Measured total production rate (open bars) vs predicted production rate (hatched bars) of angiotensin I present in the interstitial fluid during perfusion of the modified Langendorff heart with porcine renin combined with porcine angiotensinogen (means + half range, n=3). Predicted production rate was calculated on the basis of the renin-angiotensinogen reaction in the fluid phase of the interstitial compartment. Measured and predicted production rates were similar.

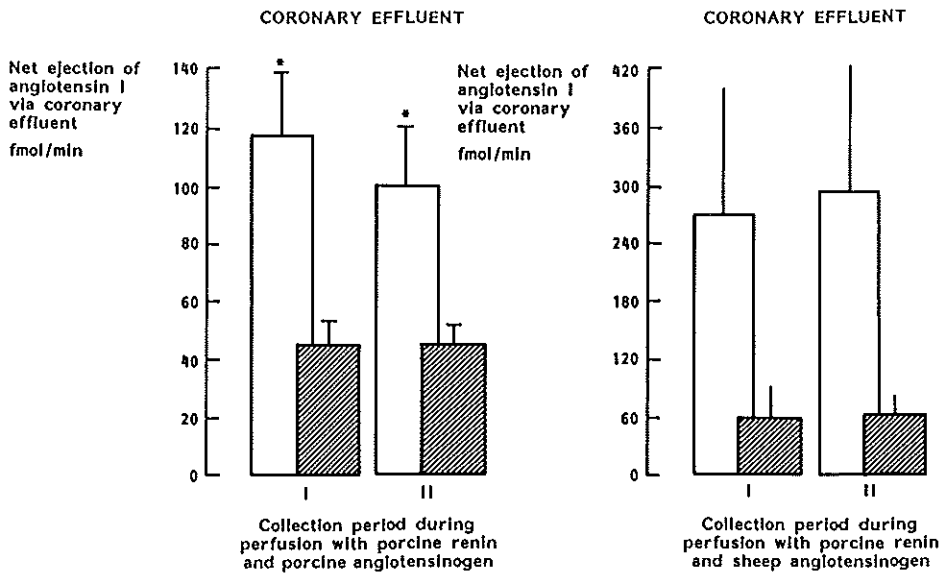

Figure 8. *Left panel.* Measured net ejection rate (open bars) vs. predicted production rate (hatched bars) of angiotensin I present in coronary effluent during perfusion of the modified Langendorff heart with porcine renin combined with porcine angiotensinogen (means and s.e.m., n=6). Predicted production rate was calculated on the basis of the renin-angiotensinogen reaction in the fluid phase of the intravascular compartment. Collection periods I and II were at 30-40 min and 45-55 min of perfusion, respectively. Measured ejection rate in each of the two collection periods was significantly higher than the predicted production (*, $p<0.05$).
Right panel. Measured net ejection rate (open bars) vs. predicted production rate (hatched bars) of angiotensin I present in coronary effluent during perfusion of the modified Langendorff heart with porcine renin combined with sheep angiotensinogen (means + half range, n=3). Predicted production rate was calculated on the basis of the renin-angiotensinogen reaction in the fluid phase of the intravascular compartment. Collection periods I and II were at 30-40 min and 45-55 min of perfusion, respectively. Measured ejection rate in each of the two collection periods was higher than predicted production in all three experiments.

The measured production rate of $AngI_{CE}$ may be somewhat higher than the true production rate, because the measured production rate accounts for the extraction of arterially delivered AngI, whereas, during combined renin/angiotensinogen perfusion, AngI is formed in the perfusate during its passage through the coronary vascular bed. Fig. 8, therefore, gives the net ejection rate of AngI via CE, as derived from equation (6), which is less than the total production of $AngI_{CE}$. It can be seen, that even with this underestimation of the true $AngI_{CE}$ production rate results were higher than predicted on the basis of the renin-angiotensinogen reaction in the fluid phase. The measured net ejection rate of AngI was 2.8 ± 1.3 times ($p<0.05$ for difference from 1.0, n=6) the predicted rate in the porcine renin/porcine angiotensinogen perfusion experiments, and 4.3(2.5-6.1) times (mean value and range, n=3) the predicted rate in the porcine renin/sheep angiotensinogen experiments. It appears therefore that part of the AngI in CE is produced at tissue sites. The $AngI_{ISF}$ production rate was much lower than the $AngI_{CE}$ production rate. The contribution of AngI from the interstitial fluid to the AngI level in CE was therefore minimal.

2.5 Discussion

This study of the cardiac uptake and production of renin-angiotensin system (RAS) components made use of a modified Langendorff heart model, that was perfused with an albumin-free buffer solution under normoxic conditions. In this model the coronary effluent (CE) is ejected by the right ventricle via the cannulated pulmonary artery, and a small amount (0.7-2%) of the infusion fluid entering the coronary arteries passes through the vascular wall, seeps through the heart tissue and reaches the epicardial surface. This transudate fluid, which is referred to in this article as interstitial transudate (IST), keeps dripping from the apex, and we assumed the levels of renin, angiotensinogen and AngI in this fluid to be representative of the levels in the cardiac interstitial fluid.

There is indeed strong evidence to support this assumption. The protein

concentration in IST is much higher than in the CE[10]. Creatine kinase, lactate dehydrogenase and malate dehydrogenase activity levels are about 100 times higher in IST than CE[19]. Glucose is taken up by the heart primarily from the interstitial fluid, whereas lactate is primarily released into this fluid rather than into CE, and this is reflected by the glucose and lactate concentrations in IST and CE[10]. The levels of renin and angiotensinogen that were reached in IST during infusions of these RAS components into the perfusion system were similar to the levels in CE. Using a Langendorff heart preparation somewhat different from ours, Wienen and Kammermeier[19] found that, during perfusion with dextran T-70 (molecular weight (mol. wt.) 70 kD), the dextran level in IST was equal to that in CE, and the same observation was made for albumin (mol. wt. 70 kD) during perfusion with 0.01% bovine serum albumin. In the experimental setup that was used by Wienen and Kammermeier[19], IST was collected by slight suction under a latex cap over the ventricles, thereby minimizing the risk of evaporation. In view of the agreement between the results obtained by these authors and the results obtained in our experiments, it is safe to conclude that evaporation had minimal effect on the measured concentrations of RAS components in IST. The half-life of AngI in IST in the collection tube was much longer than the half-life in the IST drop while it was still on the cardiac surface. Therefore, the rapid washout of AngI from the IST, while it was still on the cardiac surface (as observed after the discontinuation of perfusion with AngI), therefore, reflects the rapid disappearance of AngI from the interstitial fluid in cardiac tissue. All of these observations, taken together, support the view that the composition of IST indeed reflects the composition of the cardiac interstitial fluid. The levels of *exogenous* arterially delivered AngI are probably similar in the two fluid compartments, as are the levels of *endogenous* AngI formed in the Langendorff preparation. This is indicated by our finding that the AngI level measured in IST in the combined renin and angiotensinogen perfusion experiments was not different from the level predicted on the basis of renin-angiotensinogen reaction in the fluid phase of the interstitial compartment.

In our renin and angiotensinogen perfusion experiments, the release of renin

(mol. wt. 48 kD) and angiotensinogen (mol. wt. 65 kD) into the IST was slow as compared with the release of AngI into the IST during AngI perfusion. This is probably related to the much smaller molecular size of AngI (mol. wt. 1.297 kD). Similar observations have been published with respect to dextran-T70 (mol. wt. 70 kD) and disulfin blue (mol. wt. 0.566 kD)[19]. It should be noted that, in order to obtain sufficient amounts of IST for AngI measurement, we did not add serum or serum albumin to the perfusion fluid. Albumin, however, reduces not only the movement of water and low-molecular weight solutes from the perfusate into the IST but also the transport of other proteins, including albumin itself[23]. Our results may therefore quantitatively differ from the situation *in vivo*.

Our measurements of renin and angiotensinogen in IST and CE, together with measurements of the tissue levels during perfusion with these RAS components, indicate that most of the renin and angiotensinogen was localized in the extracellular fluid compartment. Some of the infused renin may bind to the cell surface or may have been taken up by the cells. Cardiac membrane fractions contain renin[6], and binding of renin to vascular membranes has been reported[24]. Cellular uptake of renin followed by intracellular proteolytic destruction may explain why, during perfusion with renin, the renin level in CE was lower than the arterial level.

In our perfusion experiments, approximately 40% of the infused AngI was removed from the perfusate by the heart. This is in accordance with studies in intact pigs, which demonstrated that, in the coronary vascular bed, 45-50% of the arterially delivered AngI was removed from the circulation[25], most likely by peptidases on the endothelial surface.

An important aspect of the present study is the evidence it provides for local AngI formation by the heart, outside the perfusate compartment. According to our calculations, the level of AngI in IST during combined renin and angiotensinogen perfusion can be explained by the renin-angiotensinogen reaction in the fluid phase of the interstitial compartment. In contrast, most of the AngI in CE was not formed by the renin-angiotensinogen reaction in the fluid phase of the intravascular compartment. AngI from the intravascular compartment contributed little to the AngI

level in IST, and AngI from the cardiac interstitial fluid contributed little to the AngI level in CE. Most of the AngI in CE appears to be formed at tissue sites, and the direction of AngI release from these sites seems to be toward the intravascular compartment rather than the interstitial compartment. We assume therefore that these sites are close to the luminal surface of the blood vessel wall and represent blood vessel wall-bound renin, as suggested by Swales and Thurston more than 20 years ago[26]. The finding that the AngI level in CE was not higher than in IST does not argue against this conclusion, because AngI, after its release into the intravascular fluid, is rapidly washed away by the flow of perfusate. The present study extends earlier observation in intact pigs, which suggested that the AngI concentration in coronary venous plasma was too high to be explained by the plasma renin activity[9]. With the use of biochemical and immunohistochemical methods, renin has been demonstrated in the endothelial cells of normal human gastroepiploic arteries[27]. In our experiments renin disappeared from the coronary effluent in a biphasic manner after the renin perfusion had been stopped. The slow second phase of the disappearance curve may correspond with the endothelial compartment. Such a slow second phase was not observed in the disappearance curve of angiotensinogen.

Fig. 9 presents a hypothetical scheme that is compatible with our observations. It shows two sites of AngI formation outside the perfusion fluid, namely the vascular surface and the interstitial fluid. Some renin and also the peptidases involved in AngI metabolism are bound to the vascular endothelial cells. Angiotensinogen and AngI are present in the fluid phase. Most of the AngI present in coronary effluent is produced by endothelium-bound renin. Most of the AngI present in the interstitial fluid compartment is produced by renin present in the fluid phase of this compartment.

The local formation of AngI at cardiac tissue sites depends on arterially delivered renin. In our perfusion experiments, AngI was undetectable in CE and IST collected after 30 min of perfusion with Tyrode's buffer, before the infusion of renin into the perfusion system had been started. Also, CE collected from the classical Langendorff heart model did not contain AngI, unless renin had been added to the perfusion fluid[7]. Conclusive evidence that cardiac AngI production depends on blood-

derived renin comes from our studies of the effect of nephrectomy on the cardiac tissue levels of AngI and II in pigs[6]. Both peptides became undetectable in cardiac tissue after bilateral nephrectomy. In the present study, AngI levels in IST and CE were much higher during combined renin and angiotensinogen perfusion than during perfusion with renin alone. It is, therefore, reasonable to conclude that AngI production by the heart not only depends on arterially delivered renin but also on arterially delivered angiotensinogen.

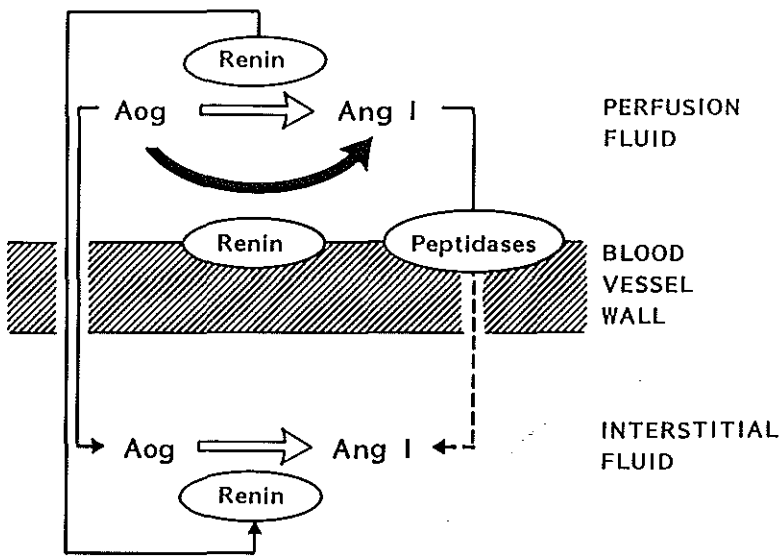

Figure 9. Proposed scheme of angiotensin I production in the heart. Circulating renin and angiotensinogen (Aog) both enter the interstitial fluid compartment, and reach concentrations in the interstitial fluid comparable to those in the circulation. Renin also binds to the vascular wall. Angiotensin I is metabolized by peptidases while passing through the vessel wall. Most of the angiotensin I in the interstitium is derived from the renin-angiotensinogen reaction in the fluid phase of this compartment. Most of the angiotensin I in coronary effluent is produced by vascular wall-bound renin.

The production of AngI at cardiac tissue sites may, via AngI to AngII conversion, lead to local concentrations of AngII that are higher than can be obtained with arterially delivered AngII. That the local formation of AngI is of physiological importance, is suggested by our recent observations in intact pigs on the effect of intracoronary administration of a specific renin inhibitor on cardiac contractility[28]. The inhibitor reduced cardiac contractility, whereas the time course of this effect was not correlated with the effect of the inhibitor on the circulating levels of AngI and II. In addition to its short-term effect on cardiac contractility, AngII also has long-term effects; it promotes left ventricular hypertrophy and the remodelling that occurs after myocardial infarction[1-3]. AngI produced locally in the heart may, after its conversion to AngII, participate in these processes. This is in keeping with the view that the long-term beneficial effects of ACE inhibitors in left ventricular hypertrophy and heart failure are determined not only by the decrease in circulating AngII but also by a decrease in the conversion of locally formed AngI.

2.6 Abbreviations

ACE	angiotensin-converting enzyme
AGA	angiotensin I-generating activity
Ang	angiotensin
$AngI_{CE}$	angiotensin I present in coronary effluent
$AngI_{IST}$	angiotensin I present in interstitial transudate
$AngI_{ISF}$	angiotensin I present in interstitial fluid
BSA	bovine serum albumin
CE	coronary effluent
HSA	human serum albumin
ISF	interstitial fluid
IST	interstitial transudate
RAS	renin-angiotensin system

2.7 References

1. Yusuf S, on behalf of the SOLVD investigators: Effect of enalapril on survival in patients with reduced left ventricular ejection fractions and congestive heart failure. *N Engl J Med.* 1991; 325: 293-302.
2. Ruzicka M, Yuan B, Leenen FHM: Effects of enalapril versus losartan on regression of volume overload-induced cardiac hypertrophy in rats. *Circulation.* 1994; 90: 484-491.
3. Schieffer B, Wirger A, Meybrunn M, Seitz S, Holtz J, Riede UN, Drexler H: Comparative effects of chronic angiotensin-converting enzyme inhibition and angiotensin II type 1 receptor blockade on cardiac remodeling after myocardial infarction in the rat. *Circulation.* 1994; 89: 2273-2282
4. van Gilst WH, de Graeff PA, Kingma JH, Wesseling H, de Langen CDJ: Captopril reduces purine loss and reperfusion arrhythmias in the rat heart after coronary artery ligation. *Eur J Pharmacol.* 1984; 100: 113-117.
5. Campbell DJ, Kladis A, Duncan AM: Nephrectomy, converting enzyme inhibition and angiotensin peptides. *Hypertension.* 1993; 22: 513-522.
6. Danser AHJ, van Kats JP, Admiraal PJJ, Derkx FHM, Lamers JMJ, Verdouw PD, Saxena PR, Schalekamp MADH: Cardiac renin and angiotensins. Uptake from plasma versus in situ synthesis. *Hypertension.* 1994; 24: 37-48.
7. Lindpaintner K, Jin M, Niedermaier N, Wilhelm MJ, Ganten D: Cardiac angiotensinogen and its local activation in the isolated perfused beating heart. *Circ Res.* 1990; 67: 564-573.
8. Schunkert H, Jackson B, Shih Tang S, Schoen FJ, Smits JFM, Apstein CS, Lorell BH: Distribution and functional significance of cardiac angiotensin converting enzyme in hypertrophied rat hearts. *Circulation.* 1993; 87: 1328-1339.
9. Danser AHJ, Koning MMG, Admiraal PJJ, Sassen LMA, Derkx FHM, Verdouw PD, Schalekamp MADH: Production of angiotensins I and II at tissue sites in intact pigs. *Am J Physiol.* 1992; 263: H429-H437.
10. De Deckere EAM, Ten Hoor P: A modified Langendorff technique for metabolic investigations. *Pflügers Arch.* 1977; 370: 103-105.
11. Jansen H, Stam H, Kalkman C, Hülsmann WC: On the dual localization of lipoprotein lipase in rat heart. Studies with a modified perfusion technique. *Biochim Biophys Res Commun.* 1980; 92: 411-416.
12. Stam H, Jansen H, Hülsmann WC: Dual localization of lipoprotein lipase in rat heart. Its relationship to chylomicron degradation. In: *Advances in myocardiology*, edited by Chazov E, Smirnov V and Dhalla NS. Plenum Publishing Corporation 1982; 3: 509-517.
13. Skinner SL, Dunn JR, Mazzetti J, Campbell DJ, Fidge NH: Purification, properties and kineties of sheep and human renin substrates. *Aust J Exp Biol Med Sci.* 1975; 53: 77-88.
14. Boulanger CM, Caputo L, Lévy BI: Endothelial AT_1-mediated release of nitric oxide decreases angiotensin II contractions in rat carotid artery. *Hypertension.* 1995; 26: 752-757.
15. Deinum J, Derkx FHM, Danser AHJ, Schalekamp MADH: Identification and quantification of renin and prorenin in the bovine eye. *Endocrinology.* 1990; 126: 1673-1682.
16. 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.
17. Hilgenfeldt U, Schott R: Differences in pattern of plasma angiotensinogen in native and nephrectomized rats. *Hypertension.* 1987; 9: 339-344.
18. Gutkowska J, Corvol P, Figueiredo AFS, Inagami T, Bouhnik J, Genest J: Kinetic studies of rat

renin and tonin on purified angiotensinogen. *Can J Biochem Cell Biol.* 1984; 62: 137-142.
19. Wienen W, Kammermeier H: Intra- and extracellular markers in interstitial transudate of perfused rat hearts. *Am J Physiol.* 1988; 254: H785-H794.
20. Fenton RA, Tsimikas S, Dobson JG: Influence of ß-adrenergic stimulation and contraction frequency on rat heart interstitial adenosine. *Circ Res.* 1990; 66: 457-468.
21. Becker BF, Bünger R, Permanetter B, Gerlach E: Compartmentation and hemodynamic effects of nicotine in isolated, perfused guinea pig hearts. *Klin Wochenschr.* 1984; 62 (suppl II): 67-75.
22. Katz J, Whang J, Boxt LM, Barts RJ: Estimation of right ventricular mass in normal subjects and in patients with primary pulmonary hypertension by nuclear magnetic resonance imaging. *J Am Coll Cardiol.* 1993; 21: 1475-1481.
23. Schnitzer JE: Update on the cellular and molecular basis of capillary permeability. *Trends Cardiovasc Med.* 1993; 3: 124-130.
24. Campbell DJ, Valentijn AJ: Identification of vascular renin-binding proteins by chemical cross-linking: inhibition of binding of renin by renin inhibitors. *J Hypertens.* 1994; 12: 879-890.
25. 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-H428.
26. Swales JD, Thurston H: Generation of angiotensin II at peripheral vascular level: studies using angiotensin II antisera. *Clin Sci Mol Med.* 1973; 45: 691-700.
27. Okamura T, Aimi Y, Kimura H, Murakami K, Toda N: Existence of renin in the endothelium of the human artery. *J Hypertens.* 1992; 10: 49-53.
28. van Kats JP, Sassen LMA, Danser AHJ, Polak MPJ, Soei LK, Derkx FHM, Schalekamp MADH, Verdouw PD. Assessment of the role of the renin-angiotensin system in cardiac contractility utilizing the renin inhibitor remikiren. *Br J Pharmacol.* 1996; 117: 891-901.

Chapter 3

Localization of arterially delivered renin in the heart

3.1 Abstract

In this study a modified Langendorff rat heart model was used to investigates 1) the localization of arterially delivered renin in the heart, and 2) whether prorenin, following its uptake in the heart, is activated to renin. In these hearts coronary effluent was recirculated because of the limited availability of (pro)renin. Following perfusion with renin, renin could be demonstrated immunohistochemically in the media of the coronary artery wall, but not in the endothelium, adventitia or cardiomyocytes. Renin was enriched 4-5 fold in a purified cardiac membrane fraction prepared from renin-perfused hearts, suggesting that binding of renin to cardiac membranes may be part of the mechanism by which renin is taken up from the circulation. Prorenin, like renin, entered the interstitial fluid (ISF) during its perfusion, reaching a steady state level in IST comparable to that in CE. No activation of prorenin could be demonstrated in either IST or CE over a 50 min period. Taken together, it appears that renin is sequestered from the blood, not only by diffusion into the cardiac ISF, but also by binding to vascular smooth muscle cell membranes. Prorenin, if activated at tissue sites, remains cell-associated and is not released into either CE or ISF.

3.2 Introduction

Angiotensin generation has been reported to occur both in the circulation and at tissue sites[1-5]. Although initially it was thought that tissue angiotensin generation depends on locally synthesized renin, recent studies have suggested that this may not be the case in heart and blood vessels. Thirty hours after a bilateral nephrectomy, renin, angiotensin I and II are no longer detectable in porcine cardiac tissue[6]. Vascular renin also disappears following bilateral nephrectomy[1] and vascular angiotensin release is directly related to the level of renin in blood plasma[2,4]. Thus, renin taken up from the circulation rather than locally synthesized renin may be involved in local cardiac and vascular angiotensin generation. Uptake of renin could occur via renin binding proteins and/or renin receptors[8-10]. Another possibility is that prorenin, the inactive precursor of renin, is taken up by tissues and locally activated to renin[11,12]. In the present study we investigated in the isolated perfused rat Langendorff heart 1) the tissue localization of arterially delivered renin and 2) the activation of arterially delivered prorenin.

3.3 Materials and methods

3.3.1 Materials

Human serum albumin (HSA) and bovine serum albumin (BSA) were from Sigma, St. Louis, MO, USA. Sodium pentobarbital was obtained from Apharma, Arnhem, The Netherlands. Recombinant human prorenin and renin and the renin inhibitor remikiren were a kind gift of Dr. W. Fischli, Hoffmann-La Roche, Basel, Switzerland. Porcine renin was prepared from porcine kidneys as described before[3]. The polyclonal anti-human renin antibody R15 was a kind gift

of Dr. J. Ménard, INSERM, Paris, France. The goat anti-(rabbit IgG) antibody, conjugated to fluorescein isothiocyanate, was obtained from Nordic, Tilburg, The Netherlands.

3.3.2 Preparation of the modified Langendorff heart

All experiments were performed under the regulations of the Animal Care Committee of the Erasmus University, Rotterdam, The Netherlands, in accordance with the 'Guiding principles in the care and use of animals, as approved by the American Physiological Society.

Male Wistar rats (Harlan, Zeist, The Netherlands; 280-400 g) were anesthetized with pentobarbital (60 mg/kg, i.p.) and heparinized (5,000 units/kg, i.v.). The hearts (1.0-1.4 g) were rapidly excised, cooled in ice-cold Tyrode's buffer (125 mmol/L NaCl, 4.7 mmol/L KCl, 1.4 mmol/L $CaCl_2$, 20 mmol/L $NaHCO_3$, 0.4 mmol/L NaH_2PO_4, 1.0 mmol/L $MgCl_2$, 10 mmol/L D-glucose, pH 7.4) until contractions stopped, and prepared for Langendorff perfusion. Continuously carbogen-gassed (95% O_2/5% CO_2) Tyrode's buffer at 37 °C was perfused immediately after cannulation of the aorta, at a constant perfusion pressure of 80 mm Hg. Coronary flow was between 4 and 8 mL/min. Subsequently, the pulmonary artery was cannulated and the caval and pulmonary veins were carefully ligated. After the ligation procedure, which took 30-45 minutes, the hearts were stabilized for 30 min.

With this modified Langendorff heart preparation it is possible to collect separately coronary effluent and interstitial transudate[3,13]. Coronary effluent (CE), ejected by the right ventricle, was collected via the cannulated pulmonary artery. Interstitial transudate (IST), which keeps dripping from the heart, was collected at the apex. IST flow was 0.7-2% of the coronary flow.

In previous experiments on renin uptake in the modified Langendorff heart we perfused hearts with porcine renin, because of the limited availability of

sufficient quantities of rat renin. In the present study we also used porcine renin. In addition, recombinant human renin and prorenin were used because 1) antibodies for immunohistochemical localization of renin are not available for porcine renin and 2) porcine prorenin is not available in sufficient quantities.

To avoid the use of large quantities of recombinant human renin and prorenin a recirculating perfusion system was developed. In this system the ejected CE is collected in a glass chamber, where oxygenation occurs under constant carbogen flow at room temperature. The oxygenated perfusion fluid is then warmed up to 37 °C and pumped back into the Langendorff system, using a roller-pump (IPS 12, Ismatec, Zurich, Switzerland). The total fluid volume within the system was 60 mL. Perfusion fluid losses due to CE and IST collection (approximately 10 mL) were not corrected. Human serum albumin was added to the perfusion fluid in order to prevent binding of renin and prorenin to the plastic tubes and glass.

3.3.3 Perfusions with porcine renin to study membrane-binding of renin

Hearts were perfused with porcine renin, diluted 1/5 with Tyrode's buffer (arterial concentration approximately 2 pmol AngI/min per mL), via a T-connection into the cannulated aorta at a speed of 0.1 mL/min. After 30 min, when a steady state had been reached, the perfusion was stopped. The hearts were then removed, rapidly minced into small pieces, and homogenized (1/2, wt/vol) with a Polytron PT10/35 in 0.01 mol/L phosphate buffer, pH 7.4, containing 0.15 mol/L NaCl. The homogenate was centrifuged at 2,000*g* for 15 min at 4 °C and the pellet was discarded. The supernatant was recentrifuged at 50,000*g* for 45 min at 4 °C. The supernatant obtained after the second centrifugation was also discarded, whereas the remaining pellet was washed two times by resuspension in phosphate buffer and subsequent centrifugation at

50,000*g* (45 min, 4°C). Renin measurements were performed in the homogenate and in the washed pellet fraction ("purified membrane fraction").

3.3.4 Perfusions with human renin to study the localization of arterially delivered renin.

Hearts were perfused with human renin (concentration 40 U/L) in a recirculating Langendorff system for 60 min. After the perfusion had been stopped, the heart was rapidly removed and cut into 3 mm thick slices. The slices were fixed in 4 % paraformaldehyde and embedded in paraffin. Slices prepared from hearts that had been perfused with Tyrode's buffer served as controls.

