

# ORIGIN AND FUNCTION OF ANGIOTENSIN IN THE HEART

OORSPRONG EN FUNCTIE VAN ANGIOTENSINE IN HET HART

PROEFSCHRIFT

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*Met de gedachte  
aan mijn ouders  
en mijn zus Esther.*

*'... alleen met je hart kun je goed zien,  
de essentie is onzichtbaar voor de ogen.'*

Antoine de Saint-Exupéry, *De kleine prins*. 1943.

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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; in press.

van Kats JP, de Lannoy LM, Danser AHJ, van Meegen JR, Verdouw PD, Schalekamp MADH. Angiotensin II type 1 (AT<sub>1</sub>) receptor mediated accumulation of angiotensin II in tissues and its intracellular half life in vivo. *Hypertension*. 1997; in press.

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## ABBREVIATIONS

ACE	angiotensin-converting enzyme
AGA	angiotensin I-generating activity
AGC	angiotensin I-generating capacity
Ang	angiotensin
Ang I <sub>CE</sub>	angiotensin I present in coronary effluent
Ang I <sub>IST</sub>	angiotensin I present in interstitial transudate
Ang I <sub>ISF</sub>	angiotensin I present in interstitial fluid
Aog	angiotensinogen
art	arterial
AT <sub>1</sub>	type 1 angiotensin II receptor
BSA	bovine serum albumin
CBF	coronary blood flow
CE	coronary effluent
CO	cardiac output
cpm	counts per minute
CVR	coronary vascular resistance
EDL	segment length at end-diastole
ESL	segment length at end-systole
HPLC	high-performance liquid chromatography
HR	heart rate
HSA	human serum albumin
i.c.	intracoronary
ISF	interstitial fluid
IST	interstitial transudate
i.v.	intravenous
LADCA	left anterior descending coronary artery
LCXCA	left circumflex coronary artery
LV	left ventricle (or left ventricular)
LVEDP	left ventricular end-diastolic pressure
MAP	mean arterial pressure
MVO <sub>2</sub>	myocardial oxygen consumption
MW	myocardial work
PRA	plasma renin activity
RAS	renin-angiotensin system
SS	systolic segment shortening
SVR	systemic vascular resistance
ven	venous

*Chapter 1*

**GENERAL INTRODUCTION**

*Thesis, 1997.*





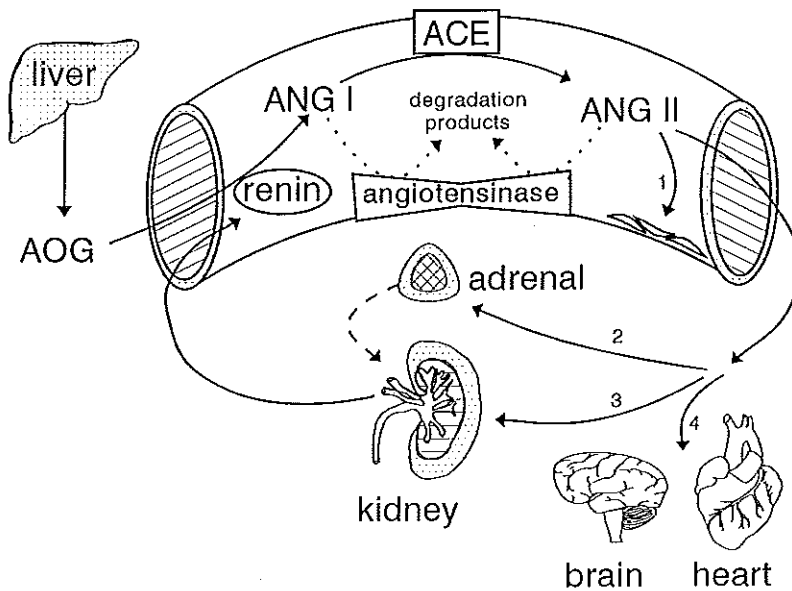
## GENERAL INTRODUCTION

*Hormones are molecules that are synthesized and secreted by specialised cells and released into the blood to exert biological effects at a distance from their site of origin. Some hormones act on one specific target tissue; others act on many tissue types. The specificity of hormone action is determined by the presence of specific hormone receptors on or in the target cells. The cellular response, however, is determined by the genetic programming of the particular cell on which the hormone acts. Thus the same hormone may have different actions on different tissues.*

## THE RENIN-ANGIOTENSIN SYSTEM AS A HORMONAL SYSTEM

Traditionally the renin-angiotensin system has been regarded as a hormonal system. However, unlike many other hormones, angiotensin II, the effector component of the system, is not released into the circulation by a group of specialized cells in one single organ, but it is produced in the circulation and the components required for angiotensin synthesis are derived from many different organs (Figure 1). The precursor protein angiotensinogen is produced in the liver. It serves as the substrate for renin, an enzyme originating from the kidney, to form the decapeptide angiotensin I. Angiotensin I is subsequently converted into the octapeptide angiotensin II by angiotensin-converting enzyme (ACE), a membrane-bound enzyme, located at the luminal side of the vascular endothelium. ACE also circulates in an active soluble form in blood plasma. Angiotensin II is a potent hormone and its synthesis and degradation are tightly controlled. Angiotensin-degrading enzymes, so-called angiotensinases, are present at the luminal surface of the vascular endothelium and in the circulating blood. These enzymes degrade angiotensin I and II into smaller, mostly inactive, fragments.

Angiotensin II is the active mediator of the cardiovascular actions of the renin-angiotensin system. It is involved in the regulation of blood pressure in several ways. Not only is angiotensin II a potent vasoconstrictor, it also regulates blood volume and sodium balance through stimulation of water- and sodium retention by the kidneys. The actions of angiotensin II in the renal water and sodium handling are mediated by the peptide's direct effect on renal tubule cells<sup>150</sup> and the renal vasculature, as well by its indirect effects on the kidney via the production and release of aldosterone from the adrenals.<sup>130,155</sup> In addition, angiotensin II regulates glomerular filtration by constriction of the efferent arterioles of the renal glomeruli.<sup>5</sup> It stimulates the release of vasopressin from the hypothalamus, and it evokes thirst when injected in the cerebral ventricle.<sup>127</sup> Recent evidence suggests that angiotensin II also acts as a growth factor.<sup>145</sup>



**Figure 1.** The classical concept of the renin-angiotensin system. Blood-borne angiotensin II causes vasoconstriction by its action on smooth muscle cells (1) and induces synthesis and release of aldosterone from the adrenal (2). In the kidney (3), angiotensin II is involved in the regulation of renal haemodynamics, water and sodium handling and release the of renin. Angiotensin II also acts on other organs, such as brain and heart (4).

ANG II:  $\text{NH}_2\text{-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-COOH}$

ANG I:  $\text{NH}_2\text{-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-COOH}$

AOG:  $\text{NH}_2\text{-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Asn-Glu-R-COOH}$

R consist of 437 amino acids; ANG: angiotensin; AOG: angiotensinogen.

Biological action of angiotensin II requires its binding to a receptor, which activates an intracellular reaction cascade that results in a cellular response. For angiotensin II, at least two different subtypes of receptors,  $\text{AT}_1$  and  $\text{AT}_2$ , have been identified, based upon different binding characteristics of non-peptide angiotensin II receptor antagonists.<sup>25,193</sup> Both subtypes belong to the seven transmembrane domain receptor superfamily. Most of the known physiological responses of angiotensin II are mediated by the  $\text{AT}_1$  receptor. In mouse, rat and man two isoforms of the  $\text{AT}_1$  subtype receptor can be distinguished on the basis of their amino acid sequences,  $\text{AT}_{1a}$  and  $\text{AT}_{1b}$ .<sup>100,112,176</sup> Binding of angiotensin II to the  $\text{AT}_1$  receptor leads, via interaction with a G-protein, to the activation of phospholipase C, which hydrolyses phosphatidylinositol biphosphate to inositol 1,4,5-triphosphate ( $\text{IP}_3$ ) and diacylglycerol (DAG).  $\text{IP}_3$  mobilises  $\text{Ca}^{2+}$  from the endoplasmatic reticulum, while DAG activates protein kinase C.<sup>15,67</sup> Additional

signal transduction pathways that are triggered by AT<sub>1</sub> receptor stimulation are the JaK/STAT pathway and the Ras/MAP kinase pathway.<sup>8</sup>

The function of the AT<sub>2</sub> receptor is not yet clear. A role in the regulation of proliferation and differentiation of cells has been postulated.<sup>176</sup> Recent evidence suggests that it induces anti-growth effects,<sup>12,163,184</sup> possibly via stimulation of phosphotyrosine phosphatase activity.<sup>13</sup> Most likely, the AT<sub>2</sub> receptor is G-protein coupled.<sup>200</sup>

In addition to the AT<sub>1</sub> and AT<sub>2</sub> plasma membrane receptors, angiotensin II binding sites have been detected in the cytosol and cell nucleus.<sup>53,72,88,133,153,172</sup> Some of these binding proteins had AT<sub>1</sub> receptor-like characteristics.<sup>53,72,172</sup> The function of these intracellular receptors is presently unknown.

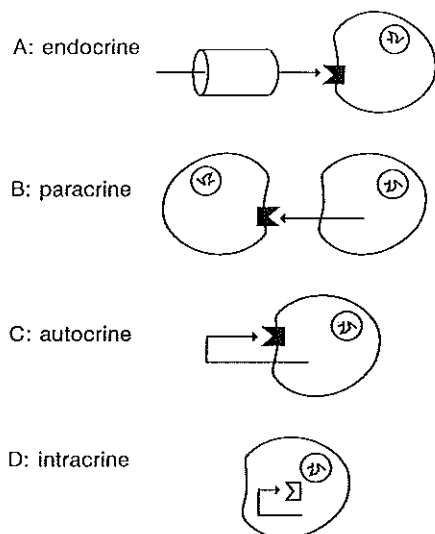
## **LOCAL RENIN-ANGIOTENSIN SYSTEMS**

The presence of components of the renin-angiotensin system in a wide variety of tissues has led to a renewed understanding of the renin-angiotensin system. More than twenty years ago the existence of a so-called local renin-angiotensin system in the bloodvessel wall was postulated to explain some discrepancies between acute changes in the circulating levels of renin and angiotensin II on the one hand and the changes in blood pressure on the other hand.<sup>157</sup> In the past two decades evidence has accumulated supporting the presence of such local renin-angiotensin systems in kidney, adrenal, brain, heart, bloodvessel wall, ovary, testis and eye.<sup>18,38,92,102,103,127</sup> There is no full agreement on how a local renin-angiotensin system should be defined. Some have defined it as a system that functions completely independently of the circulating renin-angiotensin system, whereas others support the idea that local angiotensin II generation depends, at least partly, on blood-derived components. However, irrespective of this debate, the significance of local angiotensin II formation is widely acknowledged. Locally synthesized angiotensin II can exert autocrine, paracrine, or even intracrine effects, as opposed to the endocrine effects of circulating angiotensin II (Figure 2).

## **ORIGIN OF RENIN, ANGIOTENSINOGEN AND ACE IN TISSUE**

Renin production in extrarenal tissues is a controversial issue. Renin gene expression has been reported in the adrenal<sup>47</sup> and the reproductive system.<sup>94</sup> The evidence for renin production in heart and blood vessels is less consistent.<sup>186</sup> Uptake of renin from the circulation has been postulated as the source of this enzyme in extrarenal tissues.<sup>80,109,121,158</sup> This hypothesis is supported by the disappearance of renin-activity in the vessel wall after a bilateral nephrectomy.<sup>64,166</sup> Tissue-uptake of circulating, kidney-derived renin may be realised through simple diffusion of renin into the extravascular compartment or via binding of renin to specific binding proteins or receptors.<sup>3,151</sup> Recently, the existence of such binding

proteins/receptors has been reported,<sup>21,116,152,170</sup> but the significance of these findings is still unclear.



**Figure 2.** Alternative mechanisms of action of angiotensin II in addition to its traditional function as an endocrine factor.