3.3.5 Perfusions with human prorenin to study prorenin uptake and activation.

Hearts were perfused with human prorenin (concentration 2 U/L) in a recirculating Langendorff system for 50 min. Longer perfusion periods were not possible because the condition of the heart started to deteriorate after more than 60 min of recirculation. At 5, 10, 20, 30, 40 and 50 min after the start of the prorenin perfusion, 1 mL-samples of CE and 0.6 mL-samples of ISF were collected into Eppendorf cups containing 35 µL and 21 µL, respectively, of an inhibitor mixture in order to prevent prorenin activation in the cups. The mixture contained phenylmethylsulfonyl fluoride (0.07 mol/L), disodium EDTA (0.14 mol/L), 8-hydroxyquinoline sulphate (0.10 mol/L) and aprotinin (2000 kallikrein-inhibiting units per mL). The samples were frozen on dry ice and stored at -80 °C.

3.3.6 Immunohistochemistry

Paraffin-embedded heart sections were dewaxed, rehydrated, washed in tap water for 5 min at room temperature, and subsequently washed in phosphate buffered saline (PBS) for 5 min. Sections were then incubated overnight at 4 °C with the primary polyclonal anti-human renin antibody R-15 diluted 1/50 in PBS containing 1% bovine serum albumin (BSA). On the next day, sections were washed 3 times 10 min with PBS containing 1% BSA. To localize the primary antibody, the sections were incubated with the secondary goat anti-(rabbit IgG) antibody conjugated to fluorescein isothiocyanate for 45 min. Sections were subsequently washed 4 times 20 min with PBS containing 1% BSA, and mounted with 1,4-diazobizyclo-[2,2,2]-octane (DABCO)[14].

3.3.7 Biochemical measurements

Porcine renin

The concentration of porcine renin in cardiac homogenates and purified membrane fractions was measured by enzyme-kinetic assay as described before[3]. Results are expressed as fmol AngI/min per mg protein.

Human prorenin and renin

The concentration of human prorenin and renin was measured in CE and IST by immunoradiometric assay (Nichols Institute, Wychen, The Netherlands), following the methods proposed by Derkx *et al.*[15]. The concentration of prorenin was calculated by subtracting the results obtained before activation of prorenin (i.e. renin) from those obtained after activation (i.e., renin + prorenin). Prorenin was activated non-proteolytically, using the renin inhibitor remikiren[15,16]. Results are expressed as U/L using the international human kidney renin standard MRC

68/356 (Medical Research Council, National Institute of Biological Standards and Control, London, UK) as a reference.

Total protein
Total protein was measured using the pyrogallol red molybdate complex method (Instruchemie, Hilversum, The Netherlands).

3.4 Results

3.4.1 Membrane binding of renin

The purified membrane fraction contained 5.2 ± 1.4 % of the total amount of renin present in the crude homogenate. Expressed per gram protein, renin was enriched 4.7 ± 3.5 fold in the purified membrane fraction (71.4 ± 50.7 vs. 16.9 ± 4.6 fmol AngI/min per mg protein, purified membrane fraction vs. homogenate, $p<0.05$).

3.4.2 Localization of renin with immunohistochemistry

Sections of hearts perfused with human renin showed clear renin staining in the media of the coronary arteries, whereas no staining was found in the endothelium, adventitia or cardiomyocytes (Fig 1). Sections incubated with the secondary antibody only showed no staining (data not shown). Sections of control hearts not perfused with human renin also revealed no staining, indicating that the staining in the coronary arteries was specific for human renin.

3.4.3 Prorenin uptake and activation in interstitial fluid

A steady-state prorenin concentration was reached in IST within 10 min (Fig 2), and this concentration was not significantly different from the steady-state prorenin concentration in CE. The prorenin concentrations in CE and IST remained constant over the 50-min perfusion period once a steady state had been reached. Low levels of renin were detected in both CE and IST, which did not change over the 50 min perfusion period. Since the monoclonal antibodies used to measure renin crossreact (<2%) with prorenin, the most likely explanation for these low levels of renin is that they represent falsely detected prorenin[15].

3.5 Discussion

The experiments described in this paper were performed to address the localization of arterially delivered renin in the heart. In addition, the activation of arterially delivered prorenin at cardiac tissue sites was investigated. We made use of a modified Langendorff model, which allowed the separate collection of CE and IST[3,13].

Previous studies in this model have shown that arterially delivered renin enters the interstitial fluid compartment, and disappears from the coronary vascular bed in a biphasic manner[3]. We speculated that the second, slow phase of the disappearance curve corresponded with vascular wall-bound renin. This renin might be involved in the local production and subsequent release of AngI into the coronary circulation, which has been described by us and others[2,3,17]. The immunohistochemical data obtained in the present study show that arterially delivered recombinant human renin is indeed sequestered by the coronary vascular wall. Renin appeared to be confined to the media; no staining was observed in the endothelium, adventitia or cardiomyocytes. The absence of renin-staining in cardiomyocytes suggests either that arterially delivered renin

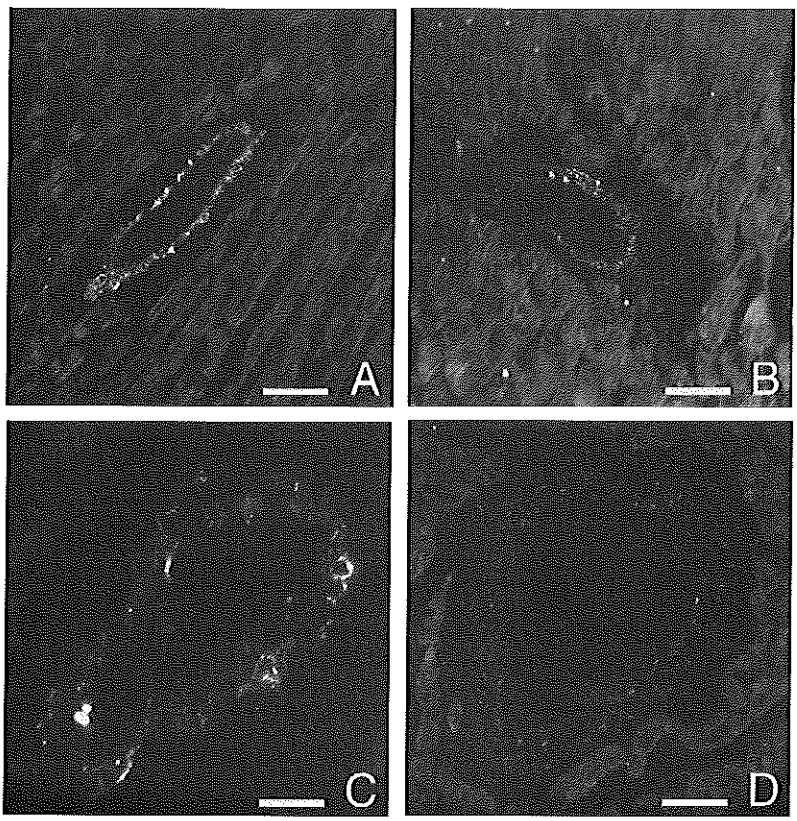

Figure 1. Immunofluorescent stained sections of a rat heart perfused for 60 min in a recirculating Langendorff system with human renin. The photomicrographs show coronary arteries in different regions of the heart. The white bar in photomicrograph A, B and D indicates 50 μm. The white bar in photomicrograph C indicates 20 μm.

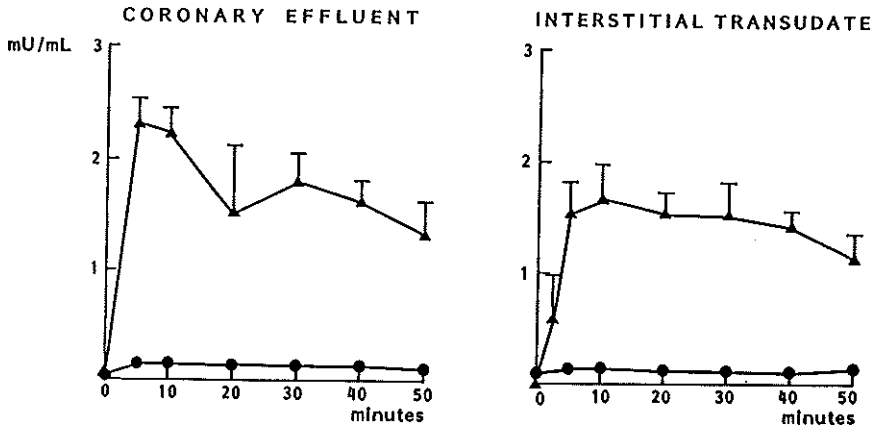

Figure 2. Human prorenin (triangles) and renin (circles) levels in coronary effluent (left panel) and interstitial transudate (right panel), during a 50 min recirculating perfusion of the modified Langedorff heart with human prorenin. Values are means ± s.d..

does not reach these cells or that cardiomyocytes are not capable of binding renin in amounts that can be detected immunohistochemically.

Our studies are in agreement with earlier investigations in nephrectomized rats demonstrating the presence of i.v. injected mouse submaxillary gland renin in the media of the aorta[18], but contrast with studies in humans where vascular renin appeared to be confined to the endothelium[19]. The reasons for this discrepancy are at present unclear. The vascular site(s) of angiotensin production may differ between humans and rats. For instance, vascular ACE in the rat is mainly present in smooth muscle cells, whereas in humans ACE is limited to the endothelial layer[20-23].

Based upon our immunohistochemical data it can not be concluded whether renin is cell-associated. We therefore prepared membrane fractions from hearts perfused with semipurified porcine renin and found renin to be enriched in these fractions, suggesting that renin may indeed be localized on or in smooth muscle cells. Such membrane-binding is in agreement with the recently described renin receptors and (pro)renin binding proteins in membranes prepared from human mesangial cells and rat tissues respectively[8-10]. Rat (pro)renin binding proteins have been described to bind both to rat and human prorenin and renin[8]. It must be kept in mind, however, that less than 10% of cardiac renin was found to be membrane-bound. This may relate to the fact that renin binding appeared to occur in the vascular wall only. Most likely therefore, the majority of cardiac renin is present in extracellular fluid[3].

Taken together, the following scenario might underlie vascular angiotensin production. Plasma renin diffuses into the interstitial fluid of the vascular wall and binds to smooth muscle cells. Both cell membrane-associated and interstitial renin may react with angiotensinogen, present in extracellular fluid, to form AngI. This locally generated AngI may directly enter the vascular lumen. Alternatively, it could be converted locally to AngII by ACE present on smooth muscle cells and/or endothelial cells. Most or all of this locally generated AngII will remain in the tissue, since AngII release from tissue sites into the coronary circulation could not be demonstrated[2,24].

It has been suggested that prorenin, the inactive precursor of renin, might also bind to the membrane-associated (pro)renin binding protein[8]. Such prorenin binding could either prevent renin binding, thereby inhibiting local AngI production[8], or it might be followed by local activation of prorenin to renin, thereby leading to enhanced local AngI production[12]. We investigated the latter possibility by perfusing rat hearts for 50 minutes with human prorenin. Prorenin, like renin, entered the ISF compartment, reaching steady-state concentrations that were comparable to its concentration in CE. Over a 50-min period, however, no prorenin activation could be demonstrated in either ISF or CE. Thus, either no

activation occurs, or prorenin, if activated, remains in the tissue and is not released into the circulation. Another possibility is that a period of 50 minutes is too short to demonstrate activation. Further studies, measuring prorenin in tissue compartments, are necessary to resolve these issues.

3.6 References

1. Admiraal PJJ, Derkx FHM, Danser AHJ, Pieterman H, Schalekamp MADH: Intrarenal de novo production of angiotensin I in subjects with renal artery stenosis. *Hypertension.* 1990; 16: 555-563.
2. Danser AHJ, Koning MMG, Admiraal PJJ, Sassen LMA, Derkx FHM, Verdouw PD, Schalekamp MADH: Production of angiotensin I and II at tissue sites in the intact pig. *Am J Physiol.* 1992; 263: H429-H437.
3. de Lannoy LM, Danser AHJ, van Kats JP, Schoemaker RG, Saxena PR, Schalekamp MADH: Renin-angiotensin system components in the interstitial fluid of the isolated perfused rat heart. Local production of angiotensin I. *Hypertension.* 1997; 29: 1240-1251.
4. Kato H, Iwai N, Inui H, Kimoto K, Uchiyama Y, Inagami T: Regulation of vascular angiotensin release. *Hypertension.* 1993; 21: 446-454.
5. Hilgers KF, Kuczera M, Wilhelm MJ, Wiecek A, Ritz E, Ganten D, Mann JFE: Angiotensin formation in the isolated rat hindlimb. *J Hypertens.* 1989; 7: 789-798.
6. Danser AHJ, van Kats JP, Admiraal PJJ, Derkx FHM, Lamers JMJ, Verdouw PD, Saxena PR, Schalekamp MADH: Cardiac renin and angiotensins. Uptake from plasma versus in situ synthesis. *Hypertension.* 1994; 24: 37-48.
7. Thurston H, Swales JD, Bing RF, Hurst BC, Marks ES: Vascular renin-like activity and blood pressure maintenance in the rat: studies of the effect of changes in sodium balance, hypertension and nephrectomy. *Hypertension.* 1979; 1: 643-649.
8. Sealey JE, Catanzaro DF, Lavin TN, Gahnem F, Pitarresi T, Hu L-f, Laragh JH: Specific prorenin/renin binding (PRoBP). Identification and characterization of a novel membrane site. *Am J Hypertens.* 1996; 9: 491-502.
9. Nguyen G, Delarue F, Berrou J, Rondeau E, Sraer JD: Specific receptor binding of renin on human mesangial cells in culture increases plasminogen activator inhibitor-1 antigen. *Kidney Int.* 1996; 50: 1897-1093.
10. Campbell DJ, Valentijn AJ: Identification of vascular renin-binding proteins by chemical cross-linking: inhibition of binding of renin by renin inhibitors. *J Hypertens.* 1994; 12: 879-890.
11. Skinner SL, Thatcher RL, Whithworth JA, Horowitz JD: Extraction of plasma prorenin by human heart. *The Lancet.* 1986; 1: 995-997.
12. Sealey JE,, Rubattu S: Prorenin and renin as separate mediators of tissue and circulating systems. *Am J Hypertens.* 1989; 2: 358-366.

13. De Deckere EAM, Ten Hoor P: A modified Langendorff technique for metabolic investigations. *Pflügers Arch.* 1977; 370: 103-105.
14. Langanger G, de Mey J, Adam H: 1,4-Diazobiclo-[2,2,2]-Octane [DABCO] is ratarding fading of immunofluorescence preparations. *Microskopie.* 1983; 40: 237-241.
15. Derkx FH, de Bruin RJ, van Gool JMG, van den Hoek MJ, Beerendonk CCM, Rosmalen F, Haima P, Schalekamp MADH: Clinical validation of renin monoclonal antibody-based sandwich assay of renin and prorenin, and use of renin inhibitor to enhance prorenin immunoreactivity. *Clin Chem.* 1996; 42: 1051-63.
16. Derkx FHM, Deinum J, Lipovski M, Verhaar M, Fischli W, Schalekamp MADH: Nonproteolytic "activation" of prorenin by active site-directed renin inhibitors as demonstrated by renin-specific monoclonal antibody. *J Biol Chem.* 1992; 267: 22837-22842.
17. Neri Serneri GG, Boddi M, Coppo M, Chechi T, Zarone N, Moira M, Poggesi L, Margheri M, Simonetti I: Evidence for the existence of a functional cardiac renin-angiotensin system in humans. *Circulation.* 1996; 94: 1886-1893.
18. Swales JD, Abramovici A, Beck F, Bing RF, Loudon M, Thurston H: Arterial wall renin. *J. Hypertens* 1983; 1 (suppl 1): 17-22.
19. Okamura T, Aimi Y, Kimura H, Murakami K, Toda N: Existence of renin in the endothelium of human artery. *J Hypertens.* 1992; 10: 49-53.
20. van der Giessen WJ, Danser AHJ, van Beusekom HMM, Derkx FHM, Verdouw PD, Lamers JMJ, Serruys PW: Enhanced angiotensin II degradation in porcine coronary neointimal hyperplasia induced by stent implantation. *Coron Artery Dis.* 1992; 3: 730-737.
21. Pipili E, Manolopoulos VG, Catravas JD, Maragoudakis ME: Angiotensin converting enzyme activity is present in the endothelium-denuded aorta. *Br J Pharmacol.* 1989; 98: 333-335.
22. Egleme C, Cressier F, Wood JM: Local formation of angiotensin II in the rat aorta: effect of endothelium. *Br J Pharmacol.* 1990; 100: 237-240.
23. 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: 1137-1142.
24. 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: 173-184.

Chapter 4

Localization and production of angiotensin II in the isolated perfused rat heart

4.1 Abstract

We used a modification of the isolated perfused rat heart, in which coronary effluent and interstitial transudate were separately collected, to investigate the localization and production of angiotensin II (AngII) in the heart. During combined renin (0.7-1.5 pmol AngI/mL per min) and angiotensinogen (6-12 pmol/mL) perfusion (4-8 mL/min) for 60 min (n=3), the steady-state levels of AngII in interstitial transudate in two consecutive 10-min periods were 4.3 ± 1.5 and 3.6 ± 1.5 fmol/mL, as compared to 1.1 ± 0.4 and 1.1 ± 0.6 fmol/mL in coronary effluent (mean ± half range). During perfusion with AngII (n=5), steady-state AngII in interstitial transudate was 32 ± 19% of arterial AngII, as compared to 65 ± 16% in coronary effluent (mean ± s.d., $p<0.02$). During perfusion with AngI (n=5), AngII in interstitial transudate was 5.1 ± 0.6% of arterial AngI, as compared to 2.2 ± 0.3% in coronary effluent ($p<0.05$). The tissue concentration of AngII in the combined renin/angiotensinogen perfusions (per g) was as high as the concentration in interstitial transudate (per mL). Addition of losartan (10^{-6} mol/L) to the renin/angiotensinogen perfusion (n=3) had no significant effect on the tissue level of AngII, whereas losartan in the perfusions with AngI (n=5) or AngII (n=5) decreased tissue AngII to undetectably low levels. The results indicate that the heart is capable of producing AngII and that this can lead to higher levels in tissue than in blood plasma. Cardiac AngII does not appear to be restricted to the extracellular fluid. This is in part due to AT_1 receptor-mediated cellular uptake of extracellular AngII, but our results also raise the possibility of intracellular AngII production.

4.2 Introduction

Angiotensin II (AngII) is the primary mediator of the physiological actions of the renin-angiotensin system (RAS), and has an important function in cardiovascular homeostasis. AngII in the circulation is produced by the conversion of AngI by angiotensin-converting enzyme (ACE) of the vascular endothelium. AngI is generated in the circulation by the action of renin from the kidney on its substrate, angiotensinogen, produced by the liver. The various RAS components have all been identified in tissues[1-4], which suggests the existence of a locally acting RAS in the tissues, apart from the RAS in the circulation.

ACE inhibitors and the more recently developed AngII AT_1-type receptor antagonists are now widely used for the treatment of hypertension and heart failure. Local AngII production in the heart is often invoked to explain the results of clinical trials, which indicate that the beneficial effects of these drugs in heart failure are partly independent of their blood pressure-lowering effect[5,6]. Knowledge of cardiac AngI and II production, however, is still fragmentary and little is known about its regulation and physiological significance[3].

In a previous study we used a modified Langendorff rat heart preparation to investigate the production of AngI in cardiac tissue[7]. This isolated heart preparation enabled us to collect interstitial transudate separately from the coronary effluent. During perfusion with renin and angiotensinogen, AngI appeared in the transudate. This AngI was produced in cardiac tissue and was not derived from AngI in the perfusate. There was no indication that the heart was capable of producing AngI independently of arterially delivered renin and angiotensinogen. Observations in nephrectomized pigs also indicated that the cardiac tissue levels of AngI and II are determined by kidney-derived renin[4].

Here we report on a study in which we used the same modified Langendorff rat heart preparation to investigate the production of AngII. The presence of AngII in tissue depends on arterial delivery of this peptide and on its local generation. AngII may be formed from arterially delivered AngI or

from in-situ synthesized AngI. Extracellular AngII is known to be taken up into cells by AT_1 receptor endocytosis[8-11]. The adrenals, the kidneys and also the heart accumulate AngII from the circulation by an AT_1 receptor-mediated process, probably endocytosis[12].

The present study addresses the question of where AngII is localized in the heart, where it is produced, and how the cardiac tissue levels are influenced by AT_1 receptor-mediated cellular uptake.

4.3 Materials and methods

4.3.1 Chemicals and reagents

[Ile^5]-Ang-(1-10) decapeptide (AngI) and [Ile^5]-Ang-(1-8) octapeptide (AngII) were obtained from Bachem, Bubendorf, Switzerland. Bovine serum albumin (BSA) was from Sigma, St. Louis, MO, USA. 1,10-Phenanthroline was from Merck, Darmstadt, Germany. Sodium pentobarbital was obtained from Apharma, Arnhem, The Netherlands. The AT_1 receptor antagonist, losartan, was a kind gift of Dr. R.D. Smith, Du Pont Merck, Wilmington, DE, USA. The renin inhibitor, remikiren, was a kind gift of Dr. P. van Brummelen, Hoffmann-La Roche, Basel, Switzerland. Renin was prepared from porcine kidneys as described before[7]. Angiotensinogen was prepared from plasma of nephrectomized pigs[7]. All other reagents were of standard laboratory grade.

4.3.2 Preparation of the modified Langendorff heart

All experiments were performed under the regulation of the Animal Care Committee of the Erasmus University, Rotterdam, The Netherlands, in

accordance with the 'Guiding principles in the care and use of animals' as approved by the American Physiological Society.

Male Wistar rats (Harlan, Zeist, The Netherlands; 280-400 g) were anesthetized with pentobarbital (60 mg/kg, i.p.), and heparinized (5,000 units/kg, i.v.). The hearts (1.0-1.4 g) were rapidly excised, cooled in ice-cold Tyrode's buffer (125 mmol/L NaCl, 4.7 mmol/L KCl, 1.4 mmol/L $CaCl_2$, 20 mmol/L $NaHCO_3$, 0.4 mmol/L NaH_2PO_4, 1.0 mmol/L $MgCl_2$, 10 mmol/L D-glucose, pH 7.4) until contractions stopped, and prepared for Langendorff perfusion. Carbogen-gassed (95% O_2/5% CO_2) Tyrode's buffer at 37 °C was continuously perfused immediately after cannulation of the aorta, at a constant perfusion pressure of 80 mm Hg. Subsequently the pulmonary artery was cannulated and the caval and pulmonary veins were carefully ligated. After the ligation procedure, which took 30-45 minutes, the hearts were allowed to stabilize for 30 min.

With this modified Langendorff heart preparation it is possible to collect separately coronary effluent and interstitial transudate[7,13]. Coronary effluent (CE), ejected by the right ventricle, was collected via the cannulated pulmonary artery. Interstitial transudate (IST), which keeps dripping from the heart, was collected at the apex. IST flow was 0.03-0.16 mL/min, corresponding with 0.7-2% of the coronary flow. An IST flow > 2% of the coronary flow was considered to be an indication of leakage, e.g. from veins that were not properly ligated[7]. Hearts with such high IST flow were therefore not used. IST is derived from the interstitial fluid (ISF) and the AngI and II concentrations in IST are considered to be representative for the AngI and II concentrations in ISF[7].

4.3.3 Perfusions with renin-angiotensin system components and collection of CE and IST

The Langendorff hearts were perfused with Tyrode's buffer via the cannulated aorta. After a 30-min stabilization period, RAS components were infused, via a T-connection, into the cannulated aorta.

CE and IST were collected during and after the infusions. One-min (4-8 mL), 4-min (16-40 mL) or 10-min samples (40-80 mL) of CE were collected into BSA-coated 10- or 50-mL polystyrene tubes. Individual drops of IST (approximately 50 µL each minute) and 4-min (approximately 200 µL) or 9- to 10-min (approximately 500 µL) samples of IST were collected into BSA-coated 1.5-mL Eppendorf cups. The Eppendorf cups and polystyrene tubes contained a mixture of inhibitors, 5, 10 or 25 µL in the Eppendorf cups (for the individual drops of IST and the 4-min and 10-min IST samples, respectively), and 250, 2500 and 5000 µL in the polystyrene tubes (for the 1-min, 4-min and 10-min CE samples), in order to prevent the ongoing formation of AngI, the conversion of AngI to AngII and the degradation of AngI and II. The mixture consisted of 0.2 mmol/L of the renin inhibitor remikiren, 125 mmol/L disodium EDTA, and 25 mmol/L 1,10-phenanthroline[7]. Remikiren is an inhibitor of human renin (IC_{50} 7×10^{-10} mol/L). It also inhibits porcine renin (IC_{50} 5×10^{-8} mol/L)[4].

The Eppendorf cups and polystyrene tubes were kept on ice during the perfusions, so that the samples were rapidly cooled during their collection and remained cold (0-4 °C) during the experiment. After the experiment had been finished, the samples for AngI and II measurements were frozen at -80 °C.

4.3.4 Perfusion with AngII to study the localization of arterially delivered AngII

Langendorff hearts were perfused for 15 min with AngII. AngII diluted with Tyrode's buffer to a concentration of 400 pmol/mL was infused into the perfusion system at a rate of 0.1 mL/min. AngII caused a transient fall in coronary flow which stabilized in 2-3 min at 4-9 mL/min. After the AngII infusion had been switched off, the heart was either quickly removed from the perfusion apparatus and frozen in liquid nitrogen, or subjected to a 10-min washout period. One-min samples of CE and individual drops of IST were collected during the infusion and wash-out periods in order to study the uptake and washout kinetics of AngII. A steady state was reached within 5 min. Samples of CE and IST that had been collected from 7 to 10 min and from 11 to 14 min were used to determine the steady-state levels of AngII. The frozen hearts that were not subjected to a wash-out period were used to measure the steady-state tissue levels of AngII.

Steady-state levels of AngII in CE, IST and cardiac tissue were also measured in perfusions with AngII in the presence of the AT_1 receptor antagonist losartan (10^{-6} mol/L) in the perfusion fluid. The frozen hearts were used to measure the steady-state tissue levels of AngII. Coronary flow in these experiments was 4-11 mL/min.

4.3.5 Perfusion with AngI to study the localization of arterially delivered AngI and locally generated AngII

Langendorff hearts were perfused for 15 min with AngI. AngI diluted with Tyrode's buffer to a concentration of 400 pmol/mL was infused into the perfusion system at a rate of 0.1 mL/min. Coronary flow in these experiments was 5-10 mL/min. The uptake and washout kinetics of AngI are known from our

previous study[7]. After the AngI infusion had been switched off, the heart was immediately removed from the perfusion apparatus and quickly frozen in liquid nitrogen. Samples of CE and IST collected from 7 to 10 min and from 11 to 14 min were used to determine the steady-state levels of AngI and II. The frozen hearts were used to measure the steady-state tissue levels of AngI and II.

Steady-state levels of AngI and II in CE, IST and cardiac tissue were also measured in perfusions with AngI in the presence of the AT_1 receptor antagonist losartan (10^{-6} mol/L) in the perfusion fluid. Coronary flow in these experiments was 4-9 mL/min.

4.3.6 Perfusions with renin combined with angiotensinogen to study the localization of locally generated AngI and II

Langendorff hearts were perfused for 60 min with renin and angiotensinogen. Porcine renin diluted with Tyrode's buffer to a concentration of approximately 60 pmol AngI/min per mL and undiluted porcine angiotensinogen (500 pmol/mL) were infused into the perfusion system both at a rate of 0.1 ml/min. Coronary flow was 4-8 mL/min. The final concentrations of renin and angiotensinogen in the perfusion fluid ranged from 0.7 to 1.5 pmol AngI/mL per min and from 6 to 12 pmol/mL, respectively. These experiments were performed in the presence or absence of the AT_1 receptor antagonist losartan (10^{-6} mol/L) in the perfusion fluid. The renin and angiotensinogen solutions were kept at 4 °C until they reached the aorta, in order to prevent angiotensin generation outside the heart[7]. After the perfusion had been switched off, the hearts were rapidly frozen in liquid nitrogen.