A: Blood-borne angiotensin II reaches its distant target organ (e.g. the vasculature or adrenal gland) via the circulation, where the peptide produces its effect.

B: Angiotensin II is released from one cell and exerts its effect on an adjacent cell.

C: Angiotensin II acts on receptors on the same cell from which it has been released.

D: Both synthesis and action of angiotensin II are located within the cell.

(Adapted from M. Paul et al.<sup>125</sup>)

Although the liver is the major site of angiotensinogen production, its mRNA and protein can also be found in the kidney, brain, vasculature and heart.<sup>16,106,142</sup> Thus for angiotensinogen, both uptake from the circulation and local synthesis are viable explanations for its presence in tissue.

While there is controversy regarding the significance of renin production, there is no doubt that ACE is synthesized at extrarenal tissue sites. As an endothelial enzyme, ACE is present in the vascular beds of all tissues. Its presence outside vascular tissue has been reported for kidney, adrenal, brain, testis, ovary and heart.<sup>58,81,148,164</sup>

Biochemical experiments have also suggested the presence of non-renin, non-ACE, pathways for angiotensin formation in vessel wall and heart.<sup>51,84,122</sup> Angiotensin II may be formed directly from angiotensinogen by cathepsin D and G, or from angiotensin I by chymase.<sup>177,195</sup> The importance of these pathways *in vivo* remains to be established.

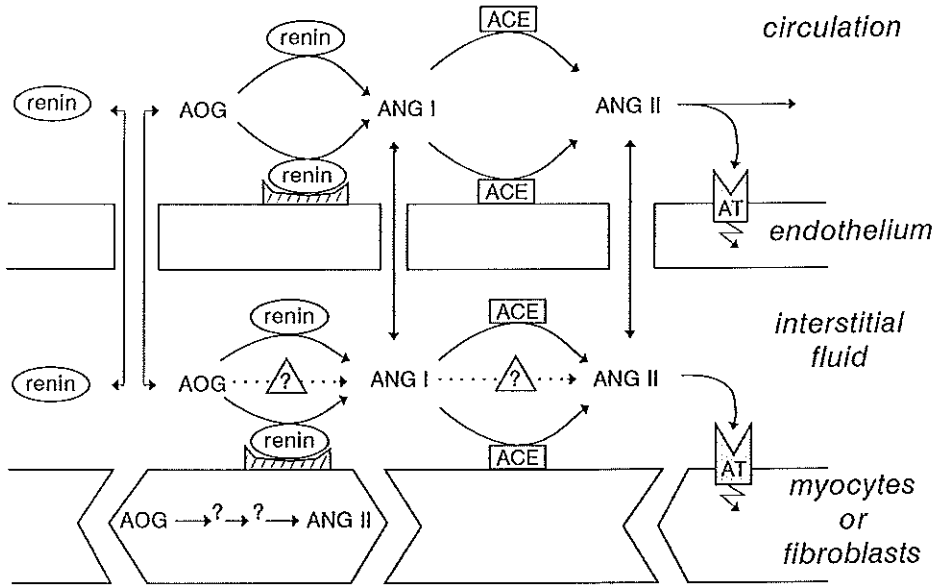
## ORIGIN OF ANGIOTENSINS IN TISSUE

While there is much debate on the origin of renin and angiotensinogen in extrarenal tissues, there is agreement that angiotensins are synthesized in these tissues. However, apart from this qualitative conclusion there is little agreement on the quantities of angiotensins that are produced in the tissues. The reliability of tissue angiotensin measurements depends on the method that is used. The stability of angiotensins in tissues is unknown and careful collection of tissue samples is therefore important. Furthermore, angiotensins have to be separated from crossreacting material before quantification by radioimmunoassay.<sup>1,18,119</sup>

The reported levels of angiotensins in cardiac tissue exceed the levels that are expected on the basis of the blood plasma content of the heart.<sup>19,103,115</sup> Therefore, uptake from the circulation and/or local synthesis seem likely. Data from *in-vitro* studies suggest that local formation of angiotensin I and II may occur. Angiotensin I and II were present in the perfusate of isolated, buffer-perfused rat hindlimbs.<sup>77,90</sup> Addition of renin to the perfusion fluid increased the angiotensin release by the hindlimbs, and the release remained elevated for some time when renin, following the discontinuation of its infusion, was no longer present in the perfusion fluid. These data indicate that renin, sequestered from the perfusion buffer by the vessel wall, may lead to local angiotensin generation. The isolated perfused rat heart did not release angiotensin I or II into the perfusate, unless renin was added to the perfusion buffer.<sup>104</sup> Apparently, cardiac angiotensin generation occurs only when renin is delivered to the heart via the circulation.

The local angiotensin formation at tissue sites has been studied *in vivo*, by measuring the arteriovenous differences for angiotensin I and II across a certain vascular bed. To make such calculations more precise, the arteriovenous differences should be corrected for regional degradation. Moreover a correction should be made for the generation of angiotensin I in circulating blood by the reaction of circulating renin with circulating angiotensinogen (plasma renin activity, PRA) while the blood is flowing through a certain vascular bed, and for the conversion of circulating angiotensin I to angiotensin II in that vascular bed. This approach has been followed in animals<sup>30,59,169</sup> and humans.<sup>1,2</sup> Regional clearance was estimated by using infusions of <sup>125</sup>I-labelled or endogenous angiotensin I or II. The advantages of radiolabelled angiotensins are that they can be distinguished from endogenous angiotensins and that they can be infused in low concentrations without significant effects on the endogenous renin-angiotensin system. These studies made it possible to calculate the amounts of *de-novo* generated angiotensin I and II that were released from tissue sites into the circulation. In the vascular beds that were studied, *de-novo* angiotensin I production could not be accounted for by PRA.<sup>1,31,59,169</sup> A high percentage of angiotensin I in the venous blood appears to be produced at tissue sites. *De-novo* produced angiotensin II in the venous blood however, is largely derived from the conversion of circulating angiotensin I.

Release of angiotensin II derived from angiotensin I that is produced at tissue sites could not be demonstrated.<sup>2,169</sup>



**Figure 3.** Angiotensin production sites. According to the classical concept angiotensin I and II are generated in the circulation. However, formation of angiotensins may also occur at tissue sites, *e. g.* in interstitial fluid, on the cell membrane, or within the cell. There may be exchange of components of the renin-angiotensin system between the intravascular compartment and the interstitial fluid compartment. Renin may bind to specific renin receptors. Dotted arrows indicate alternative (renin- and ACE-independent) angiotensin-forming pathways. ANG: angiotensin; ACE: angiotensin-converting enzyme; AT: angiotensin II receptor.

### FUNCTIONAL EVIDENCE SUPPORTING LOCAL ANGIOTENSIN SYNTHESIS IN THE HEART

The cardiac renin-angiotensin system has been focus of intensive research efforts, mainly due to the successful application of ACE inhibitors in heart failure and post-infarction remodelling.<sup>29</sup> The short-term effects of angiotensin II on the heart comprise the regulation of coronary blood flow<sup>126</sup> and the stimulation of the chronotropic and inotropic state.<sup>99,113</sup> The latter can be achieved directly by stimulation of AT<sub>1</sub> receptors on cardiomyocytes, or indirectly by stimulation of presynaptic AT<sub>1</sub> receptors that facilitate noradrenergic neurotransmission.<sup>66</sup> At the long-term, angiotensin II has growth-regulating properties in the heart both under normal<sup>7</sup> and pathological<sup>145</sup> circumstances. Via the AT<sub>1</sub> receptor, angiotensin II induces hyperplasia of fibroblasts and hypertrophy of cardiomyocytes,<sup>176</sup> angiogenesis<sup>114</sup> and extracellular matrix deposition.<sup>93</sup> Based upon studies in

endothelial cells, stimulation of the  $AT_2$  receptor is now believed to result in growth inhibition. This may also apply to cardiac fibroblasts and myocytes.<sup>12,184</sup> Thus, the cardiac  $AT_1/AT_2$  receptor ratio will determine the net growth effect of angiotensin II on the heart.

After myocardial infarction, circulating renin, angiotensin II and aldosterone are transiently elevated; they return to normal levels within a week.<sup>34,79</sup> A similar pattern is observed following aortic banding (a model for pressure overload) in animals. In contrast, ACE mRNA and ACE activity are increased in the cardiac left ventricle following coronary artery ligation (myocardial infarction model), or following aortic banding, and remain elevated for three months.<sup>79,123,128</sup> These data suggest that cardiac ACE, and therefore cardiac angiotensin II, is involved in the development of left ventricular hypertrophy following myocardial infarction or aortic banding. Renin mRNA was also found to be elevated after myocardial infarction.<sup>124</sup> However, the renin mRNA quantity that could be detected was low and mainly confined to the infarcted area. The results on cardiac angiotensinogen gene transcription following myocardial infarction are contradictory; both no change<sup>124</sup> and an increase<sup>106</sup> in angiotensinogen mRNA have been observed. Myocardial infarction causes an upregulation of  $AT_1$  receptors<sup>101,117,165</sup> and possibly  $AT_2$  receptors,<sup>117</sup> but the long-term consequences of these changes are not yet known.

## EVIDENCE FOR LOCAL ANGIOTENSIN SYNTHESIS FROM PHARMACOLOGICAL AND CLINICAL STUDIES

Pharmacological blockade of the renin-angiotensin system is an effective tool for the treatment of hypertension and heart failure. Blockade is possible at the level of renin, ACE or the  $AT_1$  receptor. ACE inhibitors, the class of renin-angiotensin system blockers that was developed first, have the disadvantage that ACE is not a specific enzyme. It hydrolyses several substrates other than angiotensin I, such as bradykinin, substance P and enkephalins.<sup>56</sup> Some side effects observed with ACE inhibitor therapy may be related to this non-specificity.

Angiotensinogen is the only known substrate for renin, and renin inhibition may therefore be devoid of the side effects observed during ACE inhibition. The non-peptide renin inhibitors that have been developed so far are effective blood pressure lowering agents.<sup>178,189</sup> However, because of their poor bioavailability following oral administration, renin inhibitors are not widely used in the clinic.

The most recently developed class of blockers of the renin-angiotensin system are the non-peptide  $AT_1$  receptor antagonists.<sup>180,190</sup> These drugs do not only offer the advantage of lacking some side effects of ACE inhibitors, they may also overcome the problems arising from the fact that long-term treatment with ACE inhibitors does not fully suppress the levels of angiotensin II, especially those at tissue sites.<sup>20</sup> This incomplete suppression of angiotensin II production may be due

to enhanced angiotensin I formation (as a consequence of stimulated renin release) or generation of angiotensin II by enzymes other than ACE.

ACE and renin inhibitors decrease blood pressure for a much longer time than circulating angiotensin II.<sup>178,187</sup> This observation has led to the assumption that inhibition of angiotensin II formation within tissues is of greater importance for the blood pressure lowering effect than suppression of circulating angiotensin II. Furthermore, the beneficial effects of ACE inhibitors in heart failure<sup>29,141,160</sup> appear to be independent, at least in part, of their effect on blood pressure.<sup>107</sup> This also supports the concept of angiotensin generation at cardiac tissue sites.

## AIM OF THE THESIS

The origin, distribution and production of components of the renin-angiotensin system in the heart has not yet been thoroughly investigated. We performed studies in pigs and rats with the following aims:

1. To measure the levels of renin-angiotensin system components in the heart under normal circumstances and following blockade of the renin-angiotensin system.
2. To determine the contribution of plasma-derived renin-angiotensin system components to the cardiac levels of these components.
3. To study the distribution and localization of renin-angiotensin system components in the heart and to assess the importance of local angiotensin synthesis for cardiac function.