Samples of CE and IST collected from 31 to 40 min and from 46 to 55 min were used to determine the steady-state levels of AngI and II. Collections from 41 to 45 and from 56 to 60 min were used for other measurements, not

reported in this paper. The frozen hearts were used to measure the steady-state tissue levels of AngI and II.

4.3.7 Measurements of AngI and II

The AngI and II concentrations in CE and IST samples, collected during AngI and II perfusions, were measured directly with sensitive radioimmunoassays[14,15]. Measurements were made in 50 μL of undiluted CE and in 50 μL IST diluted with 25 μL of 0.25 mol/L phosphate buffer, pH 7.4, containing 0.15 mol/L NaCl. The lowest measurable AngI concentration was 15 fmol/mL in CE and 40 fmol/mL in IST. The lowest measurable AngII concentration was 10 fmol/mL in CE and 25 fmol/mL in IST.

The AngI and II concentration of CE and IST samples obtained during infusion with renin combined with angiotensinogen, as well as the cardiac tissue concentrations of AngI and II both during AngI and II perfusion and during combined renin and angiotensinogen perfusion, were measured by radioimmunoassay, after SepPak extraction and reversed phase-high performance liquid chromatography (HPLC) separation[4,14,15]. To measure tissue AngI and II, the frozen hearts were minced and homogenized (1:10, weight/volume) in an iced solution of 0.1 mol/L HCl/80% ethanol. Homogenates were centrifuged at 20,000*g* for 25 min at 4 °C. Ethanol in the supernatant was evaporated under constant air flow. The remainder of the supernatant was diluted in 20 mL 1% ortho-phosphoric acid and centrifuged again at 20,000*g*. The supernatant was diluted with 1% ortho-phosphoric acid 1:1 (vol/vol). The tissue homogenate supernatants and CE and IST samples were concentrated over SepPak columns (SepPak C18, Waters, Milford, MA, USA), and the concentrated extracts were subjected to HPLC followed by radioimmunoassay.

^{125}I-labeled AngI had been added as an internal standard to the CE and IST samples before SepPak extraction and to tissue before its homogenization.

Recovery was better than 70%, and the AngI and II results were corrected for incomplete recovery. The lowest level of AngI that could be measured with the AngI radioimmunoassay after HPLC separation was 2.0 fmol/g in cardiac tissue, 0.05 fmol/mL in CE, and 2.5 fmol/mL in IST. The lowest level of AngII that could be measured with the AngII radioimmunoassay after HPLC separation was 1.0 fmol/g in cardiac tissue, 0.05 fmol/mL in CE and 1.5 fmol/mL in IST.

4.3.8 Calculations

In order to determine the localization of AngI and II in cardiac tissue, the measured tissue levels of AngI and II were compared with the levels predicted on the basis of the presence of these peptides in the cardiac extracellular fluid compartments. If the measured tissue levels are higher than predicted, angiotensin is not restricted to the extracellular fluid compartments. If the measured tissue levels are lower than predicted, angiotensin has disappeared from the extracellular fluid compartments, during the period between the moment the heart has been removed from the perfusion equipment and the moment the tissue is transferred into liquid nitrogen. The predicted tissue level (fmol/g) was calculated as follows:

Predicted tissue Ang concentration = $[Ang_{IST}]$ x ISF volume + $[Ang_{CE}]$ x IVF volume

in which $[Ang_{IST}]$ is the steady-state AngI or II concentration in IST and $[Ang_{CE}]$ is the steady-state AngI or II concentration in CE. ISF volume is the cardiac interstitial fluid volume (0.23 mL/g), determined previously as the difference between the distribution volume of ^{51}Cr-EDTA and ^{125}I-HSA[7], and IVF volume is the intravascular fluid volume (0.38 mL/g), determined previously as the distribution volume of ^{125}I-HSA[7]. In this calculation, it is assumed that $[Ang_{CE}]$

is representative for the angiotensin concentration in most of the intravascular fluid compartment (coronary venules and veins, and right ventricle).

4.3.9 Statistical analysis

The AngI and II levels in CE, IST and cardiac tissue during AngI or II perfusion are expressed as a percentage of the arterial levels of AngI (AngI perfusion) or AngII (AngII perfusion). AngI and II levels in CE, IST and cardiac tissue during renin combined with angiotensinogen perfusion are given as absolute values. Differences between the experimental groups were evaluated for statistical significance by Student's unpaired t-test. Differences within groups were evaluated for statistical significance by Student's paired t-test. Differences were assigned to be significant for values of $p < 0.05$.

4.4 Results

4.4.1 Localization of arterially delivered AngII during perfusion with AngII

During perfusion with AngII (n=10), the steady-state AngII level in CE was 65% of the arterial level (Fig 1 and Fig 2). Thus, 35% of the arterially delivered AngII was removed by the heart, during a single passage of the perfusate. The intravascular fluid volume of the Langendorff heart was measured in our previous study of this model and was found to be 0.38 mL/g. Heart weight was 1.0-1.4 g, thus the intravascular fluid volume per heart was 0.38-0.53 mL. Since the perfusate flow was 4-9 mL/min, the perfusate transit time of the isolated heart preparation was 0.04-0.13 min. It can be concluded, therefore, that 35% of the arterially delivered AngII was removed in 0.04-0.13 min, which corresponds with a $t_{½}$ of 0.06-0.21 min.

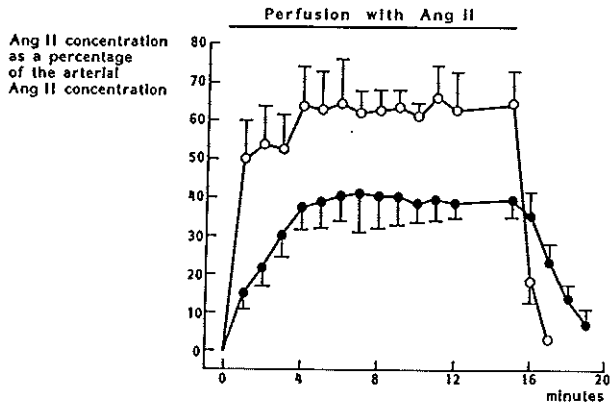

Figure 1. AngII concentration in IST (closed circles) and CE (open circles) during and after perfusion with angiotensin II (n=5). Data (mean and s.e.m.) are presented as a percentage of the arterial AngII concentration.

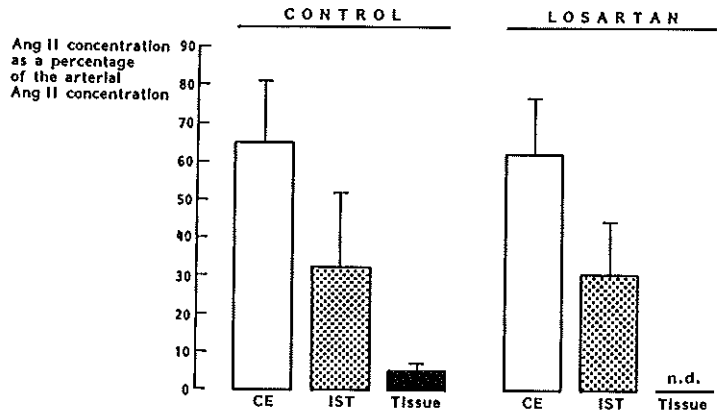

Figure 2. Steady-state AngII concentration in CE and IST during perfusion with AngII for 15 min, and the AngII concentration in cardiac tissue shortly after the perfusion. Perfusions were carried out in the absence (n=5) or presence (n=5) of losartan (10^{-6} mol/L). The mean of the levels of the 7 to 10-min and 11 to 14-min perfusion periods was taken as the steady-state concentration. Data (mean and s.d.) are presented as a percentage of the arterial AngII concentration. AngII in IST was lower than in CE ($p<0.02$) and higher than in cardiac tissue ($p<0.05$) both with and without losartan. n.d.: not detectable.

A steady-state level in IST was reached within 5 min. It was approximately 35% of the arterial level (39% in the experiments in which also the AngII washout was measured, Fig 1; 32% in the experiments in which also the tissue AngII concentration was measured, Fig 2). The steady-state AngII concentration in IST was significantly lower than in CE.

After discontinuation of the AngII perfusion, AngII disappeared from IST, in a monophasic way, with a $t_{½}$ of 1.4 ± 0.3 min (mean ± s.d., n=5).

The cardiac tissue level of AngII (per g tissue), shortly after the AngII perfusion had been switched off, was approximately 5% of the arterial level (per mL perfusate) (Fig 2). This is lower than predicted on the basis of the presence of AngII the cardiac extracellular fluid compartments (see Methods). The predicted tissue concentration of AngII would be 30-35% of the arterial concentration. This discrepancy suggests that extracellular AngII was rapidly degraded in the short period between the moment the AngII infusion had been stopped and the moment the tissue was transferred into liquid nitrogen.

During perfusion with AngII in the presence of the AT_1 receptor antagonist losartan (n=5), the steady-state AngII levels in CE and IST were not significantly different from the levels during AngII perfusion in the control situation (Fig 2). AngII, which was above the detection limit in all cardiac tissue samples, could not be detected in the tissue samples in the AngII/losartan perfusion experiments. This is likely to be due to blockade of the AT_1 receptor-mediated cellular uptake of extracellular AngII.

4.4.2 Localization of arterially delivered AngI and locally generated AngII during perfusion with AngI

During perfusion with AngI (n=5), the steady-state AngI level in CE was 48% of the arterial level (Fig 3). Thus, 52% of arterially delivered AngI was removed

by the heart during a single passage of perfusate. Part of the AngI removal was caused by conversion to AngII (Fig 4).

The steady-state AngII level in IST was higher than in CE, whereas the steady-state AngI level in IST was lower than in CE. This, together with the finding that during perfusion with AngII the AngII level in IST was also lower than in CE (see above), indicates that part of the AngII present in IST during AngI perfusion was not derived from AngII in the perfusate.

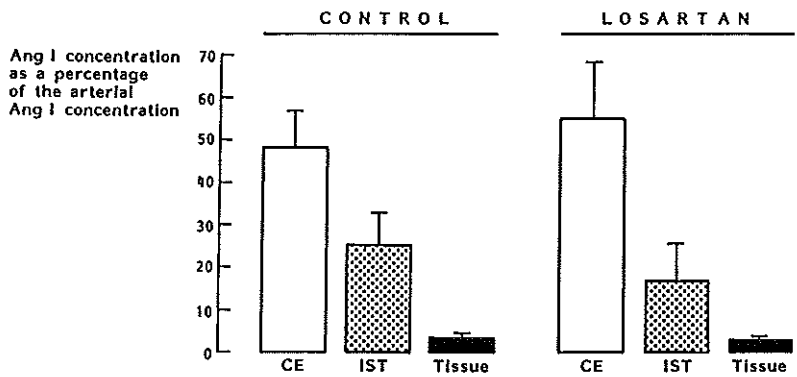

Figure 3. Steady-state AngI concentration in CE and IST during perfusion with AngI for 15 min, and the AngII concentration in cardiac tissue shortly after the perfusion. Perfusions were carried out in the absence (n=5) or presence (n=5) of losartan (10^{-6} mol/L). The mean of the levels of the 7 to 10-min and 11 to 14-min perfusion periods was taken as the steady-state concentration. Data (mean and s.d.) are presented as a percentage of the arterial AngI concentration. AngI in IST was lower than in CE ($p<0.02$) and higher than in cardiac tissue ($p<0.02$) both with and without losartan.

The cardiac tissue level of AngI (per g tissue), shortly after the AngI perfusion had been switched off, was approximately 5% of the arterial level (per mL perfusate) (Fig 3). This is lower than predicted on the basis of the presence

of AngI in the cardiac extracellular fluid compartments (see Methods). The predicted tissue concentration of AngI would be 22% of the arterial concentration. This discrepancy suggests that extracellular AngI, like extracellular AngII, was rapidly degraded in the short period between the moment the AngI infusion had been stopped and the moment the tissue was transferred into liquid nitrogen.

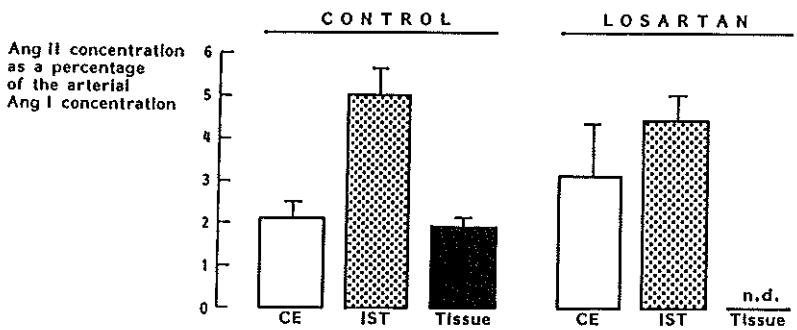

Figure 4. Steady-state AngII concentration in CE and IST during perfusion with AngI for 15 min, and the AngII concentration in cardiac tissue shortly after the perfusion. Perfusions were carried out in the absence (n=5) or presence (n=5) of losartan (10^{-6} mol/L). The mean of the levels of the 7 to 10-min and 11 to 14-min perfusion periods was taken as the steady-state concentration. Data (mean and s.d.) are presented as a percentage of the arterial AngI concentration. AngII in IST was higher than in CE ($p<0.05$) and cardiac tissue ($p<0.05$) both with and without losartan. n.d.: not detectable.

During perfusion with AngI in the presence of the AT_1 receptor antagonist losartan (n=5), the steady-state AngI and II levels in CE and IST were not significantly different from those during AngI perfusion in the control situation (Fig 3 and Fig 4). AngII, which was above the detection limit in all cardiac

tissue samples in the control AngI perfusion experiments, could not be detected in the tissue samples in the AngI/losartan perfusion experiments, which is probably due to blockade of the AT_1 receptor-mediated cellular uptake of extracellular AngII.

4.4.3 Localization of locally generated AngI and II during combined renin/angiotensinogen perfusion

Results of the combined renin/angiotensinogen perfusions are shown in Fig 5 and Fig 6. During combined renin/angiotensinogen perfusion in the absence of losartan (n=3), the AngI levels in IST in the 31- to 40-min and 46- to 55-min perfusion periods were 4.2 (1.5-8.2) and 3.2 (0.9-6.5) times higher than in CE, respectively (mean and range). The AngII levels in IST in these perfusion periods were 4.8 (2.7-6.8) and 3.8 (2.2-5.7) times the levels in CE.

The cardiac tissue level of AngI, 18.2 (11.4-22.8) fmol/g, shortly after the combined renin/angiotensinogen perfusion had been switched off, was close to the level predicted on the basis of the presence of AngI in the cardiac extracellular fluid compartments (see Methods), approximately 20 fmol/g.

The cardiac tissue level of AngII, 4.8 (2.8 - 6.4) fmol/g was higher than the level predicted on the basis of the presence of AngII in the cardiac extracellular fluid compartments, approximately 1.5 fmol/g. This suggests that the presence of the locally formed AngII was not limited to the intravascular and interstitial fluid.

During combined renin/angiotensinogen perfusion in the presence of the AT_1 receptor antagonist losartan (n=3), the AngI and II levels measured in CE, IST and cardiac tissue were similar to the levels measured in the control experiments without losartan. Thus, blockade of the AT_1 receptor-mediated uptake of extracellular AngII in these experiments did not decrease tissue AngII to an undetectably low level as it did in the AngI and II perfusion experiments.

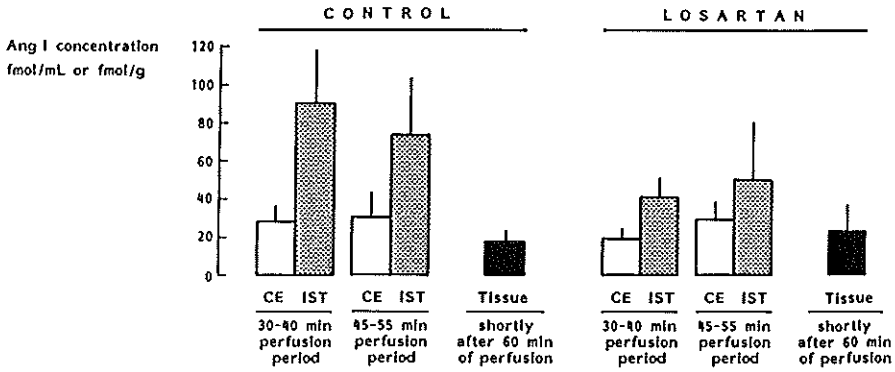

Figure 5. Steady-state AngI concentration in CE and IST during combined renin/angiotensinogen perfusion for 60 min, and the AngI concentration in cardiac tissue shortly after the perfusion. Perfusions were carried out in the absence (n=3) or presence (n=3) of losartan (10^{-6} mol/L). Data are presented as mean and half range.

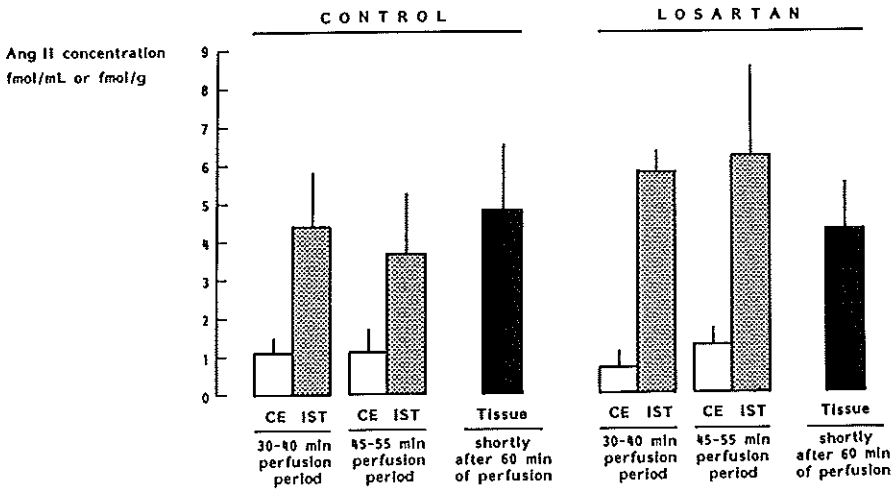

Figure 6. Steady-state AngII concentration in CE and IST during combined renin/angiotensinogen perfusion for 60 min, and the AngII concentration in cardiac tissue shortly after the perfusion. Perfusions were carried out in the absence (n=3) or presence (n=3) of losartan (10^{-6} mol/L). Data are presented as mean and half range.

4.5 Discussion

In a previous series of experiments we studied the production of AngI by the modified rat Langendorff heart, in which the interstitial fluid transudate could be collected separately from the coronary effluent[7]. The present study uses the same isolated heart model and focuses on the production of AngII.

As expected, part of the AngI present in the perfusate during the combined renin/angiotensinogen perfusions and formed by the reaction of arterially delivered renin with arterially delivered angiotensinogen, was converted to AngII and ejected via the CE. AngI-to-II conversion by the coronary vascular bed has been reported by others[16,17]. More interesting is our finding that, during these combined renin/angiotensinogen perfusions, the concentration of AngII in the IST was several times higher than in the CE, whereas during perfusions with AngII the AngII concentration in the IST was lower than in the CE. These results indicate that a substantial part of AngII in the interstitial fluid was not derived from the AngII that was present in the perfusate.

At the time we finished this manuscript, a paper was published by Oparil's group[18] on measurements of AngII in the interstitial fluid space of the dog heart by using microdialysis probes. The AngII level in the interstitial fluid was higher than in blood plasma and did not change after systemic infusion of AngI. These results indicate compartmentalization of AngII in the heart between the interstitial and intravascular compartments. Our results are in agreement with this conclusion and suggest that AngII in the interstitial fluid is formed outside the intravascular compartment.

In the combined renin/angiotensinogen perfusion experiments, the AngII level we measured in cardiac tissue (expressed per g tissue) was higher than in CE (expressed per mL of fluid). It was in fact as high as in the IST. This is an indication that the locally produced AngII is not restricted to the extracellular fluid and that a large part of this AngII might be located in the cells, since the

intravascular and interstitial fluid compartments comprise about 38 and 23 percent of tissue weight, respectively[7]. Cellular binding and uptake through AT_1-type AngII receptors is known to occur[8-11]. ^{125}I-AngII infused into the left cardiac ventricle of intact pigs is accumulated in cardiac tissue by an AT_1 receptor-dependent process, and the kinetics of AngII accumulation and disappearance are compatible with receptor-mediated endocytosis[12].

AT_1 receptor-mediated binding of AngII to cardiac cells is also indicated by our results obtained during the AngI or II perfusions, which showed that the cardiac tissue concentration of AngII during these perfusions fell to an undetectably low level when the specific AT_1 receptor antagonist losartan was added to the perfusion fluid. Losartan, however, had no significant effect on the cardiac tissue concentration of locally formed AngII during the combined renin/angiotensinogen perfusions. This is in accordance with measurements of tissue AngII in rats, which showed an increase and not a decrease in AngII after 8 days of treatment with losartan, an increase that is likely to be related to the stimulated renin release from the kidney after this drug[19]. The apparent discrepancy with respect to the observed effects of losartan, between the results of the AngI and II perfusions on the one hand and the combined renin/angiotensinogen perfusion on the other hand, raises the possibility that, during the combined renin/angiotensinogen perfusion, local AngII production in the heart is taking place is a compartment that is not reached by losartan. This compartment might be located within the cells.

The levels of locally formed AngII we measured in cardiac tissue in the combined renin/angiotensinogen perfusion experiments might be lower than the true levels at the time of these perfusions, since the tissue levels of AngII in the AngI and II perfusion experiments were lower than expected on the basis of measurements in the CE and IST. The fact that the measured levels of AngII in cardiac tissue in the AngI and II perfusion experiments were lower than calculated on the basis of their presence in the extracellular fluid, may be explained as follows. Most of the extracellular fluid in the Langendorff heart

preparation is located in the intravascular compartment, that is the coronary vascular bed and the right ventricle[7]. The half life of AngI and II is approximately 1 min in the interstitial fluid compartment[7]. In the intravascular compartment it is even shorter, approximately 0.1-0.2 min. It is therefore possible that the short period between the moment the perfusions had been stopped and the tissue had been transferred into liquid nitrogen, was long enough for the endothelial peptidases to cause substantial breakdown of intravascular AngI and II. In the combined renin/angiotensinogen perfusion experiments these losses of extracellular AngII might have been overcome, at least partly, by ongoing formation of this peptide during the time that elapsed until the tissue was frozen. Another reason why the tissue levels of AngII we measured in the combined renin/angiotensinogen perfusion experiments are probably more close to the true levels at the time of perfusion than the tissue levels we measured in the AngI and II perfusion experiments, is related to the fact that the AngII concentration in the extracellular fluid was several orders of magnitude higher during the AngI and II perfusions than during combined renin/angiotensinogen perfusion. Because of these higher levels of extracellular AngII a smaller proportion of it will be bound to the cardiac cell AT_1 receptors and taken up in the cells, where it is protected against the rapid degradation to which extracellular AngII is exposed[11,12,20]. Finally, if it is true that AngII is produced within the cells during combined renin/angiotensinogen perfusion, this intracellular AngII will similarly be protected against this rapid degradation.

The results of the renin/angiotensinogen perfusion experiments indicate that, in contrast with AngII, most of the locally produced AngI in the heart is restricted to the cardiac extracellular fluid compartments. Likely sites of cardiac AngI production are the interstitial fluid and a site closer to the blood compartment, possibly vascular surface-bound renin[7]. The question of where in the tissue the AngII is formed is incompletely answered by the present study. Part of it is formed at the vascular endothelial and endocardial surfaces[16,21-23]. Our observations in the present study on the effects of blockade of AT_1 receptor-

mediated uptake of AngII, which did not show a change in the tissue level of locally produced AngII as opposed to a decrease in the tissue levels of arterially administered AngII, raise the interesting possibility of intracellular AngII formation. There is experimental evidence that neonatal rat cardiac myocytes are capable of binding and internalizing renin and prorenin through a mannose 6-phosphate receptor and that prorenin is activated in the cells[24]. Internalized AngII has a long half life and there is growing evidence that intracellular AngII can serve important functions[25-27]. AT_1 receptor-mediated uptake of AngII and the evidence that the formation and degradation of AngI and II in cardiac tissue are highly compartmentalized has implications for the effects of drugs that interfere with AngII receptor binding (AT_1 receptor antagonists) or reduce the production of AngII (renin inhibitors, ACE inhibitors). These drugs may have different effects on the AngII levels in different cardiac tissue compartments.

4.6 References

1. Campbell DJ, Lawrence AC, Towrie A, Kladis A, Valentijn A: Differential regulation of angiotensin peptide levels in plasma and kidney of the rat. *Hypertension.* 1991; 18: 763-773.
2. Kim S, Tokuyama M, Hosoi M, Yamamoto K: Adrenal and circulating renin-angiotensin system in stroke-prone hypertensive rats. *Hypertension.* 1992; 20: 280-291.
3. Lindpaintner K, Ganten D: The cardiac renin-angiotensin system. An appraisal of present experimental and clinical evidence. *Circ Res.* 1991; 68:905-921.
4. Danser AHJ, van Kats JP, Admiraal PJJ, Derkx FHM, Lamers JMJ, Verdouw PD, Saxena PR, Schalekamp MADH: Cardiac renin and angiotensins. Uptake from plasma versus in situ synthesis. *Hypertension.* 1994; 24: 37-48.
5. Pfeffer MA, Braunwald E, Moye LA, Basta L, Brown Jr EJ, Cuddy TE, Davis BR, Geltman EM, Goldman S, Flaker GC. Klein M, Lamas DA, Packer M, Rouleau J, Rouleau JL, Rutherford J, Wertheimer JH, Hawkins CM, on behalf of the SAVE investigators: Effect of captopril on mortality and morbidity in patients with left ventricular dysfunction after myocardial infarction. *N Engl J Med.* 1992; 327: 669-677.
6. The SOLVD Investigators (1991): Effect of enalapril on mortality and the development of heart failure in asymptomatic patients with reduced left ventricular ejection fractions. *N Engl J Med.* 1991; 327: 685-691.
7. de Lannoy LM, Danser AHJ, van Kats JP, Schoemaker RG, Saxena PR, Schalekamp MADH: Renin-angiotensin system components in the interstitial fluid of the isolated perfused rat heart. Local production of angiotensin I. *Hypertension.* 1997; 29: 1240-1251.

8. Ullian ME, Linas SL: Role of receptor cycling in the regulation of angiotensin II surface receptor number and angiotensin II uptake in rat vascular smooth muscle cells. *J Clin Invest.* 1989; 84: 840-846.
9. Anderson KM, Murahashi T, Dostal DE, Peach MJ: Morphological and biochemical analysis of angiotensin II internalization in cultured rat aortic smooth muscle cells. *Am J Physiol.* 1993; 264: C179-C188.
10. Bianchi C, Gutkowska J, De Léan A, Ballak M, Anand-Srivastava MB, Genest J, Cantin M: Fate of ^{125}I-angiotensin II in adrenal zona glomerulosa cells. *Endocrinology.* 1986; 118: 2605-2607.
11. Crozat A, Penhoat A, Saez JM: Processing of angiotensin II (A-II) and (Sar1,Ala8)A-II by cultured bovine adrenocortical cells. *Endocrinology.* 1986; 118: 2312-2318.
12. van Kats JP, de Lannoy LM, Danser AHJ, Meegen JR, Verdouw PD, Schalekamp MADH: Angiotensin II type 1 (AT_1) receptor-mediated accumulation of angiotensin II in tissues and its intracellular half-life in vivo. *Hypertension.* 1997, 30: 42-49.
13. De Deckere EAM, Ten Hoor P: A modified Langendorff technique for metabolic investigations. *Pflügers Arch.* 1977; 370: 103-105.
14. Danser AHJ, Koning MMG, Admiraal PJJ, Sassen LMA, Derkx FHM, Verdouw PD, Schalekamp MADH: Production of angiotensins I and II at tissue sites in intact pigs. *Am J Physiol.* 1992; 263: H429-H437.
15. 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.
16. Danser AHJ, Koning MMG, Admiraal PJJ, Derkx FHM, Verdouw PD, Schalekamp MADH: 1992. Metabolism of angiotensin I by different tissues in the intact animal. *Am J Physiol.* 1992; 263: H418-H428.
17. Zisman LS, Abraham WT, Meixell GE, Vamvakias BN, Quaife RA, Lowes BD, Roden RL, Peacock SJ, Groves BM, Raynolds, Bristow MR, Perryman MB: Angiotensin II formation in the intact human heart. Predominance of the angiotensin-converting enzyme pathway. *J Clin Invest.* 1995; 95: 1490-1498.
18. Dell'Italia LJ, Meng QC, Balcells E, Wei C-C, Palmer R, Hageman GR, Durand J, Hankes GH, Oparil S: Compartmentalization of angiotensin II generation in the dog heart. *J Clin Invest.* 1997; 100: 253-258.
19. Campbell DJ, Kladis A, Valentijn AJ: Effects of losartan on angiotensin and bradykinin peptides and angiotensin-converting enzyme. *J Cardiovasc Pharmacol.* 1995; 26: 233-240.
20. Urata H, Khosla MC, Bumpus FM, Husain A: Evidence for extracellular, but not intracellular, generation of angiotensin II in the rat adrenal zona glomerulosa. *Proc Natl Acad Sci USA.* 1988; 85: 8251-8255.
21. Falkenhahn M, Franke F, Bohle RM, Zhy YC, Stauss HM, Bachmann S, Danilov S, Unger T: Cellular distribution of angiotensin-converting enzyme after myocardial infarction. *Hypertension.* 1995; 25: 219-226.
22. Yamada H., Fabris B, Allen AM, Jackson B, Johnston CE, Mendelsohn FAO: Localization of angiotensin converting enzyme in rat heart. *Circ Res.* 1991; 68: 141-149.
23. Hokimoto S, Yasue H, Fujimoto K, Yamamoto H, Nakao K, Kaikita K, Sakata R, Miyamoto E: Expression of angiotensin-converting enzyme in remaining viable myocytes of human ventricles after myocardial infarction. *Circulation.* 1996; 94: 1513-1518.

24. van Kesteren CAM, Danser AHJ, Derkx FHM, Dekkers DHW, Lamers JMJ, Saxena PR, Schalekamp MADH: Mannose 6-phosphate receptor-mediated internalization and activation of prorenin by cardiac cells. *Hypertension.* 1997; in press.
25. Haller H, Lindschau C, Erdmann B, Quass P, Luft FC: Effects of intracellular angiotensin II in vascular smooth muscle cells. *Circ Res.* 1996; 79: 765-772.
26. Eggena P, Zhu JH, Clegg K, Barret JD: Nuclear angiotensin receptors induce transcription of renin and angiotensinogen mRNA. *Hypertension.* 1993; 22: 496-501.
27. de Mello WC: Influence of intracellular renin on heart cell communication. *Hypertension.* 1995; 25: 1172-1177.

Chapter 5

Angiotensin II type 1 (AT_1) receptor-mediated accumulation of angiotensin II in tissues and its intracellular half life in vivo

5.1 Abstract

Angiotensin II (AngII) is internalized by various cell types via receptor-mediated endocytosis. Little is known about the kinetics of this process in the whole animal and about the half life of intact Ang II after its internalization. We measured the levels of ^{125}I-Ang II and ^{125}I-Ang I that were reached in various tissues and in blood plasma during infusions of these peptides into the left cardiac ventricle of pigs. Steady-state concentrations of ^{125}I-Ang II in skeletal muscle, heart, kidney and adrenal were 8-41%, 64-150%, 340-550% and 680-2100% of the ^{125}I-Ang II concentration in arterial blood plasma, respectively (ranges of 6 experiments). The tissue concentrations of ^{125}I-Ang I were less than 5% of the arterial plasma concentration. The ^{125}I-Ang II accumulation, seen in heart, kidney and adrenal, was almost completely blocked by a specific AngII type 1 (AT_1) receptor antagonist. Steady-state concentrations of ^{125}I-Ang II were reached within 30-60 min in the tissues, and within 5 min in blood plasma. The in-vivo half life of intact ^{125}I-Ang II in heart, kidney and adrenal was approximately 15 min, as compared with 0.5 min in the circulation. Thus, Ang II, but not AngI, from the circulation, is accumulated by some tissues, and this is mediated by AT_1 receptors. The time course of this process and the long half life of the accumulated Ang II support the contention that this Ang II has been internalized after its binding to the AT_1 receptor, so that it is protected from rapid degradation by endothelial peptidases. The results of this study are in agreement with the growing evidence of an important physiological role for internalized Ang II.

5.2 Introduction

Receptor-mediated endocytosis of the vasoactive peptide angiotensin (Ang) II is an important mechanism by which the *in-vivo* activity of the renin-angiotensin system is regulated. Two pharmacologically distinct classes of cell surface receptors have been identified for Ang II, *i.e.* type 1 and 2 (AT_1 and AT_2)[1]. Most of the classical physiological actions of the renin-angiotensin system appear to be mediated by the AT_1 receptors.

Many cell surface receptors are internalized following binding to their agonists. Endocytosis of the complex of AT_1 receptor with its agonist Ang II has been demonstrated in a number of target cells, *i.e.* vascular smooth muscle cells[2,3], renal tubule cells[4] and cells from the adrenal cortex and medulla[5-8]. This provides a mechanism for regulating the number of receptors on the cell surface. Receptor-mediated endocytosis of Ang II and its subsequent degradation in lysosomes may also serve an important function in the disposal of this peptide[3,7].

Although a plasma membrane localization is thought to be essential for Ang II receptor function, it has been suggested that internalization of the receptor is important for signal transduction. There is evidence that, in cultured vascular smooth muscle cells, the delayed accumulation of the protein kinase C activator, diacylglycerol, in response to Ang II depends on receptor-mediated endocytosis of this peptide. An early step in this process seems to be important for the second and sustained phase of diacylglycerol accumulation[9]. Receptor-mediated endocytosis of Ang II may also be important for the generation of inositol 1,4,5-triphosphate (IP_3) and transport sodium in response to stimulation of the apical AT_1 receptors of proximal renal tubule cells[4]. It has been reported that, in adrenal glomerulosa cells, the inhibition of Ang II-induced internalization reduces the sustained phase of IP_3 generation and abolishes the second phase of the cytoplasmic calcium response[10, 11]. It is known that stimulated steroidogenesis by these cells closely follows the changes in

intracellular calcium[12]. Others found that, in adrenal glomerulosa cells, internalization of the AT_1 receptor is required for protein kinase C activation but not for IP_3 release and steroidogenesis[13].

Evidence also suggests that selective intracellular delivery of internalized Ang II is necessary for an intracellular action. A high-affinity cytoplasmic Ang II-binding protein with many characteristics of a receptor has been described[14, 15]. Ang II is rapidly accumulated in vascular and cardiac muscle cell nuclei[16], and AT_1-like Ang II binding sites have been identified in liver cell nuclei[17, 18]. It has been reported that Ang II binds to chromatin and may influence transcriptional processes[19-21]. Ang II induces the expression of proto-oncogenes and has growth-promoting effects in various cells[22]. An intracellular action would require a sufficiently long half life of internalized Ang II or a biologically active Ang II metabolite.

Previous studies of the cellular uptake and intracellular half life of Ang II have been carried out *in vitro*. The *in-vitro* studies made use of ^{125}I-labeled Ang II, but did not discriminate between *intact* ^{125}I-Ang II and ^{125}I-labeled peptide fragments. In the course of studies in our laboratory, aimed at quantifying Ang II production in different regional vascular beds, ^{125}I-labeled Ang I or II was infused into pigs[23, 24]. Here we report on the *in-vivo* accumulation of *intact* ^{125}I-Ang II in cardiac, renal, and adrenal tissues during these infusions, and on the effect of a specific AT_1 receptor antagonist on the Ang II uptake by these tissues. We compared the data on the tissue accumulation and the half life of ^{125}I-Ang II with data obtained for ^{125}I-Ang I. Unlike Ang II, Ang I is not biologically active and is probably not subjected to receptor-mediated endocytosis.

5.3 Materials and methods

5.3.1 Chemicals

[Ile^5]-Ang-(1-10) decapeptide (Ang I), [Ile^5]-Ang-(1-8) octapeptide (Ang II), and [Ile^5]-Ang-(2-8) heptapeptide (Ang III) were obtained from Bachem, Bubendorf, Switzerland. [Ile^5]-Ang-(2-10) nonapeptide was from Senn Chemicals, Dielsdorf, Switzerland. [Ile^5]-Ang-(3-8) hexapeptide (Ang-(3-8)), [Ile^5]-Ang-(4-8) pentapeptide (Ang-(4-8)), and [Ile^5]-Ang-(1-7) heptapeptide (Ang-(1-7)) were from Peninsula Laboratories, Belmont, CA, USA. Mono-iodonated ^{125}I-Ang I was prepared with the chloramine-T method and purified as described previously[25]. Mono-iodinated ^{125}I-labeled preparations of Ang II, Ang III, Ang-(3-8), Ang-(4-8), Ang-(2-10), Ang-(1-7), and tyrosine were also made[25]. The specific radioactivity of the ^{125}I-Ang I and ^{125}I-Ang II preparations was approximately 3.6×10^6 cpm/pmol.

5.3.2 Antisera

Ang I and Ang II antisera prepared in New Zealand White rabbits, were used to identify the peptides in high-performance chromatography (HPLC) radioactivity peaks. Ang I antiserum crossreacted with Ang-(2-10) (100%) but not (<0.1%) with Ang II, Ang III, Ang-(3-8), Ang-(4-8) or Ang-(1-7). Ang II antiserum crossreacted with Ang III (55%), Ang-(3-8) (73%) and Ang-(4-8) (100%) but not (<0.2%) with Ang I, Ang-(2-10) or Ang-(1-7). These crossreactivity patterns show that the antibodies in both antisera were directed against the C-terminal sequences of Ang I and Ang II[24].

5.3.3 Animals

All experiments were performed under the regulations of the Animal Care Committee of the Erasmus University, Rotterdam, The Netherlands, in accordance with the 'Guiding principles in the care and use of animals' as approved by the American Physiological Society. Twenty-three female pigs (crossbred Yorkshire x Landrace, Hedelse Varkens Combinatie, Hedel, The Netherlands) with a body weight of 25-30 kg were included in the study. Some animals were also used for studies on the cardiac uptake of kidney-derived renin. For these studies, which extend our earlier observations of this subject[26] and which will be reported in a separate article, the renin release from the kidney was stimulated with the diuretic furosemide, 40 mg twice daily for 2 days before the experiments reported here. Other animals were pretreated with the ACE inhibitor captopril, 25 mg twice daily for 2 days, in order to investigate the effect of ACE inhibition on the tissue levels of ^{125}I-Ang I and ^{125}I-Ang II.

5.3.4 Instrumentation of the animals

Animals were sedated with an intramuscular injection of 20 mg/kg ketamine (AUV, Cuijk, The Netherlands), and anesthetized with 20 mg/kg sodium pentobarbital (Apharma, Arnhem, The Netherlands) administered via a dorsal ear vein. They were intubated and connected to a ventilator for intermittent positive pressure ventilation with a mixture of oxygen and nitrogen (30%/70%). Respiratory rate and tidal volume were adjusted to keep arterial blood gases within the physiological range. For maintainenance of adequate anesthesia, a 7 French (Fr) catheter was placed in the superior caval vein for administration of 8.5-10 mg/kg per hour sodium pentobarbital. Another 7Fr catheter was placed in the superior caval vein for infusion of saline to correct for fluid losses and administer the AT_1 receptor antagonist L-158,809[27, 28] (a gift of Dr. R. D. Smith,

Du Pont Merck Pharmaceutical Company, Wilmington, DE, USA). A 7Fr catheter was inserted, via the left carotid artery, into the left ventricle under X-ray control to infuse ^{125}I-Ang I or ^{125}I-Ang II (see below). An 8Fr catheter was inserted into the descending aorta, via a femoral artery, to measure central aortic pressure and to collect arterial blood.

After administration of 4 mg pancuronium bromide (Organon Teknika, Boxtel, The Netherlands) a midline thoracotomy was performed, and the heart was suspended in a pericardial cradle. An electromagnetic flow probe (Skalar, Delft, The Netherlands) was placed around the ascending aorta for measurement of ascending aortic blood flow (cardiac output). After a stabilization period of 30-45 min following completion of instrumentation, baseline measurements of systemic hemodynamic variables were made, and blood samples were collected for the determination of blood gases. The animals were then subjected to constant infusions of either ^{125}I-Ang I or ^{125}I-Ang II.

5.3.5 Infusions of ^{125}I-angiotensin I or ^{125}I-angiotensin II

^{125}I-Ang I was infused into the left cardiac ventricle at a constant rate of approximately 5 x 10^6 cpm/min. Steady-state plasma levels of ^{125}I-Ang I and ^{125}I-Ang II were reached within 10 min[23]. For determination of the time required for tissue levels to reach a steady state, heart, kidney and adrenals were removed after various time periods of ^{125}I-Ang I infusion, *i.e.* after 15, 60 or 120 min of ^{125}I-Ang I infusion. Blood samples were taken from the aorta at 10 min and 60 min of ^{125}I-Ang I infusion.

In some experiments, in which ^{125}I-Ang I had been infused for 15 min, the heart, kidney and adrenals were removed at 15 or 30 min after the infusion had been stopped, in order to obtain an estimate of the *in-vivo* tissue half-life of ^{125}I-Ang I and II. The *in-vivo* half-life of ^{125}I-Ang I and ^{125}I-Ang II in the circulation was determined by measuring the plasma levels of ^{125}I-Ang I and ^{125}I-Ang II in

blood samples taken from the aorta at 0.25, 0.5, 1, 1.5 and 2 min after the ^{125}I-Ang I infusion had been stopped.

The effect of blockade of the AT_1-receptor on the tissue levels of ^{125}I-Ang I and ^{125}I-Ang II during ^{125}I-Ang I infusion, was studied by administration of the AT_1-receptor antagonist L-158,809, 1 mg/min i.v. for 10 min , 30 min before the start of the ^{125}I-Ang I infusion. At this dose of the AT_1-receptor antagonist, the pressor effect of systemically administered Ang II (0.1-1.0 μg/kg) is completely blocked[29]. After 10 min of ^{125}I-Ang I infusion an arterial blood sample was taken, and heart, kidney and adrenals were removed at 15 min of infusion.

^{125}I-Ang II was infused into the left cardiac ventricle at a constant rate of approximately 3 x 10^6 cpm/min. Heart, kidney and adrenal were removed at 15, 60 or 120 min of ^{125}I-Ang II infusion. Blood samples were taken from the aorta at 10 min and 60 min of ^{125}I-Ang II infusion. A steady-state plasma level of ^{125}I-Ang II had been reached by that time[30].

5.3.6 Blood and tissue sampling

During ^{125}I-Ang I and ^{125}I-Ang II infusions, blood samples (10 mL) were taken from the aorta for measurement of the plasma levels of ^{125}I-Ang I and ^{125}I-Ang II. The blood was rapidly withdrawn with a plastic syringe containing the following inhibitors (0.5 mL inhibitor solution in 10 mL blood), 0.01 mmol/L remikiren (a gift of Dr. W. Fischli, Hoffmann-LaRoche, Basel, Switzerland), 6.25 mmol/L disodium EDTA and 1.25 mmol/L 1,10 ortho-phenanthroline (Merck, Darmstadt, Germany) (final concentrations in blood). It was then immediately transferred into prechilled polystyrene tubes and centrifuged at 3,000 g for 10 min at 4°C. Plasma was stored at -70°C and assayed within 3 days.

The heart was removed either immediately or at various times after the

infusion of ^{125}I-Ang I or ^{125}I-Ang II infusion had been stopped. Before its removal from the body, the heart was stopped by fibrillation, while the radiolabeled peptide infusion was still running. Immediately after the heart had been removed from the body, a piece of left ventricular free wall tissue (1-2 g) was excised and transferred into liquid nitrogen. The tissue was frozen within 15 seconds after the heart had been stopped. Subsequent to removal of the heart, the left kidney and both adrenal glands, and in some cases also part of the sternocleidomastoid muscle, were excised and a piece of each tissue (0.5-1 g) was immediately transferred into liquid nitrogen. The piece of renal tissue was mainly renal cortex. These tissues were frozen within 60 seconds after the heart had been stopped. The frozen tissues were stored at -70°C and assayed within 3 days.

For study of the *ex-vivo* degradation of ^{125}I-labeled angiotensins in tissue, remaining parts of the left ventricular wall tissue, the kidney and the adrenals were kept at 37°C. Pieces of tissue were then cut off and rapidly frozen in liquid nitrogen at various time points (0-60 min) after the heart had been stopped. The frozen tissues were stored at -70°C and assayed within 3 days.

5.3.7 Measurements of ^{125}I-angiotensin I and ^{125}I-angiotensin II in tissue and plasma

Frozen tissue samples were homogenized with a Polytron PT10/35 (Kinematica,Luzern, Switzerland) in 20 mL ice-cold ethanol / 0.1 mol/L HCl 4:1 (vol/vol)[26]. Homogenates were centrifuged at 20,000*g* for 25 min at 4°C. Ethanol in the supernatant was evaporated under constant air flow. The remainder of the supernatant was diluted in 20 mL 1% ortho-phosphoric acid and centrifuged again at 20,000*g*. The supernatant was diluted with an equal volume of 1% ortho-phosphoric acid and then concentrated by reversible adsorption to octadecylsilyl silica (Sep-Pak C18, Waters, Milford, MA, USA). Plasma was

directly applied to Sep-Pak cartridges.

The Sep-Pak cartridges were conditioned with 5 mL methanol and equilibrated with 5 mL cold water. Samples were passed through the cartridges at 4°C, followed by a wash with 10 mL cold water. Adsorbed angiotensins were eluted with 2.5 mL of 90% methanol / 10% water (vol/vol) into polypropylene tubes, and the eluted samples were dried under vacuum.

Separations were performed by reversed-phase HPLC using a Nucleosil C18 steel column of 250x4.6 mm and 10 μm particle size (Alltech, Eke, Belgium) as previously described[25]. Water for HPLC was prepared with a Milli-Q system (Waters, Milford, MA, USA). Mobile phase A was 25% methanol in 0.085% ortho-phosphoric acid, containing 0.02% sodium azide. Mobile phase B was 75% methanol in 0.085% ortho-phosphoric acid, containing 0.02% sodium azide. The flow was 1.5 mL/min, and the working temperature was 45°C. The vacuum-dried Sep-Pak extracts were dissolved in 100 μL 0.085% ortho-phosphoric acid and injected. Elution was performed as follows: 85% A / 15% B (vol/vol) from 0 to 5 min, followed by a linear gradient to 40% A / 60% B (vol/vol) until 20 min. The eluate was collected in 20-second fractions into polystyrene tubes coated with bovine serum albumin (Sigma, St. Louis, MI, USA). The concentrations of ^{125}I-labeled angiotensins and their metabolites in the HPLC fractions were measured in a gamma counter.

In previous studies, in which the levels of Ang I and II were measured in cardiac tissue , we added a known amount of ^{125}I-Ang I as an internal standard before the extraction procedure. We then used the recovery of ^{125}I-Ang I after HPLC separation to correct for losses (maximally 20-30%) occurring during extraction and separation[26]. In the present study, we did not add ^{125}I-Ang I as an internal standard because the tissue already contained ^{125}I-labeled Ang I and metabolites. We, therefore, did not correct for losses of angiotensin during the extraction and separation procedures. This may have led to an underestimation (by maximally 20-30%) of the actual tissue levels.

5.4 Results

5.4.1 Hemodynamic effects of ^{125}I-angiotensin I and ^{125}I-angiotensin II infusions

Baseline heart rate, cardiac output and mean arterial pressure were similar in furosemide- and captopril-pretreated pigs and in pigs treated with the AT_1 receptor blocker L-158,809 (Table 1). ^{125}I-Ang I and ^{125}I-Ang II infusions did not affect any of these parameters (data not shown), which is in agreement with previous studies[23, 30].

5.4.2 Identification of ^{125}I-angiotensin I, ^{125}I-angiotensin II and their ^{125}I-labeled metabolites in blood plasma and in tissue by HPLC

Satisfactory separations were obtained of ^{125}I-Ang I and ^{125}I-Ang II and of these peptides and most of their ^{125}I-labeled metabolites (Figure 1). A comparison between the retention times of the various ^{125}I-labeled peptide standards demonstrated that the ^{125}I-Ang I and II peaks were virtually free of ^{125}I-Ang III, ^{125}I-Ang-(3-8), ^{125}I-Ang-(4-8), ^{125}I-Ang-(2-10), ^{125}I-Ang-(1-7) and ^{125}I-tyrosine.

In plasma, more than 90% of the radioactivity in the peak with the same retention time as ^{125}I-Ang I was bound by Ang I antiserum, as compared with less than 5% by Ang II antiserum. This peak was therefore identified as ^{125}I-Ang I. No peak with this retention time was observed in skeletal muscle, and a very small peak with this retention time was sometimes observed in heart and kidney. Adrenal tissue showed a larger peak with a retention time similar to that of ^{125}I-Ang I, but less than 10% of the radioactivity in this peak was bound to Ang I antiserum. This peak therefore was not ^{125}I-Ang I.

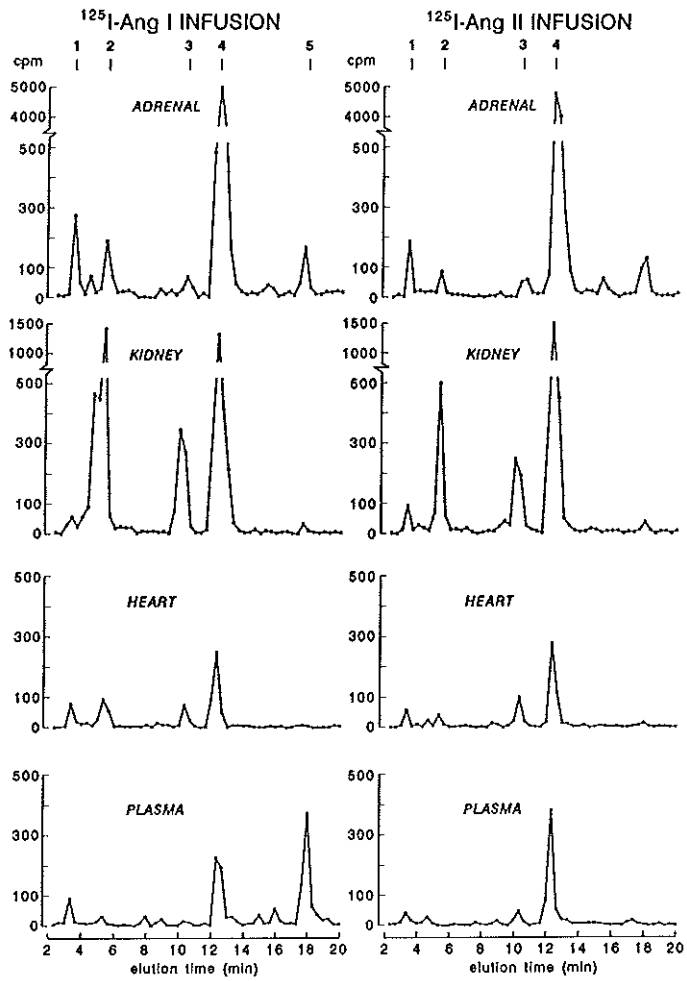

Figure 1. High-performance liquid chromatography (HPLC) elution profile of ^{125}I-labeled angiotensins in adrenal, kidney and heart tissue and blood plasma, measured by gamma counting, during infusion of ^{125}I-angiotensin I (left panel) or ^{125}I-angiotensin II (right panel). Retention times of ^{125}I-labeled standards are indicated at the top. 1, ^{125}I-tyrosine; 2, ^{125}I-Ang-(1-7); 3, ^{125}I-Ang-(4-8); 4, ^{125}I-Ang II; 5, ^{125}I-Ang I.

Table 1. Baseline hemodynamic parameters and steady-state arterial plasma levels of ^{125}I-angiotensin I and ^{125}I-angiotensin II during infusion of ^{125}I-angiotensin I or ^{125}I-angiotensin II in furosemide- or captopril-treated pigs.

treatment	Infusion	(n)	Mean Arterial Pressure (mm Hg)	Heart Rate (beats/min)	Cardiac Output ($L.min^{-1}$)	^{125}I-Ang I conc. (cpm/mL)	^{125}I-Ang II conc. (cpm/mL)	^{125}I-AngII/I conc. ratio
furosemide	^{125}I-AngI	11	81±4	105±18	2.4±0.5	1470±350	1000±350	0.67±0.14
furosemide & AT_1 receptor blockade	^{125}I-AngI	2	82-83	107-135	3.0-5.2	1150-1220	660-970	0.57-0.80
captopril	^{125}I-AngI	6	85±4	114±20	2.8±0.4	2440±410*	370±350*	0.15±0.08*
furosemide	^{125}I-AngII	4	78±13	110±15	2.3±0.7	-	1960±530	-

Data are means ± SD or individual values (AT_1 receptor blockade). Ang, angiotensin; cpm, counts per min.
* $p < 0.05$ vs furosemide-treatment (unpaired t-test).

In both plasma and in the tissues, more than 90% of the radioactivity in the peak with the same retention time as ^{125}I-Ang II was bound by Ang II antiserum, as compared with less than 3% by Ang I antiserum. This peak was therefore identified as ^{125}I-Ang II.

In addition to the ^{125}I-Ang I and ^{125}I-Ang II peaks, separate peaks with retention times corresponding to ^{125}I-tyrosine, ^{125}I-Ang-(1-7) and ^{125}I-Ang-(4-8) were observed in the tissues. More than 80% of the radioactivity in the peak with the retention time of ^{125}I-Ang-(4-8) was bound by Ang II antiserum, whereas less than 10% was bound by Ang I antiserum. We therefore concluded that this peak was indeed ^{125}I-Ang-(4-8). The radioactivity in the peaks with the retention times of ^{125}I-tyrosine and ^{125}I-Ang-(1-7) was not bound by these antisera. The conclusion that these peaks were indeed ^{125}I-tyrosine and ^{125}I-Ang-(1-7) needs further confirmation.