Chapter 2 describes the results of measurements of renin, angiotensinogen, ACE and angiotensin I and II in the porcine heart and the effect of bilateral nephrectomy on these levels. Chapter 3 describes the measurements of renin-angiotensin system components in the cardiac interstitial fluid of the isolated perfused rat heart in order to obtain some information on the intracardiac sites of angiotensin formation. Chapter 4 addresses the uptake of circulating angiotensin I and II by the heart and other organs and the role of the AT<sub>1</sub> receptor in the uptake process. Chapter 5 addresses the question of how much of the angiotensins in cardiac tissue are produced in the tissue itself and how much is derived from the circulation; these studies were performed with and without ACE inhibition. In chapter 6, the acute cardiac effects of the renin inhibitor remikiren are described and related to remikiren-induced changes in the circulating renin-angiotensin system. The results and conclusions from these studies are summarized in chapter 7.

*References are presented in the general reference list.*



*Chapter 2*

**CARDIAC RENIN AND  
ANGIOTENSINS:  
UPTAKE FROM PLASMA  
VERSUS IN SITU SYNTHESIS.**

*Hypertension*. 1994; 24: 37-48.

**SUMMARY**

*The existence of a cardiac renin-angiotensin system, independent of the circulating renin-angiotensin system, is still controversial. We compared the tissue levels of renin-angiotensin system components in the heart with the levels in blood plasma in healthy pigs and 30 hours after nephrectomy. Angiotensin I-generating activity of cardiac tissue was identified as renin by its inhibition with a specific active site-directed renin inhibitor. Precautions were taken to prevent the ex-vivo generation and breakdown of cardiac angiotensins, and appropriate corrections were made for any losses of intact angiotensin I and II during extraction and assay. Tissue levels of renin (n=11) and angiotensin I (n=7) and II (n=7) in the left and right atria were higher than in the corresponding ventricles ( $p < 0.05$ ). Cardiac renin and angiotensin I levels (expressed per g wet weight) were similar to the plasma levels, and angiotensin II in cardiac tissue was higher than in plasma ( $p < 0.05$ ). The presence of these renin-angiotensin system components in cardiac tissue therefore cannot be accounted for by trapped plasma or simple diffusion from plasma into the interstitial fluid. Angiotensinogen levels (n=11) in cardiac tissue were 10-25% of the levels in plasma, which is compatible with its diffusion from plasma into the interstitium. Like angiotensin-converting enzyme, renin was enriched in a purified cardiac membrane fraction prepared from left ventricular tissue, as compared to crude homogenate, and  $12 \pm 3\%$  (mean and SD, n=6) of renin in crude homogenate was found in the cardiac membrane fraction and could be solubilized with 1% Triton X-100. Tissue levels of renin and angiotensin I and II in the atria and ventricles were directly correlated with the plasma levels ( $p < 0.05$ ), and both in tissue and plasma the levels were undetectably low after nephrectomy. We conclude that most, if not all, renin in cardiac tissue originates from the kidney. Results support that in the healthy heart, angiotensin production depends on plasma-derived renin, and that plasma-derived angiotensinogen in the interstitial fluid is a potential source of cardiac angiotensins. Binding of renin to cardiac membranes may be part of a mechanism by which renin is taken up from plasma.*

**ACKNOWLEDGEMENTS**

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## INTRODUCTION

Angiotensin I (Ang I) is produced in the circulating blood by the action of renin from the kidney on angiotensinogen produced by the liver. Ang I is converted to Ang II, a potent vasoconstrictor, by angiotensin-converting enzyme (ACE) located on the luminal surface of the vascular endothelium. It is now well established that Ang I and II are not only produced in the blood compartment, but also locally in tissues. Recent evidence suggests that complete local renin-angiotensin systems (RAS) are present in a number of organs, for instance kidney, adrenal gland and ovary.<sup>18,92,102</sup> In such local RAS, the production of Ang I and II is thought to depend on *in-situ* synthesized renin rather than plasma-derived renin.

A local cardiac RAS has also been postulated.<sup>49,103</sup> Direct evidence for Ang I and II production in the heart by *in-situ* synthesized renin, however, is still lacking. Renin mRNA levels in the heart are usually low and can only be detected by PCR.<sup>54,85,108</sup> Early studies showed Ang I-generating activity in left ventricular tissue of the canine heart,<sup>74</sup> but it is not known whether this activity was due to renin or to renin-like enzymes, such as cathepsin D.<sup>70</sup> Recently both renin mRNA and renin itself were demonstrated in cultured myocytes and fibroblasts from neonatal rat hearts.<sup>49</sup>

Angiotensinogen mRNA and angiotensinogen have been detected in rat<sup>106</sup> and human cardiac tissue,<sup>142</sup> as well as in cultured myocytes and fibroblasts from neonatal rat hearts.<sup>49</sup> It has also been reported that angiotensinogen is released by the isolated perfused rat Langendorff heart,<sup>104</sup> but it remains to be proven that this angiotensinogen was not derived from plasma.

ACE mRNA can be readily detected in the heart.<sup>148</sup> ACE has been demonstrated in the rat heart by autoradiography, using a radiolabeled ACE inhibitor,<sup>198</sup> as well as by measuring ACE activity in cardiac tissue homogenates.<sup>57</sup> Infusion of Ang I into the coronary circulation resulted in the prompt appearance of Ang II in the coronary effluent, and this was blocked with an ACE inhibitor.<sup>30,173</sup> It is not known whether cardiac ACE is present on myocytes or is limited to the endothelium of the coronary circulation.

Attempts have been made to measure Ang I and II in cardiac tissue. Results showed a wide range, from barely detectable to very high levels.<sup>19,103,115,120</sup> These variable results are likely to be caused by methodological difficulties, related to the lack of specificity of some angiotensin assays and to angiotensin breakdown during preparation of the tissue extracts. Recently, with a very sensitive and specific technique, it was found in the rat that, after nephrectomy, Ang II was still present in cardiac tissue.<sup>19</sup> However, Ang II was also still present in plasma, and uptake of Ang II from plasma remained therefore a possibility.