5.4.3 Accumulation of ^{125}I-angiotensin II in tissue and the effect of AT_1 receptor blockade

Figure 2 shows the tissue levels of ^{125}I-Ang II after 60 min infusions of ^{125}I-Ang I or ^{125}I-Ang II. The ^{125}I-Ang II levels in the kidney and the adrenal (expressed per g tissue) were 340-550% and 680-2100% of the ^{125}I-Ang II level in plasma (expressed per mL plasma), respectively (ranges of 6 experiments). In the heart, the ^{125}I-Ang II level was 64-150% of the level in plasma. The ^{125}I-Ang II level in skeletal muscle was 8-41% of the level in plasma. Results obtained in the furosemide-pretreated pigs were similar to those in the captopril-pretreated pigs, and results obtained after ^{125}I-Ang I infusion were similar to those obtained after ^{125}I-Ang II infusion. In the kidney and the adrenal, and also in the heart, the tissue levels of ^{125}I-Ang II were too high to be explained by the presence of ^{125}I-Ang II in the extracellular fluid. The tissue levels of ^{125}I-Ang I were less than 5% of the plasma level.

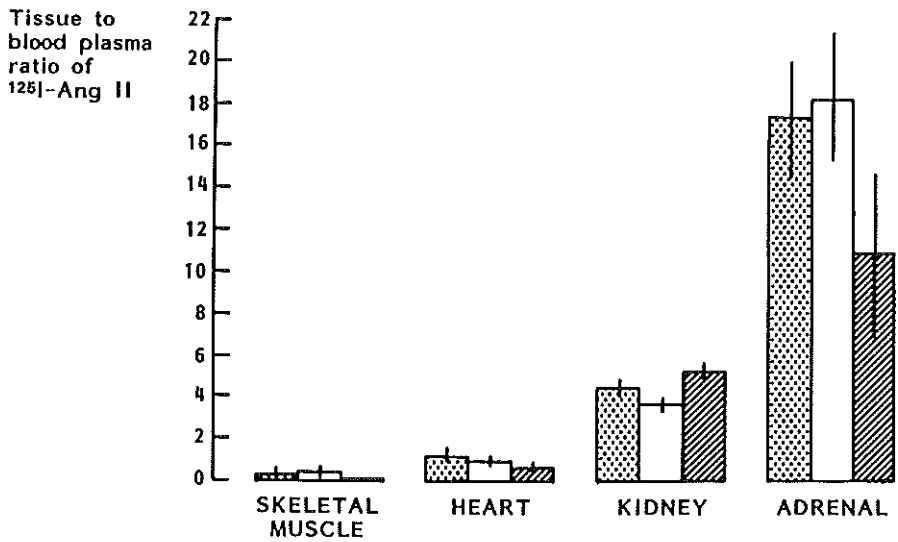

Figure 2. Tissue-to-blood plasma ^{125}I-angiotensin II concentration ratios in skeletal muscle, heart, kidney and adrenal, at 60-min infusion of infusion of ^{125}I-angiotensin I in furosemide- (stippled bars) or captopril-pretreated (open bars) pigs or during infusion of ^{125}I-angiotensin II in furosemide-pretreated pigs (hatched bars). Values are means and range (n=2).

These results demonstrate that Ang II from the circulation is accumulated by the heart, kidney and adrenal and that, in the case of the kidney and the adrenal, the tissue concentration of the accumulated Ang II is several times the plasma concentration. Ang I from the circulation is not accumulated by these tissues.

In Figure 3, the ^{125}I-Ang II levels that were reached in heart, kidney and adrenal after 60-min infusions of ^{125}I-Ang I or ^{125}I-Ang II are compared with the levels after 15- or 120-min infusions. It took between 30 and 60 min for ^{125}I-Ang II to reach steady-state levels in the tissues. This is much longer than in plasma, where it takes fewer than 10 min to reach a steady state[23,25,30].

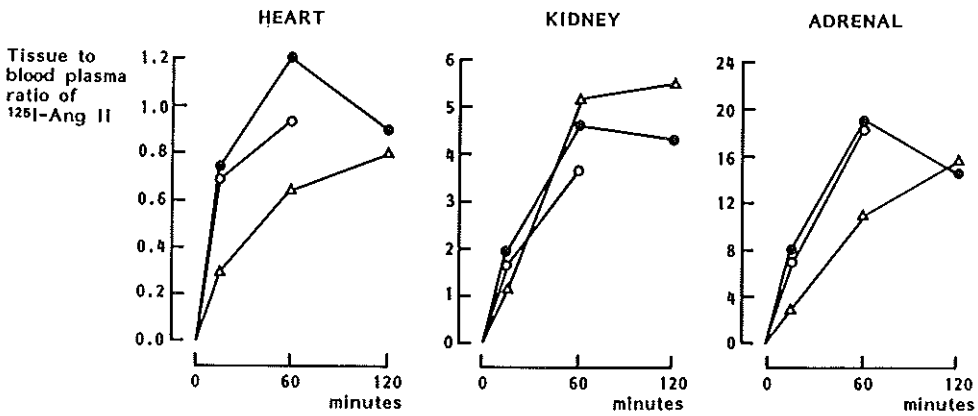

Figure 3. Tissue-to-blood plasma ^{125}I-angiotensin II concentration ratios in heart, kidney and adrenal, during infusion of ^{125}I-angiotensin I in furosemide- (closed circles) or captopril-pretreated (open circles) pigs or during infusion of ^{125}I-angiotensin II in furosemide-treated pigs (triangles). Values are means of 2 experiments (at 15 and 60 min of infusion) or single observations (120 min of infusion).

The effect of the AT_1 receptor antagonist L-158,809 on the accumulation of ^{125}I-Ang II in heart, kidney and adrenal was studied during a 15-min infusion of ^{125}I-Ang I. Skeletal muscle was not studied because of the apparent lack of ^{125}I-Ang II accumulation in this tissue (see above). As shown in Figure 4, L-158,809 caused nearly complete blockade of ^{125}I-Ang II accumulation. L-158,809 had no effect on the plasma levels of ^{125}I-Ang I and ^{125}I-Ang II (Table 1). Thus, the accumulation of Ang II from the circulation by heart, kidney and adrenal appears to depend on AT_1 receptors.

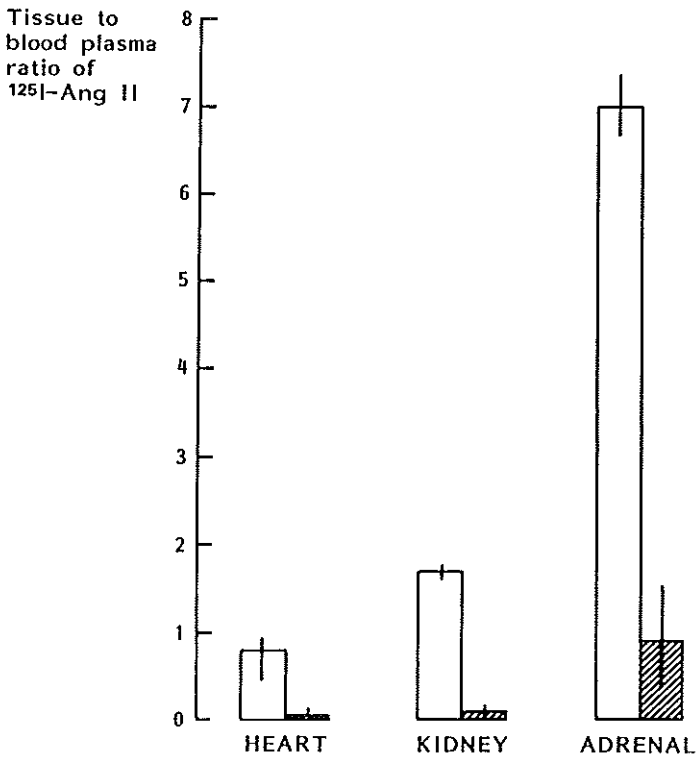

Figure 4. Tissue-to-blood plasma ^{125}I-angiotensin II concentration ratios in heart, kidney and adrenal, at 15 min of infusion of ^{125}I-angiotensin I in furosemide-pretreated pigs either with (hatched bars) or without (open bars) AT_1 receptor blockade by L-158,809. Values are means and range (n=2).

5.4.4 *Ex-vivo* and *in-vivo* half life of ^{125}I-angiotensin II in tissue

The process of tissue removal, cutting and transfer into liquid nitrogen took less than 1 min. Still, ^{125}I-Ang II metabolism might be so rapid that the measured levels of this peptide are substantially below the levels *in vivo*. We, therefore, investigated the decrease in the tissue levels of ^{125}I-Ang II in heart, kidney and

adrenal, while the pieces of tissue cut from these organs were kept at 37°C before they were transferred into liquid nitrogen. Skeletal muscle was not included in these experiments, because of the apparent lack of ^{125}I-Ang II accumulation in this tissue (see above).

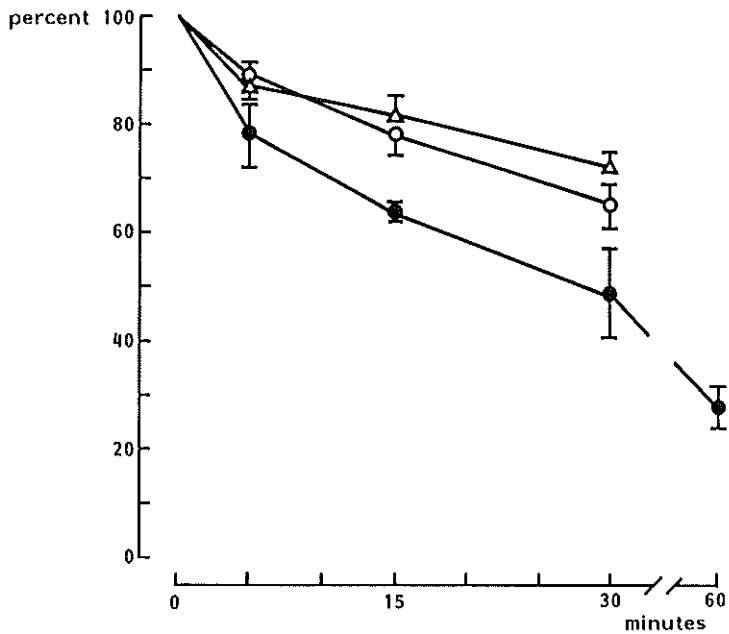

Figure 5. The *ex-vivo* decrease of ^{125}I-angiotensin II in heart (closed circles; n=6), kidney (open circles; n=4) and adrenal (triangles; n=4) tissue during storage at 37°C. Values (means ± SD) are expressed as a percentage of the levels measured immediately after removal of the tissues from the body.

As shown in Figure 5, the *ex-vivo* half life of ^{125}I-Ang II in heart, kidney and adrenal was 30 min or longer. Thus, one can conclude that the ^{125}I-Ang II levels that were measured in these tissues after they had been transferred into liquid nitrogen as quickly as possible, were indeed representative of the levels *in vivo*.

In order to get some information on the *in-vivo* half life of ^{125}I-Ang II in tissue, we measured ^{125}I-Ang II levels in heart, kidney and adrenal after these organs had been kept in the body for 15 or 30 min after the ^{125}I-Ang I infusion had been stopped. As shown in Figure 6, the tissue levels of ^{125}I-Ang II, 15 and 30 min after discontinuation of the ^{125}I-Ang I infusion were 40-70% and 20-50% of the level immediately after discontinuation of the infusion (n=2). This corresponds with an *in-vivo* half life in tissue of approximately 15 min. The half life of ^{125}I-Ang II in the circulation was approximately 0.5 min, which is in agreement with earlier studies[23]. Thus, it appears that Ang II that is taken up from the circulation by the heart, the kidney and the adrenal and is accumulated in these organs, has a much longer half life in tissue than in circulating plasma.

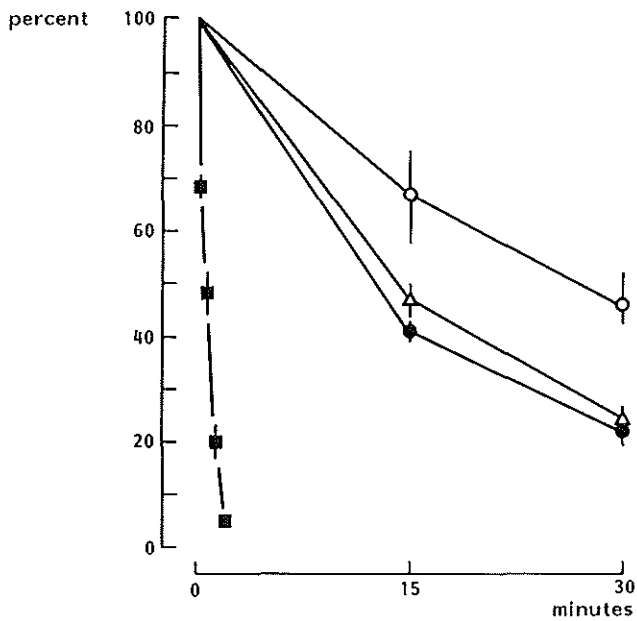

Figure 6. Levels of ^{125}I-angiotensin II in arterial plasma (squares) and heart (closed circles), kidney (open circles) and adrenal (triangles) during the elimination phase after discontinuation of a 15 min ^{125}I-angiotensin I infusion. Plasma was collected at 15, 30, 60 90 and 120 seconds after discontinuation of the infusion. Values (means and range; n=2) are expressed as a percentage of the steady-state ^{125}I-angiotensin II levels in arterial plasma.

5.5 Discussion

In the present experiments, we infused ^{125}I-Ang I and ^{125}I-Ang II at a constant rate into the left cardiac ventricle of pigs, to investigate the uptake and degradation of Ang I and II in the tissues. The use of radiolabeled angiotensins is based on the assumption that the body does not distinguish between labeled and unlabeled peptides. In a previous study, also in pigs, the arterial and venous plasma levels of ^{125}I-Ang I and ^{125}I-Ang II and unlabeled Ang I and II were measured in a number of regional vascular beds in animals receiving infusions of ^{125}I-Ang I combined with unlabeled Ang I, for comparison of the regional extraction rates of the arterially delivered labeled and unlabeled angiotensins[23, 24]. Ang I in that study was infused in quantities that were sufficiently high to ignore the levels of endogenous Ang I and II. The results showed little difference in regional extraction between labeled and unlabeled angiotensins. There was a difference in conversion rate, the ^{125}I-Ang I-to-II conversion rate being two times the Ang I-to-II conversion rate, but the degradation of ^{125}I-Ang I into peptides other than ^{125}I-Ang II occurred at the same rate as the degradation of Ang I. The ^{125}I-Ang II degradation rate was also not different from the Ang II degradation rate[30].

Another important methodological aspect of our study is the possibility that the measured tissue levels of ^{125}I-Ang I and II differed from the levels *in vivo*, because of rapid degradation of these peptides after the ^{125}I-Ang I or ^{125}I-Ang II infusions had been stopped. We addressed this issue by investigating the *ex-vivo* degradation of ^{125}I-Ang I and ^{125}I-Ang II when the tissues were kept at 37°C. On the basis of the results of these experiments, it can be concluded that the measured tissue levels of ^{125}I-Ang II are probably representative of the levels present *in vivo*. The measured tissue level of ^{125}I-Ang I was too low to permit any conclusion about its level *in vivo*.

The main new findings of the present study were: 1) the time-dependent

accumulation of arterially delivered *intact* Ang II in heart, kidney and adrenal gland of the *intact* animal and the importance of AT_1 receptors for this process, and 2) the long *in-vivo* half life of blood-derived *intact* Ang II in these tissues, as opposed to the short half life of Ang II in the circulation.

The tissue levels of ^{125}I-Ang II we measured were about half maximal after 15-30 min of ^{125}I-Ang I or II infusion, and the maximum appeared to be reached within one hour of infusion. This time course of Ang II accumulation in the tissues is similar to that observed for the AT_1 receptor-dependent accumulation of radioactivity in isolated bovine adrenocortical and chromaffin cells, when incubated with ^{125}I-Ang II[7, 8]. In these studies of isolated cells, surface-bound radioactivity was rapidly internalized. Within 15 min of incubation at 37°C more than 50 % of the total radioactivity of the adrenal cells was derived from internalized ^{125}I-Ang II. In addition, the increase in the total specific (AT_1 receptor-related) radioactivity of the cells during the first 30 min of incubation with ^{125}I-Ang II represented the increase of the internalized fraction, the surface-bound fraction remaining more or less constant. Such rapid internalization was also observed in monolayer cultures of rat vascular smooth muscle cells[2, 3] and in COS-7 cells expressing the rat AT_1 receptor[31]. Also in our *in-vivo* experiments, the progressive accumulation of ^{125}I-Ang II in heart, kidney and adrenal appeared to be mediated by AT_1 receptors, since it was inhibited by the AT_1 receptor antagonist L-158,809. Because of this and because of the similarities between the kinetics of the ^{125}I-Ang II accumulation process in the *intact* animal and the kinetics of ^{125}I-Ang II internalization by cells in culture, we conclude that the increase in the tissue levels of ^{125}I-Ang II in heart, kidney and adrenal that we observed during ^{125}I-Ang I and ^{125}I-Ang II infusion is mainly determined by AT_1 receptor-mediated internalization and, therefore, reflects the increase in intracellular ^{125}I-Ang II.

The steady-state tissue level of ^{125}I-Ang II in the heart (expressed per g tissue) was similar to that in plasma (expressed per mL plasma). In kidney and adrenal, the tissue levels of ^{125}I-Ang II were several times those in plasma. In

contrast, the tissue levels of ^{125}I-Ang I were less than 5 % of levels in plasma. These results illustrate that arterially delivered Ang I is not accumulated and is probably not subjected to receptor-mediated internalization. The order of steady-state tissue levels of ^{125}I-Ang II, i.e. adrenal > kidney > heart, is in agreement with the Ang II receptor densities in these organs[32-36]. Since most of the ^{125}I-Ang II in tissue seems to be localized in the cells, after its internalization through AT_1 receptors, it is indeed logical to assume that the tissue-to-plasma ^{125}I-Ang II concentration ratio is proportional to the AT_1 receptor density. This ratio is not influenced by the level of endogenous Ang II to which the receptors are exposed, as long as the receptor occupancy is low. This may explain why the tissue-to-plasma ^{125}I-Ang II concentration ratios after captopril-treatment were similar to those after furosemide.

Recently Zou et al.[37] reported that [Val^5]-Ang II, during low-dose infusion of this peptide for 14 days in uninephrectomized rats, was accumulated by the kidney (the endogenous Ang II of the rat is [Ile^5]-Ang II). These authors suggested that renal accumulation of circulating Ang II might be caused by AT_1 receptor-mediated endocytosis. In a subsequent study from the same laboratory[38], also in uninephrectomized rats, it was found that chronic low-dose infusion of [Ile^5]-Ang II increased the level of this peptide in the kidney, and that this increase was prevented by the AT_1 receptor antagonist losartan. From this the authors concluded that chronic Ang II infusion leads to either receptor-mediated internalization of Ang II, enhancement of intrarenal Ang II formation, or both. Our results indicate that the findings of Zou et al.[37, 38] are indeed explained, at least in part, by AT_1 receptor-mediated endocytosis.

There was little difference in the tissue levels of ^{125}I-Ang II between the animals subjected to ^{125}I-Ang I infusions after furosemide treatment and the animals subjected to ^{125}I-Ang I infusions after captopril treatment. There was also little difference between the results obtained after ^{125}I-Ang I infusion and those after ^{125}I-Ang II infusion. This indicates that most of the ^{125}I-Ang II in the tissues was derived from arterially delivered ^{125}I-Ang II and not, via conversion,

from arterially delivered ^{125}I-Ang I.

An important difference between our experiments and the studies reported so far, lies in the fact that we relied on measurements of the tissue levels of *intact* ^{125}I-Ang II rather than *total* tissue radioactivity. This enabled us to provide an estimate of the *in-vivo* intracellular half life of *intact* ^{125}I-Ang II, by measuring its tissue levels at different time intervals after the discontinuation of ^{125}I-Ang I infusion. The intracellular half life of ^{125}I-Ang II (approximately 15 min) was much longer than its half life in the circulation (0.5 min). Endocytosis of Ang II may protect the peptide from rapid degradation by endothelial peptidases.

Activation of cell membrane-bound receptors is crucial for the physiological actions of Ang II. AT_1 receptor antagonist drugs block this activation process, and it is generally believed that this is the mechanism that underlies the beneficial effects of these drugs in hypertension and heart failure. However, these drugs also interfere with the receptor-mediated endocytosis of Ang II, and, as our study demonstrates, the AT_1 receptor antagonist L-158,809 reduces the tissue concentrations of blood-derived Ang II to very low levels. There is growing evidence that AT_1 receptor-mediated endocytosis of Ang II is important for some physiological responses to Ang II[4, 9-11]. Our observations, which indicate that internalized Ang II has a much longer half life than Ang II in the circulation, are in agreement with the concept that intracellular Ang II has indeed functional significance. This raises the possibility that reduced endocytosis of Ang II may contribute to the therapeutic effects of AT_1 receptor antagonists.

5.6 Acknowledgements

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5.7 References

1. de Gasparo M, Husain A, Alexander W, Catt KJ, Chiu AT, Drew M, Goodfriend T, Harding JW, Inagami T, Timmermans PBMWM: Proposed update of angiotensin receptor nomenclature. *Hypertension.* 1995; 25: 924-927.
2. Ullian ME, Linas SL: Role of receptor cycling in the regulation of angiotensin II surface receptor number and angiotensin II uptake in rat vascular smooth muscle cells. *J Clin Invest.* 1989; 84: 840-846.
3. Anderson KM, Murahashi T, Dostal DE, Peach MJ: Morphological and biochemical analysis of angiotensin II internalization in cultured rat aortic smooth muscle cells. *Am J Physiol.* 1993; 264: C179-C188.
4. Schelling JR, Hanson AS, Marzec R, Linas SL: Cytoskeleton-dependent endocytosis is required for apical type 1 angiotensin II receptor-mediated phospholipase C activation in cultured rat proximal tubule cells. *J Clin Invest.* 1992; 90: 2471-2480.
5. Bianchi C, Gutkowska J, De Léan A, Ballak M, Anand-Srivastava MB, Genest J, Cantin M: Fate of ^{125}I-angiotensin II in adrenal zona glomerulosa cells. *Endocrinology.* 1986; 118: 2605-2607.
6. Bianchi C, Gutkowska J, Charbonneau C, Ballak M, Anand-Srivastava MB, De Léan A, Genest J, Cantin M: Internalization and lysosomal association of ^{125}I-angiotensin II in norepinephrine-containing cells of the rat adrenal medulla. *Endocrinology.* 1986; 119: 1873-1875.
7. Crozat A, Penhoat A, Saez JM: Processing of angiotensin II (A-II) and (Sar1,Ala8)A-II by cultured bovine adrenocortical cells. *Endocrinology.* 1986; 118: 2312-2318.
8. Wang JM, Llona I, De Potter WP: Receptor-mediated internalization of angiotensin II in bovine adrenal medullary chromaffin cells in primary culture. *Regulatory Peptides.* 1994; 53: 77-86.
9. Griendling KK, Delafontaine R, Rittenhouse SE, Gimbrone MA, Alexander WA: Correlation of receptor sequestration with sustained diacylglycerol accumulation in angiotensin-II stimulated cultured vascular smooth muscle cells. *J Biol Chem.* 1987; 262: 14555-14562.
10. Hunyady L, Merelli F, Baukal AJ, Balla T, Catt KJ: Agonist-induced endocytosis and signal transduction in adrenal glomerulosa cells. A potential mechanism for receptor-operated calcium entry. *J Biol Chem.* 1991; 266: 2783-2788.
11. Vinson GP, Ho MM, Puddefoot JR, Teja R, Barker S, Kapas S. Hinson JP: Internalization of the type I angiotensin II receptor (AT1) and angiotensin II function in the rat adrenal zona glomerulosa cell. *Endocrine Research.* 1995; 21: 211-217.
12. Pratt JH, Rothrock JK, Dominguez JH: Evidence that angiotensin-II and potassium collaborate to increase cytosolic calcium and stimulate the secretion of aldosterone. *Endocrinology.* 1989; 125: 2463-2469.
13. Kapas S, Hinson JP, Puddefoot JR, Ho MM, Vinson GP: Internalization of the type I angiotensin II receptor (AT1) is required for protein kinase C activation but not for inositol trisphosphate release in the angiotensin II stimulated rat adrenal zona glomerulosa cell. *Biochem Biophys Res Commun.* 1994; 204: 1292-1298.

14. Sen I, Bull HG, Soffer RL: Isolation of an angiotensin II-binding protein from liver. *Proc Natl Acad Sci USA.* 1984; 81: 1679-1683.
15. Kiron MA, Soffer RL: Purification and properties of a soluble angiotensin II-binding protein from rabbit liver. *J Biol Chem.* 1989; 264: 4138-4142.
16. Robertson AL, Khairallah PA: Angiotensin II: Rapid localization in nuclei of smooth and cardiac muscle. *Science.* 1971; 172: 1138-1139.
17. Tang S-S, Rogg H, Schumacher R, Dzau VJ: Characterization of nuclear angiotensin-II-binding sites in rat liver and comparison with plasma membrane receptors. *Endocrinology.* 1992; 131: 374-380.
18. Jiménez E, Vinson GP, Montiel M: Angiotensin II (AII)-binding sites in nuclei from rat liver: partial characterization of the mechanism of AII accumulation in nuclei. *J Endocrinol.* 1994; 143: 449-453.
19. Re RN, Vizard DC, Brown J, Byran SE: Angiotensin II receptors in chromatin fragments generated by micrococcal nuclease. *Biochem Biophys Res Commun.* 1984; 119: 220-227.
20. Re RN, Parab M: Effect of angiotensin II on RNA synthesis by isolated nuclei. *Life Sci.* 1984; 34: 647-651.
21. Eggena P, Zhu JH, Clegg K, Barrett JD: Nuclear angiotensin receptors induce transcription of renin and angiotensinogen mRNA. *Hypertension.* 1993; 22: 496-501.
22. Schelling P, Fischer H, Ganten D: Angiotensin and cell growth: a link to cardiovascular hypertrophy? *J Hypertens.* 1991; 9: 3-15.
23. 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-H428.
24. Danser AHJ, Koning MMG, Admiraal PJJ, Sassen LMA, Derkx FHM, Verdouw PD, Schalekamp MADH: Production of angiotensins I and II at tissue sites in intact pigs. *Am J Physiol.* 1992; 263: H429-H437.
25. 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.
26. Danser AHJ, van Kats JP, Admiraal PJJ, Derkx FHM, Lamers JMJ, Verdouw PD, Saxena PR, Schalekamp MADH: Cardiac renin and angiotensins. Uptake from plasma versus in situ synthesis. *Hypertension.* 1994; 24: 37-48.
27. Chang RSL, Siegl PKS, Clineschmidt BV, Mantlo NB, Chakravarty PK, Greenlee WJ, Patchett AA, Lotti VJ: In vitro pharmacology of L-158,809, a new highly potent and selective angiotensin II receptor antagonist. *J Pharmacol Exp Ther.* 1992; 262: 133-138.
28. Siegl PKS, Chang RSL, Mantlo NB, Chakravarty PK, Ondeyka DL, Greenlee WJ, Patchett AA, Sweet CS, Lotti VJ: In vivo pharmacology of L-158,809, a new highly potent and selective nonpeptide angiotensin II receptor antagonist. *J Pharmacol Exp Ther.* 1992; 262: 139-144.
29. van Kats JP, Sassen LMA, Danser AHJ, Polak MPJ, Soei LK, Derkx FHM, Schalekamp MADH, Verdouw PD. Assessment of the role of the renin-angiotensin system in cardiac contractility utilizing the renin inhibitor remikiren. *Br J Pharmacol.* 1996; 117: 891-901.
30. 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: 173-184.
31. Hunyady L, Baukal AJ, Balla T, Catt KJ: Independence of type I angiotensin II receptor endocytosis from G protein coupling and signal transduction. *J Biol Chem.* 1994; 269: 24798-24804.

32. Whitebread S, Mele M, Kamber B, de Gasparo M: Preliminary biochemical characterization of two angiotensin II receptor subtypes. *Biochem Biophys Res Commun.* 1989; 163: 284-291.
33. Regitz-Zagrosek V, Auch-Schwelk W, Hess B, Klein U, Duske E, Steffen C, Hildebrandt A, Fleck E: Tissue- and subtype-specific modulation of angiotensin II receptors by chronic treatment with cyclosporin A, angiotensin-converting enzyme inhibitors and AT1 antagonists. *J Cardiovasc Pharmacol.* 1995; 26: 66-72.
34. Zhuo J, Song K, Abdelrahman A, Mendelsohn FA: Blockade by intravenous losartan of AT1 angiotensin II receptors in rat brain, kidney and adrenals demonstrated by in vitro autoradiography. *Clin Exp Pharmacol Physiol.* 1994; 21:557-567.
35. Nozawa Y, Haruno A, Oda N, Yamasaki Y, Matsuura N, Yamada S, Inabe K, Kimura R, Suzuki H, Hoshino T: Angiotensin II receptor subtypes in bovine and human ventricular myocardium. *J Pharmacol Exp Ther.* 1994; 270: 566-571.
36. Regitz-Zagrosek V, Friedel N, Heymann A, Bauer P, Neuss M, Rolfs A, Steffen C, Hildebrandt A, Hetzer R, Fleck E: Regulation, chamber localization, and subtype distribution of angiotensin II receptors in human hearts. *Circulation.* 1995; 91: 1461-1471.
37. Zou LX, Hymel A, Imig JD, Navar LG: Renal accumulation of circulating angiotensin II in angiotensin II-infused rats. *Hypertension.* 1996; 27: 658-662.
38. Zou LX, Imig JD, Von Thun AM, Hymel A, Ono H, Navar LG: Receptor-mediated intrarenal angiotensin II augmentation in angiotensin II-infused rats. *Hypertension.* 1996; 28: 669-677.

Chapter 6

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

6.1 Abstract

Objectives and methods: Circulating angiotensin (Ang) I is converted to Ang II by angiotensin-converting enzyme (ACE) at the luminal surface of the vascular endothelium. To address the possibility that AngI-to-II conversion may also take place deeper in the vascular wall, the metabolism of [^{125}I]-AngI was studied in isolated perfused porcine coronary and carotid arteries, after luminal administration of the labelled peptide (via perfusion fluid) and after adventitial administration (via organ bath). To investigate whether enzymes other than ACE are involved in Ang I-to-II conversion, measurements were made both in the presence and absence of captopril. **Results:** [^{125}I]-AngII was a major metabolite and its formation was virtually completely blocked with captopril, both after luminal and adventitial administration of [^{125}I]-AngI. In coronary arteries (n=8), the [^{125}I]-AngI-to-II conversion rate after adventitial administration was about half the conversion rate after luminal administration. In carotid arteries (n=6) the conversion rate after adventitial administration was 10-20 times lower than the conversion rate after luminal administration. Degradation of [^{125}I]-Ang I into peptides other than [^{125}I]-AngII was also observed, both with luminal and adventitial administration. No [^{125}I]-AngI and II were released into the organ bath after luminal administration of [^{125}I]-AngI, and very little [^{125}I]-AngI and II entered the lumen after adventitial administration of [^{125}I]-AngI.
Conclusions: It appears that vascular AngI-to-II conversion is not limited to the endothelial surface, and that ACE is the most important, if not the only, enzyme responsible for vascular AngI-to-II conversion. The results further indicate that, if AngI and II are formed in the adventitia or media, little of these peptides will enter the vascular lumen.

6.2 Introduction

The classical view of angiotensin II (AngII), the most important biologically active product of the renin-angiotensin system (RAS), as a circulating hormone has been challenged in recent years. Previously it was thought that AngII was derived solely from the conversion of circulating angiotensin I (AngI) 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 RAS[1-3] and that AngI and AngII may also be generated locally, outside the circulating plasma[4-7]. There is even good evidence that in a number of vascular beds AngI production at tissue sites may contribute to the circulating levels of AngI and II[5,7]. AngII produced in vascular tissue from *in situ* synthesized AngI or from AngI 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 AngII in the vessel wall may not depend on endothelial ACE only. Endothelium-denuded rat aorta 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 AngI as substrate[10]. In addition, AngI has been found to contract endothelium-denuded rat aorta 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 AngI to AngII *in vitro*[13-16]. A chymostatin-sensitive angiotensin II-generating enzyme (CAGE), capable of catalyzing AngI-to-II conversion, has been isolated from aortic tissue of dog, monkey and man[17,18]. This enzyme is localized mainly in the adventitia[18], and is thought to be identical with an AngII-forming chymase, which has recently been discovered in human heart tissue[19,20]. Human heart chymase is a serine proteinase with high specificity

for AngI. Chymase mRNA is expressed in endothelial cells and in mast cells[21] and may be induced by vascular injury[22].

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

6.3 Materials and methods

6.3.1 Blood vessel preparation

Carotid and left anterior descending coronary arteries were obtained from 20 pigs (weight 16-24 kg, crossbred Yorkshire x Landrace, H.V.C., Hedel, The Netherlands) during anesthesia with 160 mg/kg α-chloralose (Merck, Darmstadt, Germany), which was given via the superior caval vein, followed by continuous i.v. infusion of low dose sodium pentobarbitone (5 mg/kg per h). 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 mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2,

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 (Ismatec IPS, Zürich, Switzerland). The flow was kept at 1 ml/min. 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 reservoir ('perfusate') and organ bath ('bath fluid') after the addition of [^{125}I]-AngI to either the perfusate reservoir or the organ bath.

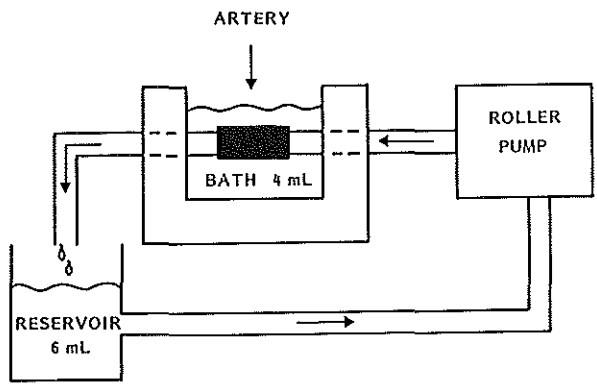

Figure 1. Schematic presentation of the experimental set-up. A 1-2 cm vessel segment is mounted in a double-jacketed organ bath that contains Krebs buffer at 37 °C. The buffer is continuously gassed with 95 % O_2 and 5 % CO_2. The vessel lumen is constantly perfused with gassed Krebs buffer from a reservoir kept in a waterbath 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').

6.3.2 Preparation of radiolabelled angiotensins

Mono-iodinated [^{125}I]-AngI was prepared with the chloramine-T method and purified as described previously[5]. The specific radioactivity of the [^{125}I]-AngI

preparation was 3.6 x 10^6 counts per minute (cpm)/pmol (74 kBq/pmol). [^{125}I]-labelled preparations of AngII, AngIII, ANG-(3-8), ANG-(4-8), ANG-(2-10), ANG-(1-7) and tyrosine were also made[5].

6.3.3 Separation and measurement of radiolabelled angiotensins

[^{125}I]-AngI and its metabolites were extracted from the samples obtained during the experiment (see below) by reversible adsorption to octadecylsilyl silica (SepPak C18, Waters, Millford, MA, USA). The SepPak cartridges were conditioned with 4 ml methanol and equilibrated with 2 times 4 ml of cold water. Samples were passed through the cartridge at 4 °C, followed by a wash with 2 times 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 (Savant Instruments, Farmingdale, NY, USA).

[^{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 250x4.6 mm and 10 μm particle size. Mobile phase A was 0.085 % ortho-phosphoric acid containing 0.02 % sodium azide. Mobile phase B was methanol. The flow was 1.5 ml/min 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-sec fractions into polypropylene tubes, and the concentrations of [^{125}I]-AngI 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]-AngI or II that had been added

to SepPak extracts was recovered after HPLC separation. Results of [^{125}I]-AngI and II measurements were therefore not corrected for the small losses during extraction and HPLC separation.

6.3.4 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]-AngI was 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]-AngI, 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]-AngI (approximately 10^6 cpm) 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 prior to the addition of [^{125}I]-AngI. This concentration of captopril had previously been shown to inhibit ACE but not other enzymes involved in the metabolism of AngI[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 ± 7 mg and 99 ± 11 mg, respectively; mean and s.e.mean) and the lengths (1.5 ± 0.1 cm and 1.6 ± 0.2 cm) were likewise similar.

6.3.5 Chemicals

[Ile5]-ANG-(1-10) decapeptide (AngI), [Ile5]-ANG-(1-8) octapeptide (AngII), and [Ile5]-ANG-(2-8) heptapeptide (AngIII) were from Bachem (Bubendorf, Switzerland). [Ile5]-ANG-(2-10) nonapeptide (ANG-(2-10)) was from Senn Chemicals (Dielsdorf, Switzerland). [Ile5]-ANG-(3-8) hexapeptide (ANG-(3-8)), [Ile5]-ANG-(4-8) pentapeptide (ANG-(4-8)), [Ile5]-ANG-(1-7) heptapeptide (ANG-(1-7)) and tyrosine were from Peninsula Laboratories (Belmont, CA, USA). Captopril was obtained from Bristol-Myers Squibb, Woerden, The Netherlands. Methanol, ortho-phosphoric acid (both analytical grade) and 1,10-phenantroline were purchased from Merck, Darmstadt, Germany. Water for high performance liquid chromatography (HPLC) was prepared with a Milli-Q system from Waters (Millford, MA, USA).

6.3.6 Calculations

[^{125}I]-AngI is eliminated either by conversion to [^{125}I]-AngII 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]-AngI[24]. The first order rate constants for degradation and conversion of [^{125}I]-AngI are denoted as k_1 and k_2, respectively. The rate constant for [^{125}I]-AngI 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]-AngI 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:

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

in which $[[^{125}I]\text{-AngI}]_t$ is the concentration of $[^{125}I]$-AngI at time t and $[[^{125}I]\text{-AngI}]_0$ is the concentration of $[^{125}I]$-AngI at t=0 (immediately after the addition of $[^{125}I]$-AngI).
The elimination of $[^{125}I]$-AngII involves degradation only. Experiments in pigs, in which $[^{125}I]$-AngI was infused into the left cardiac ventricle[24], and experiments in humans, in which $[^{125}I]$-AngI and $[^{125}I]$-AngII were infused in a peripheral vein[25], did not show a difference between the rate constants for degradation of infused $[^{125}I]$-AngI and infused $[^{125}I]$-AngII. Results of these experiments were also compatible with the assumption that the rate constants of infused $[^{125}I]$-AngI and $[^{125}I]$-AngII generated from infused $[^{125}I]$-AngI are not different. Applying these assumptions to the present experiments, k_1 can be calculated as follows:

$$k_1 = -1/t \cdot \ln (([[^{125}I]\text{-AngII}]_t + [[^{125}I]\text{-AngI}]_t) \,/\, [[^{125}I]\text{-AngI}]_0), \qquad (2)$$

in which $[[^{125}I]\text{-AngII}]_t$ is the concentration of $[^{125}I]$-AngII] at time t.
Finally, k_2 can be obtained by subtracting k_1 (calculated with formula (2)) from k_{el} (calculated with formula (1)).

$[^{125}I]$-AngI 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 [^{125}I]-AngI metabolism due to conversion is defined as follows:

contribution of conversion to metabolism (%) = $[k_2 / (k_1 + k_2)] \cdot 100$. (3)

6.3.7 Statistics

All data are reported as mean ± s.e.mean. Differences between carotid and coronary arteries and differences between experiments with and without captopril were evaluated for statistical significance ($p < 0.05$) by using Mann-Whitney's U-test for unpaired observations. Differences in metabolism between luminal and adventitial administration of [^{125}I]-AngI were evaluated for statistical significance by using Mann-Whitney's U-test for paired observations.

6.4 Results

6.4.1 Vascular metabolites of [^{125}I]-AngI during luminal or adventitial administration of [^{125}I]-AngI

With luminal administration of [^{125}I]-AngI, radioactivity (i.e., [^{125}I]-AngI 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 [^{125}I]-AngI or II in the vessel wall.

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

6.4.2 Release of [^{125}I]-AngI and II into the lumen after adventitial administration of [^{125}I]-AngI

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

No intact [^{125}I]-AngI or [^{125}I]-AngII could be detected in the perfusate during adventitial administration of [^{125}I]-AngI 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).

6.4.3 Vascular [^{125}I]-AngI metabolic clearance rates during luminal or adventitial administration of [^{125}I]-AngI

The decrease in [^{125}I]-AngI and the increase in [^{125}I]-AngII in the perfusion

fluid after luminal [^{125}I]-AngI administration are shown in Fig. 3. The changes in [^{125}I]-AngI and II in the bath fluid after adventitial [^{125}I]-AngI administration are shown in Fig. 4. No major differences were detected between coronary and carotid arteries. [^{125}I]-AngI levels followed first order kinetics. The changes in [^{125}I]-AngI and II levels were analyzed as described under *Calculations*, and the best fitting curves are shown in Figs. 3 and 4.

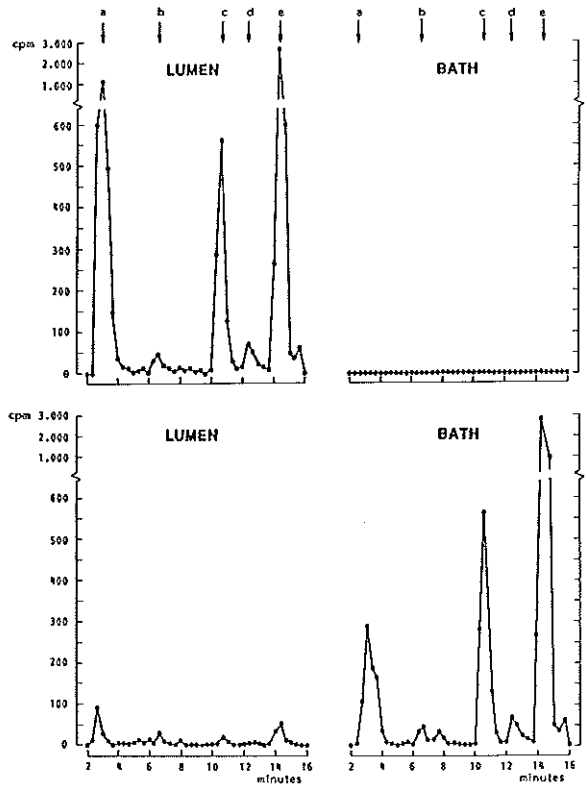

Figure 2. Examples of high-pressure liquid chromatography (HPLC) elution profiles of [^{125}I]-labelled angiotensins. Top panels, perfusate ('lumen') and bath fluid ('bath') taken from a carotid artery 20 min after the addition of [^{125}I]-AngI to the reservoir ('luminal administration'). Bottom panels, perfusate and bath fluid taken from a coronary artery 40 min after the addition of [^{125}I]-AngI to the bath ('adventitial administration'). a, [^{125}I]-tyrosine; b, [^{125}I]-ANG-(4-8); c, [^{125}I]-AngII; d, [^{125}I]-ANG-(2-10); e, [^{125}I]-AngI.

The metabolic clearance rate (MCR) of [^{125}I]-AngI was higher with luminal [^{125}I]-AngI administration than with adventitial administration, and this was true both for the coronary (Fig. 5 and Table 1) and carotid arteries (Fig. 6 and Table 1). In the coronary arteries the MCRs of [^{125}I]-AngI due to degradation and conversion were both significantly lower with adventitial [^{125}I]-AngI administration than with luminal administration (Fig. 5 and Table 1). In the carotid arteries the MCR of [^{125}I]-AngI due to degradation during luminal [^{125}I]-AngI 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 1).

The percentage of [^{125}I]-AngI metabolism that could be attributed to [^{125}I]-AngI-II conversion amounted to 45 ± 4 % (luminal administration; mean and s.e.mean, 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.

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

Captopril reduced the [^{125}I]-AngII peak in the HPLC fractions to values close to the background (Figs. 3 and 4). The peak corresponding with [^{125}I]-ANG-(4-8) became undetectable. In the presence of captopril, the peaks corresponding with [^{125}I]-tyrosine and [^{125}I]-ANG-(2-10) were the main metabolites of [^{125}I]-AngI, both with luminal and adventitial [^{125}I]-AngI administration.

Consequently, the MCR of [^{125}I]-AngI due to conversion fell to very low levels after captopril, both with luminal and adventitial [^{125}I]-AngI administration (Figs. 5 and 6). Captopril had little effect on [^{125}I]-AngI degradation.

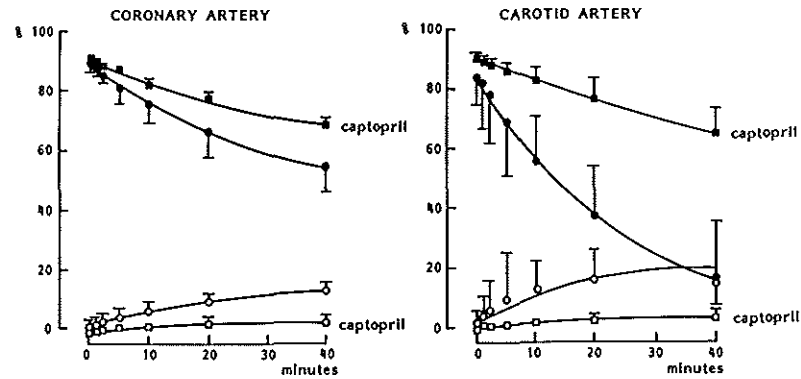

Figure 3. Decrease in [^{125}I]-AngI (closed symbols) and increase in [^{125}I]-AngII (open symbols) in the perfusate of coronary (left panel) and carotid arteries (right panel) after luminal administration of [^{125}I]-AngI in the presence (n=8) or absence (n=3) of captopril. Values are mean with s.e.mean; where no s.e.mean. is given, it was smaller than the symbol. The solid lines are the best fitting curves describing the decrease in [^{125}I]-AngI and the increase in [^{125}I]-AngII.

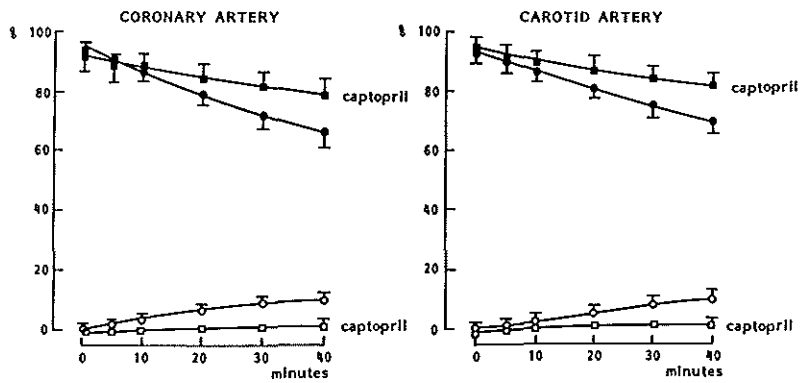

Figure 4. Decrease in [^{125}I]-AngI (closed symbols) and increase in [^{125}I]-AngII (open symbols) in the bath fluid of coronary (left panel) and carotid arteries (right panel) after adventitial administration of [^{125}I]-AngI in the presence (n=6) or absence (n=3) of captopril. Values are mean with s.e.mean; where no s.e.mean is given, it was smaller than the symbol. The solid lines are the best fitting curves describing the decrease in [^{125}I]-AngI and the increase in [^{125}I]-AngII.

With captopril, the percentage of [^{125}I]-AngI metabolism that could be attributed to [^{125}I]-AngI-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]-AngI metabolism in the coronary and carotid arteries was due to degradation.

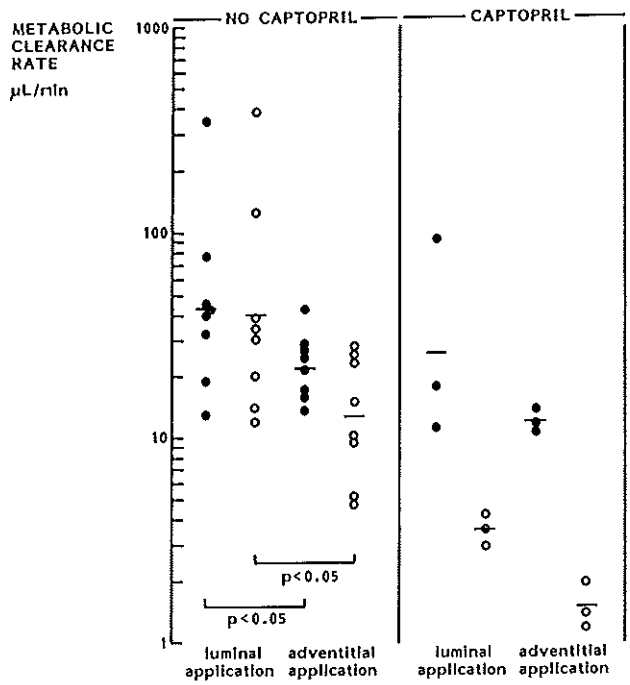

Figure 5. Metabolic clearance rate of [^{125}I]-AngI due to degradation (closed symbols) and conversion (open symbols) in coronary arteries after luminal or adventitial [^{125}I]-AngI administration in the absence (left panel) and presence (right panel) of captopril. Geometric means are shown with horizontal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

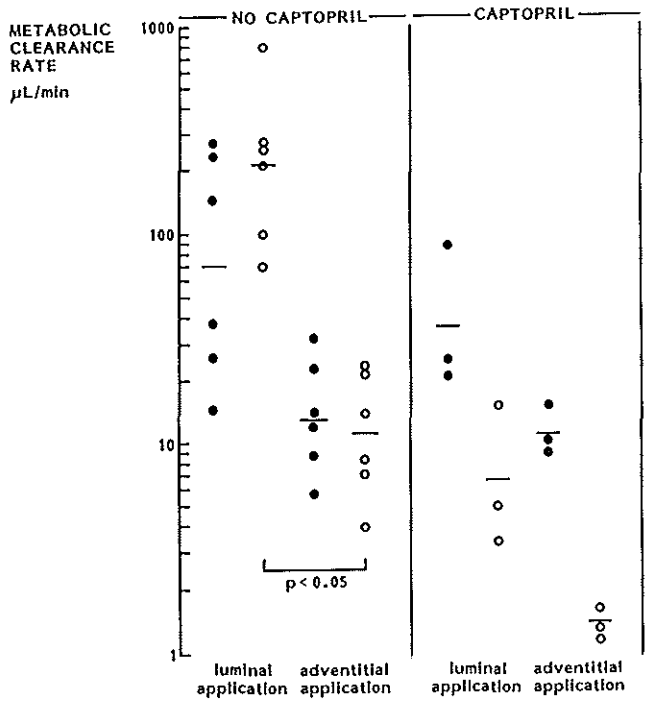

Figure 6. Metabolic clearance rate of [^{125}I]-AngI due to degradation (closed symbols) and conversion (open symbols) in carotid arteries after luminal or adventitial [^{125}I]-AngI administration in the absence (left panel) and presence (right panel) of captopril. Geometric means are shown with horizontal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

Table 1. Ratio between the vascular metabolic clearance rate (MCR) of [125I]-AngI during its adventitial administration and the MCR of [125I]-AngI during its luminal administration.

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

Values are expressed as mean and s.e.mean.
* $p < 0.05$, for difference from 1.00 (Mann-Whitney U-test for paired observations).

6.5 Discussion

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

In the present study we investigated AngI 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 AngI to the blood vessel via the perfusion fluid (luminal application) or via the surrounding bath fluid (adventitial application). With luminal application AngI is primarily exposed to the vascular endothelium. Adventitially administered AngI, which most likely corresponds with AngI that, in the *in vivo* situation, is formed in the vessel wall, 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 AngI metabolites would appear in the perfusate.

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

These findings lend support to the concept that vascular conversion of AngI is not limited to the luminal endothelial surface. Our present observation that little AngI 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, are compatible with the idea that the exchange mainly occurs 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 AngII 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 AngI.

The evidence provided by our observations on perfused arteries that ACE-dependent AngI-to-II conversion is not limited to the endothelium extends previous studies, in which the formation of [^{125}I]-AngII was followed during incubation of tissue pieces from endothelium-denuded porcine coronary arteries with [^{125}I]-AngI[10]. From these studies it was calculated that approximately 25 % of the vascular ACE was extra-endothelial. Similar findings have been reported for the rat aorta; de-endothelialization 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 extra-endothelial sites to AngI-to-II conversion in the rat aorta might be related to the localization of ACE in the smooth muscle layer[12]. Radioligand binding studies have shown that ACE in the porcine carotid artery and in the human internal mammary artery is found mainly in the endothelial layer and in the adventitia[29,30]. These species differences in ACE localization 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]-AngI, little of the peptide entered into the vascular perfusate and that [^{125}I]-AngII 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]-AngII found in the bath fluid.

The [^{125}I]-AngI-to-II conversion rates we measured after adventitial

administration of [^{125}I]-AngI may not be representative of the *in vivo* conversion rates. In the normal *in vivo* situation AngI within the vascular tissue may have been taken up from the circulating blood or may have been formed locally in the tissue at extra- and intracellular sites. Blood-derived AngI and locally formed AngI are most likely metabolized differently, since blood-derived AngI 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 are the noradrenergic nerve endings which are located at the adventitial-medial border. AngII produced by ACE in the vicinity of the nerve endings could act presynaptically to facilitate noradrenaline release. AngII-mediated facilitation of neurotransmitter release has been demonstrated in a number of vascular preparations[35,36]. It is further possible that AngII formed locally within the vascular media is involved in the induction of medial hypertrophy[8].

6.6 References

1. Lindpaintner K, Wilhelm MJ, Jin M, Unger T, Lang RE, Scholkens BA, Ganten D: Tissue renin-angiotensin systems: focus on the heart. *J Hypertens.* 1987; 5 (suppl 2): S33-S38.
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: 763-773.
3. Kim S, Tokuyama M, Hosoi M, Yamamoto K: Adrenal and circulating renin-angiotensin system in stroke-prone hypertensive rats. *Hypertension.* 1992; 20: 280-291.
4. Hilgers KF, Kuczera M, Wilhelm MJ, Wiecek A, Ritz E, Ganten D, Mann FE: Angiotensin formation in the isolated rat hindlimb. *J Hypertens.* 1989; 7: 789-798.
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: 266-277.
7. Danser AHJ, Koning MMG, Admiraal PJJ, Derkx FHM, Verdouw PD, Schalekamp MADH: Production of angiotensin I and II at tissue sites in intact pigs. *Am J Physiol.* 1992; 263: H429-H437.
8. Griffin SA, Brown WCB, MacPherson F, McGrath, Wilson VG, Korsgaard N, Mulvany MJ: Angiotensin II causes vascular hypertrophy in part by a non-pressor mechanism. *Hypertension.* 1991; 17: 626-635.