The favorable effects of ACE inhibitors in heart failure, which, at least in part, do not appear to be related to their effect on blood pressure,<sup>107</sup> have stimulated interest in the possible role of a cardiac RAS. Because of this possibility, and in

view of the uncertainties about the evidence of such a local system, the present study was undertaken to further assess the origin of the RAS components in the heart. Measurements of prorenin, renin, angiotensinogen and Ang I and II were made in hearts from anesthetized pigs. The difficulties in measuring tissue levels of these RAS components were addressed. The levels in cardiac tissue were compared with those in plasma. To assess to what extent RAS components in the heart depend on the kidney, the effects of nephrectomy on both the cardiac and circulating levels were studied.

## MATERIALS AND METHODS

### *Collection of cardiac tissue*

Hearts were obtained from pigs (crossbred Yorkshire x Landrace, Hedelse Varkens Combinatie) with a body weight of 22-30 kg. All procedures followed were in accordance with institutional guidelines. The pigs (n=24) had either been used for a series of acute pharmacological experiments, in which the cardiac effects of dobutamine after a 30-minute occlusion of the left anterior descending coronary artery were studied, or had served as a control (neither coronary occlusion nor drug administration). Two additional pigs were bilaterally nephrectomized in order to determine whether cardiac renin originates from the kidney. These animals were then allowed to recover, and were kept alive for 30 hours. The animals were anesthetized with 160 mg/kg  $\alpha$ -chloralose (Merck) injected into the superior caval vein, followed by continuous i.v. infusion of low dose pentobarbitone (5 mg/kg per hour).

The heart was quickly removed and the right and left atrium and parts of the right and left ventricle (pieces of 2-3 g) were excised on the spot. The tissues were immediately frozen in liquid nitrogen and stored at  $-70^{\circ}\text{C}$  until assay. In order to examine whether angiotensin levels remained stable for some time after the heart had been removed from the body, the various tissue pieces from two hearts were divided into two portions; one was frozen immediately and the other after it had been kept for 30 min at room temperature.

### *Collection of blood samples*

Two peripheral venous blood samples were obtained immediately before the heart was removed, one to measure angiotensins and one to measure renin and angiotensinogen. The blood sample for angiotensin measurements was collected in a prechilled polystyrene tube containing the following inhibitors (0.5 mL inhibitor solution in 10 mL blood), 0.01 mmol/L of the renin inhibitor Ro 42,5892 (a kind gift from dr W. Fischli, Hoffmann-La Roche, Basel, Switzerland), 6.25 mmol/L disodium EDTA and 1.25 mmol/L 1,10-phenanthroline (final concentrations in blood). With this inhibitor mixture virtually complete inhibition of renin, ACE and angiotensinases is achieved.<sup>30</sup> The blood sample was immediately transferred into a polystyrene tube and centrifuged at 3,000g for 10 min at  $4^{\circ}\text{C}$ . Plasma was stored at  $-70^{\circ}\text{C}$  and assayed within two days. The second blood sample, which was taken for renin and angiotensinogen measurements, was collected into a polystyrene tube containing trisodium citrate (0.2 mL citrate solution in 10 mL blood, final citrate concentration in blood 0.013 mol/L). The sample was centrifuged at 3,000g for 10 min at room temperature. Plasma was

stored at  $-20^{\circ}\text{C}$  and assayed within one week.

*Extraction, high-performance liquid chromatographic separation and measurement of angiotensins*

Liquid nitrogen-frozen cardiac tissue (2-3 g) was rapidly minced with scissors into small pieces and homogenized (1:10, wt/vol) with a Polytron (PT10/35, Kinematica) in an iced solution of 0.1 mol/L HCl/80% ethanol, as described by Chappell et al.<sup>24</sup> Homogenates were centrifuged at 20,000g for 20 min at  $4^{\circ}\text{C}$ , and the supernatant was stored for 12 hours at  $-20^{\circ}\text{C}$ . The pellet was discarded. The supernatant obtained after a second centrifugation step (20,000g, 20 min,  $4^{\circ}\text{C}$ ) was diluted 1:1 (vol/vol) with 0.1% ortho-phosphoric acid and stored for 4-6 hours at  $4^{\circ}\text{C}$ . The diluted supernatant was again centrifuged (20,000g, 20 min,  $4^{\circ}\text{C}$ ). The final supernatant was further diluted 1:1 (vol/vol) with 0.02% ortho-phosphoric acid and then concentrated by reversible adsorption to octadecylsilyl silica (SepPak C18, Waters). Plasma was directly applied to the SepPak cartridges.

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  $4^{\circ}\text{C}$ , 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  $4^{\circ}\text{C}$ , using a Speed Vac concentrator (Savant Instruments). Separations were performed by reversed-phase high-performance liquid chromatography (HPLC), according to the method of Nussberger et al.<sup>119</sup> using a Nucleosil C18 steel column of 250x4.6 mm and 10  $\mu\text{m}$  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  $45^{\circ}\text{C}$ . SepPak methanol extracts were dissolved in 100  $\mu\text{L}$  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 coated with BSA. The fractions containing Ang I and Ang II were neutralized with 0.5 mol/L sodium hydroxide and vacuum dried at  $4^{\circ}\text{C}$ . The concentrations of Ang I and Ang II were measured by radioimmunoassay as described previously.<sup>1</sup> The lower limit of detection was 0.4 fmol per fraction for the Ang II assay and 1.0 fmol per fraction for the Ang I assay.

Recovery of angiotensins after homogenization, extraction and HPLC separation was determined by adding  $^{125}\text{I}$ -Ang I or  $^{125}\text{I}$ -Ang II (specific activity  $3.6 \times 10^6$  cpm/pmol) to the tissue (approximately 10,000 cpm/g) before homogenization. The concentrations of *intact*  $^{125}\text{I}$ -Ang I and its radiolabeled metabolites in the HPLC fractions were measured in the gamma counter. Similar experiments were performed in which Ang I and Ang II were added to the tissue (approximately 100 fmol/g) before homogenization.

*Extraction of renin and prorenin*

Liquid nitrogen-frozen cardiac tissue (2-3 g) was 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 crude homogenate was centrifuged at 20,000g for 30 min at  $4^{\circ}\text{C}$ . The supernatant was stored at  $-20^{\circ}\text{C}$  ('supernatant 1'). The pellet was washed with phosphate buffer (1:2, wt/vol) and centrifuged again at 20,000g for 30 min at  $4^{\circ}\text{C}$ . The second supernatant was also stored at  $-20^{\circ}\text{C}$  ('supernatant 2'). The pellet was resuspended in phosphate buffer (1:2, wt/vol) using a Potter homogenizer (Heidolph Elektro KG) and was stored at  $-20^{\circ}\text{C}$  ('pellet').

## Chapter 2

Kidney renin was prepared from freshly obtained porcine kidneys. Kidney tissue was homogenized (1:1, wt/vol) in isotonic phosphate buffer, pH 7.4, and dialyzed at 4°C for 48 hours in 0.05 mol/L glycine buffer, pH 3.3, containing 0.001 mol/L disodium EDTA and 0.095 mol/L NaCl. This was followed by dialysis for 24 hours against a 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 20,000g for 20 min at 4°C. The resulting supernatant ('semi-purified porcine kidney renin') contained renin at a concentration of 650 pmol Ang I/min per mL, as assessed by incubation at pH 7.4 with sheep renin substrate.<sup>43</sup>

Recombinant human prorenin, which was used in recovery experiments (see below) was a kind gift of dr. W. Fischli (Hoffmann-La Roche, Basel, Switzerland). Traces of renin present in this preparation were removed by Cibacron Blue affinity chromatography.<sup>45</sup>

The pellet fraction always contained some renin activity, despite the fact that the pellet was washed by resuspension in isotonic phosphate buffer and recentrifugation. The pellet fraction, prepared as described above, contained most of the intracellular structures and organelles, such as myofibrils, cell nuclei, parts of the mitochondria, and lysosomes. In order to study whether any renin is bound to cardiac membranes, we modified the centrifugation procedure in such a way that the pellet fraction was almost devoid of myofibrils, nuclei, and mitochondria, and was mainly composed of plasma membranes and sarcoplasmic reticulum. It should be noted that *intact* cardiac tissue contains not only myocytes, but endothelial and vascular smooth muscle cells as well. Freshly obtained cardiac left ventricular tissue (20 g) was minced into small pieces and homogenized (1:10, 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 2,000g for 15 min at 4°C. The supernatant was recentrifuged at 50,000g 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. Renin measurements were performed in the washed pellet fraction ('purified cardiac membrane fraction') and the crude homogenate. ACE, as a marker of membrane-bound enzymes, was measured in the purified membrane fraction with a commercial kit (ACEcolor, Fujirebio), containing *p*-hydroxybenzoyl-glycyl-L-histidyl-L-leucine as synthetic substrate. Total protein was measured according to the method of Lowry.<sup>110</sup>

In order to assess whether any loss of renin had occurred during homogenization and extraction of cardiac tissue, known amounts of semi-purified porcine kidney renin or trypsin-activated recombinant human prorenin were added to frozen cardiac tissue before homogenization. Experiments in which known amounts of recombinant human prorenin were added to frozen tissue, were also carried out to check for inadvertent activation of prorenin.

### *Measurement of renin*

#### *'Routine' enzyme-kinetic assay.*

Samples were assessed in duplicate by measuring the rate of Ang I generation at pH 7.4 during incubation at 37°C with an excess of sheep renin substrate, in the presence of inhibitors of angiotensinases, ACE and serine proteases.<sup>43</sup> Ang I was measured by radioimmunoassay. Incubation time was maximally 6 hours. The incubation mixture consisted of 100 µL sample, 200 µL renin substrate, 14 µL inhibitor solution and 100 µL 0.01 mol/L

phosphate buffer, pH 7.4, containing 0.15 mol/L NaCl. Renin substrate was prepared from plasma of nephrectomized sheep.<sup>43</sup> The renin activity of the renin substrate preparation was less than 1.5 fmol Ang I/min per mL. Two different inhibitor solutions were used, one with the specific renin inhibitor Ro 42,5892 and one without renin inhibitor. Both solutions contained phenylmethylsulphonyl fluoride (0.0024 mol/L), disodium EDTA (0.005 mol/L), 8-hydroxyquinoline sulphate (0.0034 mol/L) and aprotinin (100 kallikrein-inhibitory units per mL) (final concentrations in the incubation mixture). The renin inhibitor Ro 42,5892 was used in a final concentration of  $10^{-5}$  mol/L. Inhibition of porcine kidney renin is virtually complete at this concentration.<sup>30</sup> Any remaining Ang I-generating activity may be assumed to be caused by enzymes other than renin.

With plasma samples, the Ang I generation during incubation was linear over time. The crude cardiac homogenates, the supernatants 1 and 2, and the pellet however, showed no detectable increase of Ang I over time. <sup>125</sup>I-Ang I added to a crude homogenate of left ventricular tissue (1:10 diluted in phosphate buffer, see under "Extraction of renin and prorenin") was rapidly broken down at 37°C ( $t_{1/2}$ =0.9 min, mean of two experiments). A small quantity of <sup>125</sup>I-Ang II was formed (less than 5% of added <sup>125</sup>I-Ang I in 30 min). The formation of <sup>125</sup>I-Ang II could be completely prevented by captopril (0.04 mmol/L), and the half life of added <sup>125</sup>I-Ang I with captopril was similar to that without captopril ( $t_{1/2}$ =1.0 min, mean of two experiments). This indicates that Ang I breakdown in the homogenates was mainly caused by degradation into fragments other than Ang II. Addition of the inhibitor mix that was used in the 'routine' renin measurements prolonged the  $t_{1/2}$  of <sup>125</sup>I-Ang I (and unlabeled Ang I) in the crude homogenates but it was still less than 10 min. This was also true for the supernatants 1 and 2 and the pellet fraction. Thus, the lack of an increase of Ang I during incubation with angiotensinogen in the 'routine' enzyme-kinetic assay was due to angiotensinase activity, which could not be blocked by the inhibitor mix. To solve these problems we used an acidification step prior to incubation.<sup>38,45</sup> One mL of sample was dialyzed at 4°C for 48 hours in 0.05 mol/L glycine buffer, pH 3.3, containing 0.001 mol/L disodium EDTA and 0.095 mol/L NaCl. This was followed by dialysis at 4°C for 24 hours against a 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 bags was then collected and the volumes were adjusted to 1 mL with phosphate buffer. With the acid-pretreated extracts, the recovery of Ang I that was added to the extracts prior to incubation with renin substrate was better than 98% (n=4) after 6 hours of incubation at 37°C. Acid-pretreatment therefore led to effective removal of angiotensinase activity.