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: 333-335.
10. Van der Giessen WJ, Danser AHJ, van Beusekom HMM, Derkx FHM, Verdouw PD, Lamers JMJ, Serruys PW: Local angiotensin II production in porcine coronary arteries: reduction of release during neointimal hyperplasia induced by stent implantation. *Cor Art Disease.* 1992; 3: 730-737.
11. Egleme C, Cressier F, Wood JM: Local formation of angiotensin II in the rat aorta: effect of endothelium. *Br J Pharmacol.* 1990; 100: 237-240.
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: 1137-1142.
13. Klickstein LB, Kaempfer CE, Wintroub BU: The granulocyte angiotensin system: angiotensin I converting enzyme activity of cathepsin G. *J Biol Chem.* 1982; 257: 1504-1506.
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: 336-339.
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: 1122-1129.
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: 277-284.
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: 1186-1192.
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: 22348-22357.
20. Shiota N, Okunishi H, Fukamizu A, Sakonjo H, Kikumori M, Nishimura T, Nukagawa T, Murakami K, Miyazaki M: Activation of two angiotensin-generating enzymes in the balloon-injured artery. *FEBS Letters.* 1993; 323: 239-242.
21. Urata H, Boehm KD, Philip A, Kinoshita A, Gabrovsek J, Bumpus FM, Husain A: Cellular localization and regional distribution of an angiotensin II-forming chymase in the heart. *J Clin Invest.* 1993; 91: 1269-1281.
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: 476-482.
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-H428.
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: 173-184.
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: 540-544.
27. Okunishi H, Miyazaki M, Toda N: Evidence for a putatively new angiotensin II-generating enzyme in the vascular wall. *J Hypertens.* 1984; 2: 277-284.
28. Danser AHJ, van Kats JP, Admiraal PJJ, Derkx FHM, Lamers JMJ, Verdouw PD, Saxena PD,

Schalekamp MADH: 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: 615-620.
30. Johnston CI: Renin-angiotensin system: a dual tissue and hormonal system for cardiovascular control. *J Hypertens.* 1992; 10 (suppl 7): S13-S26.
31. Powell JS, Clozel JP, Muller RKM, Kuhn H, Hefti F, Hosang M, Baumgartner HR: Inhibitors of angiotensin-converting enzyme prevent myointimal proliferation after vascular injury. *Science.* 1989; 245: 186-188.
32. Lam JYT, Lacoste L, Bourassa MG: Cilazapril and early atherosclerotic changes after balloon injury of porcine carotid arteries. *Circulation.* 1992; 85: 1542-1547.
33. MERCATOR Study Group: Does the new angiotensin converting enzyme inhibitor cilazapril prevent stenosis after percutaneous transluminal coronary angioplasty. Results of the MERCATOR study: a multicenter, randomized, double-blind placebo-controlled trial. *Circulation.* 1992; 86: 100-110.
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: 385-392.
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.

Chapter 7

General discussion

The studies described in this thesis were performed in order to gain a better understanding of the site of angiotensin production in the heart. We did not only study the cardiac uptake and clearance of arterially delivered renin, angiotensinogen, AngI and AngII, but also the localization and clearance of locally generated AngI and II in the heart.

Renin

Renin could not be demonstrated in CE, IST or tissue of the isolated buffer-perfused rat heart. This suggests that the renin which can normally be detected in cardiac tissue immediately after removal of the heart from the body[1] is taken up from the circulation. Our measurements in the isolated rat heart were made after approximately one hour of buffer perfusion. Apparently this period is long enough to wash away all blood-derived renin. When renin was added to the perfusion buffer, we observed that renin entered the interstitium, most likely by passive diffusion. Under steady-state conditions the renin concentrations in CE and IST were similar and the tissue concentration of renin was approximately as high as expected on the basis of its presence in extracellular fluid (= coronary effluent + interstitial fluid). However, following discontinuation of the renin perfusion, renin disappeared from CE, but not IST, in a biphasic manner, suggesting that it may be present in an additional compartment. Our immunohistochemical data suggest that this compartment might be the vascular wall, specifically the vascular smooth muscle cells of the media. We cannot exclude that renin was also bound to other cells. If so, the levels of renin in these cells were too low to be detected immunohistochemically. It must be

stressed here that, in order to detect renin immunohistochemically in the vascular wall, the heart was exposed to renin concentrations that were 1000 times above normal. Thus, immunohistochemical demonstration of renin in normal hearts might be difficult.

Most likely, only a small percentage of cardiac renin is cell-associated. This conclusion is based on measurements in membrane fractions prepared from renin-perfused hearts. Renin was enriched 4-5 fold in these fractions and approximately 5 % of the total amount of cardiac renin appeared to be membrane-bound. Since our membrane preparation contained both plasma membranes and sarcoplasmic reticulum, it is unclear whether renin binding is specific for plasma membranes.

Taken together, arterially delivered renin does not only diffuse into the interstitium, but also binds to vascular smooth muscle cells. Vascular, cell-associated renin might be involved in vascular angiotensin production. The amount of AngI released by the heart via CE during combined renin and angiotensinogen perfusion, was too high to be explained by the renin-angiotensinogen reaction occurring in intravascular fluid during coronary passage. Moreover, AngI release via CE reached a steady-state level after 30-40 min of combined renin and angiotensinogen perfusion, whereas renin and angiotensinogen in CE had reached a steady-state level within 5 minutes after the start of their infusion into the perfusion buffer (Fig 1). These data may be explained by assuming that 1) renin binding is a relatively slow process, reaching a maximum after about 30-40 minutes, and 2) vascular renin rather than circulating renin is largely responsible for the high AngI levels in CE. The latter assumption would also imply that renin is located on the cell-surface rather than in the cell. In the interstitium the situation was somewhat different. AngI release via IST reached a steady state in parallel with renin and angiotensinogen (Fig 1) and the amount of AngI present in IST was as high as expected on the basis of the reaction of renin with angiotensinogen in interstitial fluid.

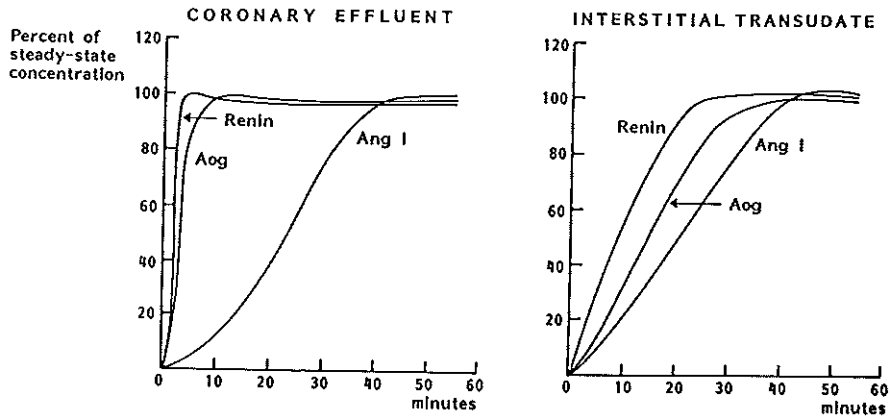

Figure 1. Time required for renin, angiotensinogen (Aog) and angiotensin I (AngI) to reach a steady-state level in CE (left) and IST (right) after starting a combined renin and angiotensinogen perfusion of the modified Langendorff heart. Note that the release of AngI via CE reaches a steady state approximately 30 min after renin and angiotensinogen have reached steady-state levels in CE, whereas the moment on which the AngI release via IST reaches a steady state coincides with the moment at which renin and angiotensinogen have reached steady-state levels in IST.

How might renin binding occur? More than a decade ago, an intracellular renin-binding protein (RnBP) has been described[2]. However, renin is inactive when bound to this RnBP. Moreover, this RnBP was recently found to be equal to *N*-acyl-D-glucosamine 2-epimerase[3], an enzyme which catalyzes the interconversion of *N*-acetylglucosamine and *N*-acetylmannosamine, and may therefore be involved in the intracellular processing of renin rather than the uptake of extracellular renin. Campbell and Valentijn identified a renin-binding protein in membranes prepared from vascular smooth muscle cells[4]. This finding is in agreement with our immunohistochemical data on vascular renin. Finally, using radiolabeled renin, high affinity (K_d ~ 1 nM) renin receptors/binding proteins have been described in membrane fractions prepared from rat tissues[5]

and in human mesangial cells[6]. Rat renin receptors were found to bind renin and prorenin equally well. Since activation of prorenin to renin did not occur in rat isolated membrane preparations, it has been suggested that prorenin, which is normally present in blood plasma in concentrations 10 times those of renin, is a natural antagonist of renin[5].

We could not demonstrate prorenin activation in isolated perfused intact hearts, that is: renin was not detectable in CE or IST during prorenin perfusion. This does not exclude prorenin activation at tissue sites. It is possible that prorenin, following binding to the renin receptor, is activated intracellularly and remains cell-associated rather than being released back into the extracellular fluid[7]. Further studies on tissue prorenin are required to investigate this in more detail.

The above remarks apply to normal healthy animals. It is possible that local synthesis of renin does occur in the heart under pathological circumstances, for instance following myocardial infarction[8].

Angiotensinogen

Cardiac angiotensinogen, like cardiac renin, appears to be largely blood-derived. Angiotensinogen cannot be detected in CE of the isolated perfused heart after approximately 30 min of buffer perfusion. Very low levels of angiotensinogen were present at that time in cardiac IST and in cardiac tissue (< 1% of the normal plasma levels in vivo). The interstitial levels decreased to levels below the detection limit during prolonged buffer perfusion. Since the in-vivo cardiac tissue levels of angiotensinogen are 10-25% of its levels in plasma[1], it seems that buffer perfusion results in a rapid washout of cardiac angiotensinogen. A tissue level equalling 10-25% of the plasma level is compatible with the diffusion of angiotensinogen into the interstitium. Our results obtained during angiotensinogen perfusion of the isolated heart support this assumption. Arterially delivered angiotensinogen diffused into the interstitium, reaching

interstitial steady-state levels comparable to those in CE. The tissue levels of angiotensinogen at steady state were also compatible with its presence in extracellular fluid. Thus, although angiotensinogen mRNA has been demonstrated in the normal heart[9], local production of angiotensinogen does not appear to make a significant contribution to the cardiac tissue levels of angiotensinogen. This may be different under pathological conditions[9].

Following discontinuation of the angiotensinogen perfusion, angiotensinogen disappeared monophasically from both CE and IST, suggesting that angiotensinogen is probably not located in a second compartment in addition to the extracellular fluid. Binding of angiotensinogen to cardiac or vascular cells is also unlikely, since angiotensinogen was not found to be enriched in membrane fractions[1,4].

Summarizing, under normal circumstances most or all cardiac angiotensinogen is plasma- (and therefore liver-) derived and restricted to the cardiac extracellular fluid. To form AngI at cardiac tissue sites it may either react with renin in this fluid or with cell membrane-associated renin.

ACE

ACE has been demonstrated by many others in cardiac tissue, both in intact hearts and in heart homogenates[10,11]. Our data showing AngII release during perfusion with AngI and during perfusion with renin combined with angiotensinogen also support the contention that ACE is present and active in the isolated perfused rat heart. The low conversion rate (<10%) of arterially delivered AngI in the rat coronary vascular bed, which is in agreement with many other reports on AngI-to-II conversion in the isolated rat heart[12,13], contrasts with the much higher conversion rates (~25%) in the human[14] and porcine coronary vascular beds[15]. Similarly, the cardiac tissue levels of AngII during combined renin and angiotensinogen perfusion were lower than the cardiac tissue levels of AngI. This is not in agreement with reports on the in-

vivo cardiac tissue levels of AngII in the rat[16] and other animals[1], which are several-fold higher than those of AngI. Since cardiac AngII is largely derived from AngI synthesis at cardiac tissue sites[17] and since renin and angiotensinogen are both present in cardiac tissue during their combined perfusion, it seems that AngI-to-II conversion in the isolated rat heart may not occur as efficiently as in vivo, for instance because the site(s) of AngI generation differ(s) between the two situations. AngI is rapidly degraded in extracellular fluid and conversion to AngII may only occur when AngI is generated in close proximity to ACE. One possibility is that under in-vivo circumstances relatively more angiotensin synthesis occurs within cells.

Alternatively, AngII receptor-binding and/or receptor recycling might be altered in the isolated buffer-perfused heart. For instance, AT_1 receptor desensitization does not occur readily in vivo[18], whereas it is a well-known phenomenon in in-vitro preparations[19]. If receptor binding is partly responsible for the high in-vivo cardiac tissue levels of AngII, the AngII levels would be expected to decrease under in-vitro conditions when receptor-related processes are altered.

Our findings on AngI-to-II conversion in porcine isolated coronary and carotid arteries suggest that in the pig, like in humans[20], ACE is restricted to the endothelium and adventitia. In contrast, vascular ACE in the rat is localized predominantly in the media rather than the endothelium[21,22]. Localization of ACE in the media (i.e. on vascular smooth muscle cells) would coincide with the renin uptake we observed in this layer. Apparently, clear differences exist between rats on the one hand, and humans and pigs on the other hand with respect to the localization of vascular ACE.

Both during AngI perfusion and during combined renin and angiotensinogen perfusion the AngII levels were higher in IST than in CE. Since we did not observe active transport of AngII into the interstitium, these differences in AngII levels might indicate that conversion occurs by extravascular ACE. ACE has been reported to be synthesized by

cardiomyocytes[23], fibroblasts[24] and valvular interstitial cells[25]. An alternative explanation for the high interstitial AngII levels is that AngI-to-II conversion is restricted to the vascular wall and that the high interstitial AngII levels more accurately reflect the vascular AngII concentrations than the low AngII levels in CE. AngII in CE is subject to rapid dilution because of the 50-100 times higher flow rate in the coronary vascular bed as compared to the interstitial fluid flow rate.

Angiotensin I and II

Angiotensin synthesis by the isolated heart depends on the presence of renin and angiotensinogen in the perfusion fluid. Angiotensin generation occurs in intravascular fluid and at tissue sites, for instance in interstitial fluid and on or within cells of the vascular wall.

Arterially delivered angiotensins are rapidly cleared by the heart. Diffusion into the interstitial fluid does not contribute importantly to this clearance process, because the interstitial fluid flow is < 2% of the CE flow. Based upon a coronary extraction of AngI and II in the order of 30-50% and a coronary transit time of ~ 6 sec, it can be calculated that the half life of both AngI and II in the coronary circulation is < 15 seconds.

Diffusion of *intact* AngI and II into the interstitial fluid is limited, most likely because of the rapid metabolism of angiotensins in the vascular wall. Consequently, AngI and II, unlike renin and angiotensinogen, reach steady-state levels in IST that are much lower than CE. The half life of angiotensins in the interstitium is approximately 1-2 minutes.

Due to the rapid metabolism of angiotensins in CE and, to a lesser extent, in interstitial fluid, the cardiac tissue levels of arterially delivered AngI and II were 10 to 20-fold lower than expected on the basis of their presence in extracellular fluid. Apparently, the removal and freezing procedure, which took approximately 15-30 seconds, is long enough to allow almost complete

degradation of extracellular AngI and II. The tissue levels of AngII were even lower when studied in the presence of the AT_1 receptor antagonist losartan. This suggests that, in agreement with our in-vivo observations on AT_1 receptor-mediated uptake of ^{125}I-AngII in the pig, receptor-binding may protect AngII against rapid degradation. The in-vivo half life of receptor-bound arterially delivered AngII is ~15 min. It appears therefore that the half life of AngII decreases upon tissue penetration, from < 15 sec in the circulation to 1-2 min in interstitial fluid and 15 min within the cells.

Significant amounts of ^{125}I-AngI could not be demonstrated in cardiac tissue during its infusion in vivo. This is not surprising on the basis of our findings in the Langendorff heart. In the isolated heart, during perfusion with AngI, the interstitial levels of AngI were approximately 5 fold lower than the arterial levels of the peptide. Thus, in a piece of left ventricular tissue (containing ~20% extracellular fluid of which 5% is blood) one would expect the ^{125}I-AngI level to be (0.05 + 0.15*1/5)*100% or less than 10% of its arterial level. It should be noted that the predicted levels in the Langendorff heart were much higher than in a piece of left ventricular tissue because of the high percentage of extracellular fluid (approximately 60%) in this whole heart preparation.

Our findings do not support the contention that AngI generated at tissue sites is located outside the extracellular fluid. During combined renin and angiotensinogen perfusion the tissue levels of AngI were as high as expected on the basis of its presence in extracellular fluid. Apparently, despite its rapid metabolism, the AngI levels can be maintained in hearts removed during renin and angiotensinogen perfusion, most likely because trapped renin and angiotensinogen continue AngI generation after removal of the heart from the Langendorff apparatus. In addition, the AngI levels in IST during combined renin and angiotensinogen perfusion were higher than in CE, which contrast with the situation during AngI perfusion, where AngI was higher in CE than in IST. Thus, during combined renin and angiotensinogen perfusion relatively more

cardiac AngI was present in a compartment where its half life is longer.

The tissue levels of locally generated AngII during combined renin and angiotensinogen perfusion were 4-5 fold higher than expected on the basis of its presence in extracellular fluid. Thus, AngII is also located outside the extracellular fluid. It may be present within cells, either because it has been internalized via AT_1 receptors following its extracellular synthesis, or because it has been synthesized intracellularly. AngII may have important intracellular functions. High affinity AngII binding sites have been detected in nuclei[26] and cytosol[27] of liver cells and AngII rapidly accumulates in nuclei of cardiomyocytes[28] and vascular smooth muscle cells[29]. AngII binds to chromatin and may influence transcriptional processes[30].

Future studies

Although our studies offer a better insight in the production of AngI and II at cardiac tissue sites under normal circumstances, many unsolved questions remain, for instance:

1. *Is ACE the only enzyme involved in cardiac AngI-to-II conversion?* In-vitro experiments have shown that other enzymes, such as human heart chymase[31], may also convert AngI to AngII. Possibly such enzymes play a role in AngII generation at tissue sites (e.g. in interstitial fluid) rather than in the intravascular compartment, since ACE inhibitors effectively block AngI-to-II conversion in the coronary vascular bed[32,33]. The modified Langendorff heart allows the investigation of ACE-dependent AngII generation in IST and CE during AngI perfusion and during combined renin and angiotensinogen perfusion.

2. *How important is the endothelium as a site of angiotensin generation and angiotensin metabolism?* The endothelium is an important barrier, both

physically and metabolically, preventing angiotensin from reaching similar levels in IST and CE. Moreover, according to some studies renin-binding is restricted to the endothelium, thus allowing AngI generation to occur on the same cells where AngI-to-II conversion by ACE occurs. On the other hand, in the rat ACE might be located predominantly at extra-endothelial sites. The importance of the endothelium can be studied in hearts pretreated with triton X-100, which effectively disrupts the endothelium but leaves the underlying vascular smooth muscle layer intact[34].

3. *Is prorenin activated to renin at cardiac tissue sites?* We found no renin in CE and IST when the heart was perfused with prorenin. In order to determine whether prorenin activation occurs in cardiac tissue (for example after uptake in cardiac cells[7]) an accurate detection method has to be developed, allowing the simultaneously measurement of inactive prorenin and activated prorenin in cardiac tissue homogenates. This method must be carefully validated, since prorenin activation may occur during preparation of the tissue for prorenin and renin measurement.

4. *Where outside the extracellular fluid is AngII situated in the heart?* Since our data have raised the possibility that AngII is AT_1 receptor-bound and/or located intracellularly, autoradiographic studies are required to investigate where arterially delivered and locally generated AngII accumulate in the isolated heart. Such studies might also address the importance of receptor-binding in the uptake process and the reason(s) for the long tissue half life of AngII.

5. *How do pathological conditions (e.g. myocardial infarction, heart failure) affect local angiotensin generation in the heart?* It is possible that, following a myocardial infarction, angiotensin synthesis in the heart occurs (partly) independently of kidney and liver. Renin mRNA has been detected in the infarcted heart in the area around the scar tissue[7], whereas the cardiac angiotensinogen mRNA levels are increased during post-infarction remodelling[9]. ACE protein and mRNA are increased following myocardial infarction[23,24,35], as well as during pressure overload-induced left ventricular hypertrophy[11]. The

modified rat Langendorff heart is a unique model to investigate the acute and long-term consequences of myocardial infarction for local angiotensin synthesis.

References

1. Danser AHJ, van Kats JP, Admiraal PJJ, Derkx FHM, Lamers JMJ, Verdouw PD, Saxena PR, Schalekamp MADH: Cardiac renin and angiotensins. Uptake from plasma versus in situ synthesis. *Hypertension.* 1994; 24: 37-48.
2. Takahashi S, Ohsawa T, Miura R, Miyake Y: Purification of high molecular weight (HMW) renin from porcine kidney and direct evidence that the HMW renin is a complex of renin with renin binding protein (RnBP). *J Biochem.* 1983; 93: 265-274.
3. Maru I, Ohta Y, Murata K, Tsukada Y: Molecular cloning and identification of *N*-acyl-D-glucosamine 2-epimerase from porcine kidney as a renin-binding protein. *J Biol Chem.* 1996; 271: 16294-16299.
4. Campbell DJ, Valentijn AJ: Identification of vascular renin-binding proteins by chemical cross-linking: inhibition of binding of renin by renin inhibitors. *J Hypertens.* 1994; 12: 879-890.
5. Sealey JE, Catanzaro DF, Lavin TN, Gahnem F, Pitarresi T, Hu L-f, Laragh JH: Specific prorenin/renin binding (PRoBP). Identification and characterization of a novel membrane site. *Am J Hypertens.* 1996; 9: 491-502.
6. Nguyen G, Delarue F, Berrou J, Rondeau E, Sraer JD: Specific receptor binding of renin on human mesangial cells in culture increases plasminogen activator inhibitor-1 antigen. *Kidney Int.* 1996; 50: 1897-1093.
7. van Kesteren CAM, Danser AHJ, Derkx FHM, Dekkers DHW, Lamers JMJ, Saxena PR, Schalekamp MADH: Mannose 6-phosphate receptor-mediated internalization and activation of prorenin by cardiac cells. *Hypertension.* 1997, in press.
8. Passier RCJJ, Smits JFM, Verluyten MJA, Daemen MJAP: Expression and localization of renin and angiotensinogen in rat heart after myocardial infarction. *Am J Physiol.* 1996; 271: H1040-H1048.
9. Lindpaintner K, Lu W, Niedermaier N, Schieffer B, Just H, Ganten D, Drexler H: Selective activation of cardiac angiotensinogen gene expression in post-infarction ventricular remodelling in the rat. *J Mol Cell Cardiol.* 1993; 23: 133-143.
10. Yamada H., Fabris B, Allen AM, Jackson B, Johnston CE, Mendelsohn FAO: Localization of angiotensin converting enzyme in rat heart. *Circ Res.* 1991; 68: 141-149.
11. Schunkert H, Dzau VJ, Tang SS, Hirsch AT, Apstein CS, Lorell BH: Increased rat cardiac angiotensin converting enzyme activity and mRNA expression in pressure overload left ventricular hypertrophy. Effects on coronary resistance, contractility, and relaxation. *J Clin Invest.* 1990; 86: 1913-1920.
12. Lindpaintner K, Wilhelm MJ, Jin M, Unger T, Lang RE, Schölkens BA, Ganten D:

Tissue renin-angiotensin systems: focus on the heart. *J Hypertens.* 1987; 5: S33-S38.
13. Schunkert H, Jackson B, Tang SS, Schoen FJ, Smits JFM, Apstein CS, Lorell BH: Distribution and functional significance of cardiac angiotensin converting enzyme in hypertrophied rat hearts. *Circulation.* 1993; 87: 1328-1339.
14. Neri Serneri GG, Boddi M, Coppo M, Chechi T, Zarone N, Moira M, Poggesi L, Margheri M, Simonetti I: Evidence for the existence of a functional cardiac renin-angiotensin system in humans. *Circulation.* 1996; 94: 1886-1893.
15. Danser AHJ, Koning MMG, Admiraal PJJ, Sassen LMA, Derkx FHM, Verdouw PD, Schalekamp MADH: Production of angiotensin I and II at tissue sites in the intact pig. *Am J Physiol.* 1992; 263: H429-H437.
16. Campbell DJ, Kladis A, Duncan AM: Nephrectomy, converting enzyme inhibition and angiotensin peptides. *Hypertension.* 1993; 22: 513-522.
17. van Kats JP, Danser AHJ, van Meegen JR, Sassen LMA, Verdouw PD, Schalekamp MADH: Uptake and production of angiotensins by the heart. A quantitative study in pigs with the use of radiolabeled angiotensin infusions. *Circulation.* 1997; submitted.
18. Sim MK, Radhakrishnan R: In vivo study of angiotensin II tachyphylaxis in freely moving normo- and hypertensive rats. *Pharmacol Toxicol.* 1994; 74: 223-227.
19. Sasamura H, Dzau VJ, Pratt RE: Desensitization of angiotensin receptor function. *Kidney Int.* 1994; 46: 1499-1501.
20. Johnston CI: Renin-angiotensin system: a dual tissue and hormonal system for cardiovascular control. *J Hypertens.* 1992; 10: 615-620.
21. Pipili E, Manolopoulos VG, Catravas JD, Maragoudakis ME: Angiotensin converting enzyme activity is present in the endothelium-denuded aorta. *Br J Pharmacol.* 1989; 98: 333-335.
22. Eglème C, Cressier F, Wood JM: Local formation of angiotensin II in the rat aorta: effect of endothelium. *Br J Pharmacol.* 1990; 100: 237-240.
23. Hokimoto S, Yasue H, Fujimoto K, Yamamoto H, Nakao K, Kaikita K, Sakata R, Miyamoto E: Expression of angiotensin-converting enzyme in remaining viable myocytes of human ventricles after myocardial infarction. *Circulation.* 1996; 94: 1513-1518.
24. Sun Y, Cleutjens JPM, Diaz-Arias AA, Weber KT: Cardiac angiotensin converting enzyme and myocardial fibrosis in the rat. *Cardiovasc Res.* 1994; 28: 1423-1432.
25. Katwa LC, Ratajska A, Cleutjens JPM, Sun Y, Zhou G, Lee SJ, Weber KT: Angiotensin converting enzyme and kininase-II-like activities in cultured valvular interstitial cells of the rat heart. *Cardiovasc Res.* 1995; 29: 57-64.
26. Tang S-S, Rogg H, Schumacher R, Dzau VJ: Characterization of nuclear angiotensin-II-binding sites in rat liver and comparison with plasma membrance receptors. *Endocrinology.* 1992; 131: 372-380.
27. Rosenberg E, Kiron MA, Soffer RL: A soluble angiotensin II-binding protein from rabbit liver. *Biochem Biophys Res Commun.* 1988; 151: 466-472.
28. Haller H, Lindschau C, Erdmann B, Quass P, Luft FC: Effects of intracellular angiotensin II in vascular smooth muscle cells. *Circ Res.* 1996; 79: 765-772.
29. Robertson AL, Khairallah PA. Angiotensin II: Rapid localization in nuclei of smooth and cardiac muscle. *Science.* 1971; 172: 1138-1139.

30. Eggena P, Zhu JH, Seerevinyayut S, Giordani M, Clegg K, Andersen PC, Hyun P, Barret JD: Hepatic angiotensin II nuclear receptors and transcription of growth-related factors. *J Hypertens.* 1996; 14: 961-968.
31. 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; 265: 22348-22357.
32. Zisman LS, Abraham WT, Meixell GE, Vamvakias BN, Quaife RA, Lowes BD, Roden RL, Peacock SJ, Groves BM, Raynolds, Bristow MR, Perryman MB: Angiotensin II formation in the intact human heart. Predominance of the angiotensin-converting enzyme pathway. *J Clin Invest.* 1995; 95: 1490-1498.
33. Danser AHJ, Koning MMG, Admiraal PJJ, Derkx FHM, Verdouw PP, Schalekamp MADH: Metabolism of angiotensin I by different tissues in the intact animal. *Am J Physiol.* 1992; 263: H418-H428.
34. Fort S, Lewis MJ: A factor released from coronary vascular endothelium inhibits myocardial contractile performance. *Am J Physiol.* 1993; 264: H830-H836.
35. Falkenhahn M, Franke F, Bohle RM, Zhu YC, Stauss HM, Bachmann S, Danilov S, Unger T: Cellular distribution of angiotensin-converting enzyme after myocardial infarction. *Hypertension.* 1995; 25: 219-226.

Appendix I

Summary

Chapter 1 describes the circulating renin-angiotensin system (RAS) versus the local cardiac RAS, and the aim of the thesis. All renin-angiotensin system components are present in cardiac tissue, and both angiotensin (Ang) I and II may be produced in the heart, either in cardiac interstitial fluid, on the surface of cardiac cells or within cardiac cells. The renin responsible for this local angiotensin production, at least under normal circumstances, originates from the circulation and is therefore kidney-derived. Thus, a local RAS in the sense that the RAS components necessary for AngII production are synthesized in situ, does not exist in the normal heart. In spite of the fact that cardiac angiotensin production depends on renin from the kidney, there are still many ways by which the heart may regulate its own AngII production. Membrane-binding could be a mechanism by which kidney-derived renin is sequestered in the heart. A renin receptor might be involved in this process. The density of such receptors may vary, and this could modify the tissue production of AngI and II. Local concentrations of ACE may also vary. The ACE levels in human heart are, in part, determined by the insertion/deletion ACE gene polymorphism. Also, enzymatic degradation of AngII and AT_1-receptor-mediated endocytosis could influence the AngII concentrations at the cellular and subcellular level. Finally, under pathological conditions renin may be produced in the heart itself, and this would create the possibility to synthesize AngII at cardiac tissue sites independent of the kidney.

Chapter 2 addresses the modified Langendorff heart model and its suitability for studying angiotensin generation in the heart. This model allows the separate

collection of interstitial transudate (IST) and coronary effluent (CE). The composition of IST was considered to be representative for the composition of interstitial fluid. We validated this model by showing that red blood cells added to the perfusion buffer do not enter the interstitial space. The intravascular, extracellular and interstitial fluid volumes were determined with ^{125}I-labeled human serum albumin and ^{51}Cr-EDTA. We used the modified Langendorff heart to investigate the uptake and clearance of arterially delivered renin, angiotensinogen and AngI as well as the cardiac production of AngI.

During perfusion with buffer, renin and AngI could not be detected in CE, IST or cardiac tissue, whereas low levels of angiotensinogen (<1% of its normal plasma concentration) were present in IST and cardiac tissue. During perfusion with renin or AngI, the steady-state levels of renin and AngI in CE were significantly lower than their arterial levels, indicating that renin and AngI are extracted by the heart. Such extraction could not be demonstrated for arterially delivered angiotensinogen. At steady state, the levels of renin and angiotensinogen in IST were not significantly different from those in CE, whereas the level of AngI in IST was lower than in CE. This indicates that AngI is degraded when entering the interstitium from the intravascular compartment. The tissue levels of renin, angiotensinogen and AngI were consistent with their location in extracellular fluid.

AngI was not detectable during angiotensinogen infusion. It was very low in IST (and undetectable in CE) during perfusion with renin, and rose to much higher levels during combined renin/angiotensinogen perfusion. The total production of AngI in IST could be attributed to the reaction of renin with angiotensinogen present in this fluid. In contrast, the total production of AngI in CE was several times higher than could be explained by AngI formation in intravascular fluid. It appears therefore that AngI tissue generation occurs in ISF and at or in the coronary vessel wall, and depends on renin and angiotensinogen from the circulation.

Chapter 3 investigates 1) the localization of arterially delivered renin in the heart, and 2) whether prorenin, following its uptake in the heart, is activated to renin. Studies were performed in modified Langendorff hearts recirculating their CE because of the limited availability of (pro)renin. Following perfusion with renin, renin could be demonstrated immunohistochemically in the media of the coronary artery wall, but not in the endothelium, adventitia or cardiomyocytes. Renin was enriched 4-5 fold in a purified cardiac membrane fraction prepared from renin-perfused hearts, suggesting that binding of renin to cardiac membranes may be part of the mechanism by which renin is taken up from the circulation. Prorenin, like renin, entered the interstitial fluid (ISF) during its perfusion, reaching a steady state level in IST comparable to that in CE. No activation of prorenin could be demonstrated in either IST or CE over a 50 min period. Taken together, it appears that renin is sequestered from the blood, not only by diffusion into the cardiac ISF, but also by binding to vascular smooth muscle cell membranes. Prorenin, if activated at tissue sites, remains cell-associated and is not released into either CE or ISF.

Chapter 4 addresses the localization of arterially delivered and locally generated AngI and II using the modified Langendorff heart model. AngI and II were measured in CE, IST and cardiac tissue during AngII, AngI or combined renin and angiotensinogen perfusion in the presence and absence of the AT_1 receptor antagonist losartan.

CE and IST. During AngI perfusion, AngI was 4 times lower in IST than in CE, whereas AngII was higher in IST than in CE. During AngII perfusion, AngII was 2 times lower in IST than CE. During combined renin and angiotensinogen perfusion, AngI and II were 2-3 fold higher in IST than CE. Losartan did not affect the angiotensin levels in CE and ISF.

Tissue. During AngI perfusion, tissue AngI was close to the detection limit, and the tissue level of AngII could be explained on the basis of its presence in extracellular fluid. Losartan did not affect tissue AngI and decreased tissue

AngII to undetectable levels. During AngII perfusion, tissue AngII was lower than predicted on the basis of its presence in extracellular fluid and the tissue levels went down to below the detection limit with losartan. During combined renin and angiotensinogen perfusion, both with and without losartan, tissue AngI was as high as predicted on the basis of its presence in extracellular fluid, whereas tissue AngII was 4-5 fold higher than expected on the basis of its presence in extracellular fluid.

We conclude that extracellular AngI and II are rapidly metabolized in the isolated heart. AngI and II levels are maintained longer after combined renin and angiotensinogen perfusion than after AngI or II perfusion, presumably due to ongoing angiotensin generation. AngI is limited to the extracellular fluid, whereas AngII is present also outside this compartment. An AT_1 receptor-dependent mechanism (receptor-mediated internalization) appears to protect AngII against rapid degradation.

In chapter 5 studies are described investigating the uptake of arterially delivered AngI and II in various tissues in vivo. We measured the levels of ^{125}I-AngII and ^{125}I-AngI that were reached in blood plasma, skeletal muscle, heart, kidney and adrenal during infusions of these peptides into the left cardiac ventricle of pigs. The steady-state ^{125}I-AngI tissue concentrations were <5% of the ^{125}I-AngI concentrations in plasma. The steady-state ^{125}I-AngII tissue concentrations were lower (skeletal muscle), or higher (kidney and adrenal) than, or equal (heart) to the ^{125}I-AngII concentrations in plasma. The tissue accumulation of ^{125}I-AngII was almost completely blocked by the specific AT_1 receptor antagonist L-158,809. Steady-state concentrations of ^{125}I-AngII were reached within 30-60 min in the tissues, and within 5 min in blood plasma. The in-vivo half life of intact ^{125}I-AngII in heart, kidney and adrenal was approximately 15 min, as compared with 0.5 min in the circulation. Thus, AngII from the circulation, but not AngI, is accumulated by some tissues and this is mediated by AT_1 receptors. The time course of this process and the long half life of the accumulated AngII

support the contention that this AngII has been internalized after its binding to the AT_1 receptor, so that it is protected from rapid degradation by endothelial peptidases.

Chapter 6 deals with in-vitro experiments in isolated porcine coronary and carotid arteries perfused with ^{125}I-AngI. These experiments were performed 1) to quantify AngI-to-II conversion both on the endothelial surface and at deeper sites in isolated arteries, 2) to assess whether the AngII 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 AngI-to-II conversion. ^{125}I-AngI was administered at the luminal (via the perfusion fluid) or adventitial (via the organ bath) side of the arteries. In coronary arteries, the ^{125}I-AngI-to-II conversion rate after adventitial administration was about half the conversion rate after luminal administration, whereas in carotid arteries it was 10-20 times lower after adventitial administration than after luminal administration. The formation of ^{125}I-AngII was almost completely blocked in the presence of captopril, both after luminal and adventitial administration of ^{125}I-AngI. No ^{125}I-AngI and II were released into the organ bath after luminal administration of ^{125}I-AngI, and very little ^{125}I-AngI and II entered the lumen after adventitial administration of ^{125}I-AngI. We conclude from these results that vascular AngI-to-II conversion is not limited to the endothelial surface, and that ACE is the most important, if not the only, enzyme responsible for vascular AngI-to-II conversion. The results further indicate that, if AngI and II are formed in the adventitia or media, little of these peptides will enter the vascular lumen.

Nederlandse samenvatting

In hoofdstuk 1 wordt een beschrijving gegeven van het circulerende renine-angiotensine systeem (RAS), en het doel van dit proefschrift. Alle RAS componenten zijn aanwezig in hartweefsel, en angiotensine (Ang) I en II zouden in het hart kunnen worden gevormd in interstitiële vloeistof, aan de buitenkant van de cellen of in de cellen. Onder normale omstandigheden is het renine dat verantwoordelijk is voor lokale angiotensine produktie afkomstig uit de nier. Zodoende is van een lokaal RAS, in die zin dat alle RAS componenten die nodig zijn voor AngII produktie lokaal gesynthetiseerd worden, in het normale hart geen sprake. Desondanks zijn er nog steeds vele manieren waarop het hart zijn eigen AngII produktie zou kunnen reguleren. Membraan-binding zou een mechanisme kunnen zijn waarop uit de nier afkomstig renine wordt opgenomen in het hart. Hierbij is wellicht een renine-receptor betrokken zijn. De dichtheid van deze receptoren kan verschillen, en dit beïnvloedt de weefsel produktie van AngI en II. De ACE concentraties in het hart kunnen variëren. In het humane hart worden deze gedeeltelijk bepaald door het zogenaamde insertion/deletion ACE gen polymorfisme. Daarnaast zal enzymatische afbraak van AngII en AT_1-receptor gemediëerde endocytose de AngII concentraties op cellulair en subcellulair niveau beïnvloeden. Onder pathologische omstandigheden wordt renine wellicht wel in het hart geproduceerd, hetgeen de mogelijkheid creëert om AngII in hartweefsel te synthetiseren onafhankelijk van de nier.

Hoofdstuk 2 behandelt het gemodificeerde Langendorff hart model en de mogelijkheid om hiermee angiotensine vorming in het hart te bestuderen. Met dit model is het mogelijk om interstitiëel transudaat (IST) en coronair effluent (CE) apart op te vangen. Aangenomen werd dat de samenstelling van IST representatief is voor de samenstelling van interstitiële vloeistof. We hebben het model gevalideerd door aan te tonen dat erythrocyten de interstitiële ruimte niet

bereiken. De intravasculaire, extracellulaire en interstitiële vloeistof volumes werden bepaald met ^{125}I-gelabeld humaan serum albumine en ^{51}Cr-EDTA. Het gemodificeerde Langendorff hart is gebruikt om de opname en klaring van arterieel aangeleverd renine, angiotensinogeen en AngI te bepalen, alsmede de AngI produktie in het hart.

Tijdens perfusie met buffer konden renine en AngI niet worden aangetoond in CE, IST of hartweefsel, terwijl lage concentraties angiotensinogeen (<1% van de normale plasma concentratie) aanwezig waren in IST en hartweefsel. Tijdens perfusie met renine of AngI waren de steady-state concentraties van renine en AngI in CE significant lager dan hun arteriële spiegels, wat aangeeft dat renine en AngI door het hart worden geëxtraheerd. Een dergelijke extractie kon niet worden aangetoond voor arterieel aangeleverd angiotensinogeen. Tijdens steady state waren de renine en angiotensinogeen concentraties in IST niet significant verschillend van de concentraties in CE, terwijl de AngI concentratie in IST lager was dan in CE. Dit geeft aan dat AngI afgebroken wordt tijdens het transport van het vasculaire compartiment naar het interstitium. De weefselspiegels van renine, angiotensinogeen en AngI konden worden verklaard uit hun aanwezigheid in extracellulaire vloeistof.

AngI kon niet worden aangetoond tijdens perfusie met angiotensinogeen. De AngI concentratie was erg laag in IST (en niet detecteerbaar in CE) tijdens perfusie met renine, en steeg tot veel hogere waarden tijdens gecombineerde renine/angiotensinogeen perfusies. De totale AngI produktie in IST kon worden toegeschreven aan de reactie van renine en angiotensinogeen in deze vloeistof. Daarentegen was de totale produktie van AngI in CE vele malen hoger dan kon worden verklaard op grond van AngI vorming in de intravasculaire vloeistof. Het lijkt er dus op dat weefsel AngI produktie plaatsvindt in de interstitiële vloeistof en op of in de coronaire vaatwand. Deze produktie hangt af van renine en angiotensinogeen uit de circulatie.

Hoofdstuk 3 onderzocht 1) de lokalisatie van arterieel aangeleverd renine in het

hart, en 2) of prorenine wordt geactiveerd naar renine nadat het is opgenomen door het hart. Deze studies werden uitgevoerd in een gemodificeerd Langendorff hart waarin het CE werd gerecirculeerd vanwege de beperkt beschikbare hoeveelheid (pro)renine. Na perfusie met renine kon renine door middel van immunohistochemie worden aangetoond in de media van de coronaire vaatwand, maar niet in het endotheel, de adventititia of de cardiomyocyten. Renine was 4 tot 5 keer verrijkt in gezuiverde cardiale membraan fracties die uit renine geperfundeerde harten waren geïsoleerd. Dit geeft aan dat renine binding aan cardiale membranen onderdeel zou kunnen zijn van een opname mechanisme voor renine uit de circulatie. Tijdens prorenine perfusie wordt prorenine net als renine naar de intersititiële vloeistof getransporteerd, waarbij een IST steady-state concentratie wordt bereikt die vergelijkbaar is met de concentratie in CE. Prorenine activatie kon niet worden aangetoond in IST en CE tijdens de 50 min perfusie. Samenvattend: renine wordt vanuit het bloed opgenomen, niet alleen door diffusie naar de cardiale interstitiële vloeistof, maar ook door binding aan vasculaire gladde spiercelmembranen. Prorenine zou eventueel geactiveerd kunnen worden in het weefsel, maar dit geactiveerde prorenine wordt niet afgegeven aan CE of IST.

In hoofdstuk 4 wordt onderzocht waar arterieel aangeleverd en lokaal gevormd AngI en II in het hart gelokaliseerd zijn, gebruik makend van het gemodificeerde Langendorff model. AngI en II werden bepaald in CE, IST en hartweefsel tijdens AngII, AngI of gecombineerde renine/angiotensinogeen perfusie in de aan- en afwezigheid van de AT_1 receptor antagonist losartan.
CE en IST. Tijdens perfusie met AngI was AngI 4 keer lager in IST dan in CE, terwijl AngII in IST hoger was dan in CE. Tijdens AngII perfusie was AngII 2 keer lager in IST vergeleken met CE. Tijdens gecombineerde renine/angiotensinogeen perfusie waren AngI en II 2-3 keer hoger in IST dan in CE. Losartan had geen effect op de angiotensine spiegels in CE en IST.
Hartweefsel. Tijdens perfusie met AngI was de AngI weefsel spiegel onder de

detectie limiet en de AngII weefsel spiegel was even hoog als voorspeld op basis van zijn aanwezigheid in extracellulaire vloeistof. Losartan had geen effect op weefsel AngI en verlaagde de weefsel AngII spiegels tot niet detecteerbare waarden. Tijdens AngII perfusie met AngII was weefsel AngII lager dan voorspeld op basis van zijn aanwezigheid in extracellulaire vloeistof, en daalde weefsel AngII onder de detectie limiet met losartan. Tijdens gecombineerde renine/angiotensinogeen perfusie met en zonder losartan was weefsel AngI even hoog als voorspeld op basis van aanwezigheid in extracellulaire vloeistof, terwijl weefsel AngII 4-5 keer hoger was dan voorspeld.

We concluderen dat extracellulair AngI en II snel worden afgebroken in het geïsoleerde hart. AngI en II spiegels blijven langer gehandhaafd na gecombineerde renine/angiotensinogeen perfusie dan na perfusie met AngI of AngII perfusie, waarschijnlijk vanwege aanhoudende angiotensine vorming. AngI is beperkt tot de extracellulaire vloeistof, terwijl AngII zich ook daarbuiten bevindt. Een AT_1 receptor-afhankelijk mechanisme (receptor-gemediëerde internalisatie) lijkt AngII te beschermen tegen snelle afbraak.

Hoofdstuk 5 geeft een beschrijving van studies waarin de opname van arterieel aangeleverd AngI en II in verschillende weefsels in vivo wordt onderzocht. We hebben de ^{125}I-AngI spiegels gemeten in bloed en in skeletspier-, hart-, nier- en bijnier-weefsel tijdens infusen van deze peptide in het linker ventrikel van varkensharten. De steady-state ^{125}I-AngI weefsel concentraties waren meer dan 20 keer lager dan de ^{125}I-AngI weefsel concentraties in bloed. De steady-state ^{125}I-AngII weefsel concentraties waren lager (skeletspier), of hoger (nier en bijnier) dan, of gelijk (hart) aan de ^{125}I-AngII concentraties in plasma. De weefsel ophoping van ^{125}I-AngII kon bijna volledig geblokkeerd worden door de specifieke AT_1 receptor antagonist L-158,809. Steady-state concentraties van ^{125}I-AngII werden bereikt na 30-60 min in weefsel, en binnen 5 min in bloed. De in-vivo halfwaardetijd van intact ^{125}I-AngII in hart, nier en bijnier was ongeveer 15 min, vergeleken met 0.5 min in de circulatie. Kennelijk wordt AngII afkomstig

uit de circulatie, maar niet AngI, opgehoopt in sommige weefsels via een AT_1 receptor-gemediëerd proces. De tijdspanne van dit proces en de lange halfwaarde tijd van opgehoopt AngII ondersteunen de opvatting dat dit AngII geïnternaliseerd is na binding aan de AT_1-receptor, zodat het wordt beschermd tegen snelle afbraak door endotheliale peptidases.

Hoofdstuk 6 behandelt experimenten waarin geïsoleerde coronaire en carotis arteriën van varkens met ^{125}I-AngI geperfundeerd werden. De experimenten werden uitgevoerd 1) om de AngI-tot-II conversie te kwantificeren op het endotheel oppervlak en dieper in de wand, 2) om uit te zoeken of AngII dat dieper in de vaatwand is gevormd aan het vasculaire lumen wordt afgegeven, en 3) om na te gaan of andere angiotensine converterende enzymen dan ACE betrokken zijn in de vasculaire AngI-tot-II conversie. ^{125}I-AngI werd hetzij luminaal toegediend (via de perfusie vloeistof) hetzij adventitiaal (via het orgaan bad). In coronair arteriën was de ^{125}I-AngI-tot-II conversie snelheid na toediening aan de kant van de adventitia ongeveer de helft van de conversie na toediening via het lumen, terwijl de conversiesnelheid in carotis arteriën na adventiale toediening 10-20 keer lager was dan na luminale toediening. De ^{125}I-AngII vorming kon bijna volledig geremd worden door captopril, zowel na ^{125}I-AngI toediening via het lumen als na ^{125}I-AngI toediening aan de kant van de adventitia. ^{125}I-AngI en II werden niet aan het orgaan bad afgegeven na toediening via het lumen van ^{125}I-AngI via het lumen, en een kleine hoeveelheid ^{125}I-AngI en II bereikte het lumen na toediening van ^{125}I-AngI aan de kant van de adventitia. We concluderen hieruit dat vasculaire AngI-tot-II conversie zich niet beperkt tot het endotheel oppervlak en dat ACE het meest belangrijke, zo niet het enige, enzym is dat verantwoordelijk is voor vasculaire AngI-tot-II conversie. De resultaten geven ook aan dat, indien AngI en II gevormd worden in de adventitia of media, weinig van deze peptiden het vasculaire lumen bereiken.

Appendix II

Publications

Full papers

Schipper NGM, de Lannoy LM, Romeyn SG, Verhoef J, Merkus FWHM: Nasal administration of an ACTH(4-9) analogue in rats: In D. Duchêne (Ed.), Buccal and nasal administration as an alternative to parenteral administration, Éditions de Santé Publishers, Paris 1992, pp. 297-302.

Schipper NGM, Verhoef J, de Lannoy LM, Romeyn SG, Brakkee JH, Wiegant VH, Gispen WH, Merkus FWHM: Nasal administration of an ACTH(4-9) analogue with dimethyl-ß-cyclodextrin as an absorption enhancer: Pharmacokinetics and dynamics. *Br J Pharmacol.* 1993; 110: 1335-1340.

Ponec M, Kempenaar J, Weerheim A, de Lannoy L, Kalkman I, Jansen H: Triglyceride metabolism in human keratinocytes cultured at the air-liquid interface. *Arch Dermatol Res.* 1995; 287: 723-30.

Danser AHJ, Chowdury S, de Lannoy LM, van der Giessen WJ, Saxena PR, Schalekamp MADH: Conversion and degradation of ^{125}I-labelled angiotensin I in isolated perfused porcine coronary and carotid arteries. *Cardiovasc Res.* 1995; 29: 789-795.

de Lannoy LM, Danser AHJ, van Kats JP, Schoemaker RG, Saxena PR, Schalekamp MADH: Renin-angiotensin system components in the interstitial fluid of the isolated perfused rat heart. Local production of angiotensin I. *Hypertension.* 1997; 29: 1240-1251.

van Kats JP, de Lannoy LM, Danser AHJ, van Meegen JR, Verdouw PD, Schalekamp MADH: Angiotensin II type 1 (AT_1) receptor-mediated accumulation of angiotensin II in tissues and its intracellular half life in

vivo. *Hypertension.* 1997; 30: 42-49.

de Lannoy LM and Danser AHJ: The site of angiotensin generation: focus on the heart", In "Angiotensin II receptor blockade: physiological and clinical implications. ed. by N.S. Dhalla, P. Zahradka, I.M.C. Dixon and R.E. Beamish, Kluwer Acedemic Publishers, Boston. 1997. In press.

de Lannoy LM, Danser AHJ, Bouhuizen AMB, Saxena PR, Schalekamp MADH: Localization and production of angiotensin II in the isolated perfused rat heart. Submitted

de Lannoy LM, Danser AHJ, Willemsen R, Broos LAM, Saxena PR, Schalekamp MADH: Localization of arterially delivered renin in the heart. In preparation.

Abstracts

de Lannoy LM, Danser, Schoemaker RG, Saxena PR, Schalekamp MADH: Tissue-uptake of renin-angiotensin system components: a study in the isolated rat Langendorff heart. *Pharm World Sci.* 1993; 15 (Suppl. I): I-5.

de Lannoy LM, Danser AHJ, Schoemaker RG, Saxena PR, Schalekamp MADH: Uptake of renin, angiotensinogen and angiotensin I and II by the isolated rat heart. *Br J Pharmacol.* 1994; 112: 42P.

Danser AHJ, de Lannoy LM, Schoemaker RG, Saxena PR, Schalekamp MADH: Renin-angiotensin system components in cardiac interstitial fluid. Uptake from plasma and local production. *Circulation.* 1994; 90 (suppl. I): I-342.

de Lannoy LM, Danser AHJ, Schoemaker RG, Saxena PR, Schalekamp MADH: Uptake of renin-angiotensin system components by the isolated rat heart. *J Hypertens.* 1994; 12 (suppl. 3): S130.

de Lannoy LM, Danser AHJ, Bouhuizen AMB, Schoemaker RG, Saxena PR, Schalekamp MADH: Generation of angiotensin I and II in the isolated rat heart. *Pharm World Sci.* 1994; 16 (suppl. J): J6.

de Lannoy LM, Danser AHJ, Bouhuizen AMB, Schoemaker RG, Saxena PR, Schalekamp MADH: Generation of angiotensin I and II in the isolated rat heart. *Br J Pharmacol.* 1995; 114: 167P.

de Lannoy LM, Danser AHJ, Bouhuizen AMB, Schoemaker RG, Saxena PR, Schalekamp MADH: Angiotensin generation in cardiac interstitial fluid. *Circulation.* 1995; 92 (suppl. I): I-28.

de Lannoy LM, Danser AHJ, Saxena PR, Schalekamp MADH: Generation and localization of angiotensin II in the isolated rat heart. *Circulation.* 1996; 94 (suppl. I): I-145.

de Lannoy LM, Danser AHJ, Saxena PR, Schalekamp MADH: AT_1-receptor-mediated sequestration of angiotensin II by the heart. *Br J Pharacol.* 1997; 120: 117P.

Nawoord

Een AIO blijft geen vier jaar AIO zonder de interesse, stimulatie en steun van de mensen in de omgeving. Iedereen die deze periode voor mij aangenaam heeft gemaakt wil ik bedanken, een aantal personen in het bijzonder:

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Curriculum vitae

Larissa Maria de Lannoy werd op 17 februari 1968 geboren te Amsterdam. Na behalen van het VWO diploma aan het Bonhoeffer College te Castricum, studeerde zij Farmacie aan de Rijksuniversiteit Utrecht, alwaar zij in augustus 1987 haar propaedeuse behaalde. Zij vervolgde haar opleiding aan de Rijksuniversiteit Leiden, waar zij de bovenbouwstudie Bio-Farmaceutische Wetenschappen in augustus 1992 afrondde met een doctoraaldiploma. Als hoofdstage deed zij onderzoek naar de nasale toediening van een ACTH(4-9) analogon met behulp van cyclodextrinen als absorptie bevorderaar onder Dr. J. Verhoef bij de subvakgroep Farmaceutische Technologie/Biofarmacie aan de Rijksuniversiteit Leiden. Na een korte stage bij Dermatologie op het Academisch Ziekenhuis Leiden onder begeleiding van Dr. M. Ponec, waarbij het lipidemetabolisme in gereconstrueerde epidermis werd bestudeerd, liep zij gedurende 9 maanden een industriestage bij Sandoz Pharma te Bazel, onder supervisie van Dr. J. van Bree. Hier bestudeerde zij de farmacokinetiek/ farmacodynamiek van een LHRH antagonist en ontwikkelde een PK/PD model voor dit peptide.
Vanaf januari 1993 is zij werkzaam geweest bij de vakgroep Farmacologie en Inwendige Geneeskunde I van de Erasmus Universiteit Rotterdam. Onder supervisie van de promotoren Prof. Dr M.A.D.H. Schalekamp, Prof. Dr P.R. Saxena en co-promotor Dr. A.H.J. Danser deed zij onderzoek naar het lokale cardiale renine-angiotensine systeem, hetgeen staat beschreven in dit proefschrift. Sinds augustus 1997 is zij werkzaam als Medical Information Officer bij het farmaceutische bedrijf Eli Lilly Nederland te Nieuwegein.