#### *Antibody-trapping enzyme-kinetic assay*

In this assay Ang I degradation is prevented by the addition of an excess of Ang I antibody prior to incubation at 37°C with renin substrate.<sup>129</sup> We used the same Ang I antiserum as in the 'routine' assay described above. The incubation mixture consisted of 100 µL sample, 200 µL renin substrate, 10 µL Ang I antiserum (final dilution 1:4800), 14 µL inhibitor solution and 90 µL 0.01 mol/L phosphate buffer, pH 7.4, containing 0.15 mol/L NaCl. Incubation time was maximally 60 min. To quantify the trapped Ang I, the incubate was diluted 20-fold with icecold 0.15 mol/L Tris buffer, pH 7.4, containing <sup>125</sup>I-Ang I.<sup>46</sup> The antiserum dilution (1:96,000) in the diluted incubate was similar to the dilution that was used to measure Ang I in the 'routine' renin assay. The diluted incubate was kept at 4°C for 18 hours. By dilution and cooling, Ang I generation is stopped, Ang I dissociates from the antibody and can be

displaced by  $^{125}\text{I}$ -Ang I, and bound and free radioactivity can be measured. Recovery of Ang I that was added to acid-pretreated cardiac tissue extracts ( $n=4$ ) prior to the incubation at  $37^\circ\text{C}$  was better than 98% after 30 and 60 min of incubation. Recovery of Ang I added to non-pretreated cardiac tissue extracts was less complete; it was 75-83% after 30 min of incubation and 63-69% after 60 min of incubation ( $n=4$ ). Recovery of Ang I added to plasma was better than 98% both after 30 and 60 min of incubation ( $n=4$ ).

#### *Measurement of prorenin*

Prorenin was measured with the 'routine' enzyme-kinetic assay as described above, after it had been activated to renin. Different activation procedures were investigated. As described above, renin in the cardiac tissue fractions could not be measured with the 'routine' enzyme-kinetic assay, because of the high angiotensinase activity of these extracts. Acid-pretreatment of the extracts led to destruction of angiotensinases and enabled us to make accurate measurements of Ang I-generating activity. Acid-pretreatment however also causes (partial) activation of prorenin.<sup>45</sup> To activate prorenin by acid-pretreatment, the samples were acidified at pH 3.3 followed by neutralization to pH 7.4, as described under "Measurement of renin".

A second method was acidification to pH 3.3 followed by incubation with plasmin at pH 7.4. At low pH, plasmin inhibitors are destroyed,<sup>45</sup> and the added plasmin will cause activation of prorenin at neutral pH.<sup>38</sup> After dialysis for 48 hours at  $4^\circ\text{C}$  in 0.05 mol/L glycine buffer, pH 3.3, containing 0.001 mol/L disodium EDTA and 0.095 mol/L NaCl, the samples were quickly adjusted to pH 7.4 with 1 mol/L NaOH. Then 0.2 volume of plasmin solution (final concentration 1 casein unit per mL) in 0.15 mol/L NaCl was added, and the mixture was incubated for 48 hours at  $4^\circ\text{C}$ .

A third activation method consisted of incubation of 1000  $\mu\text{L}$  of sample for 48 hours at  $4^\circ\text{C}$  with 200  $\mu\text{L}$  of trypsin coupled to Sepharose (final concentration of trypsin  $8.8 \times 10^3$  N-benzoyl-L-arginine ethyl ester units per mL of sample).<sup>43</sup> After incubation the trypsin was removed by centrifugation.

In order to investigate whether the activation of prorenin with the various procedures we attempted was complete, we added purified recombinant human prorenin to the tissue extracts prior to the activation step.

#### *Identification of cardiac angiotensin I-generating activity as renin*

Renin inhibitors with high specificity can be applied to distinguish between "true" renin and "pseudorenin" (for instance cathepsin D). We used the competitive specific renin inhibitor Ro 42,5892 (molecular weight 631), which has an  $\text{IC}_{50}$  of  $7 \times 10^{-10}$  mol/L for human renin and  $3.5 \times 10^{-5}$  mol/L for bovine cathepsin D.<sup>61</sup>

We measured percent renin inhibition by Ro 42,5892 in the 'routine' enzyme-kinetic assay, at inhibitor concentrations ranging from  $10^{-11}$  to  $10^{-5}$  mol/L. The inhibition curves for the various cardiac tissue extracts were compared with those for plasma and kidney renin. Porcine kidney renin was prepared as described under "Extraction of renin and prorenin", and was used in a 1:1000 dilution.

#### *Measurement of angiotensinogen*

Angiotensinogen was measured in the same samples as were used for the renin measurement. The concentration of angiotensinogen was determined as the maximum quantity of Ang I that was generated during incubation at  $37^\circ\text{C}$  and pH 7.4 with an excess of porcine kidney



renin in the presence of inhibitors of ACE and angiotensinases.<sup>44</sup> The incubation volume consisted of 25  $\mu$ L cardiac tissue extract or plasma (the latter diluted 1:10 in phosphate buffer), 150  $\mu$ L of semipurified porcine kidney renin (see under "Extraction of renin and prorenin"), which was diluted 1:50 in phosphate buffer, and 10  $\mu$ L of inhibitor solution (see under "Measurement of renin"). The conditions of the assay were chosen in such a way that Ang I formation was complete within 30 min.

## RESULTS

### *Recovery experiments*

*Angiotensins.* Losses of Ang I and II during the homogenization, extraction and HPLC separation of cardiac tissue were measured by adding known amounts of both radiolabeled and unlabeled Ang I and II to the tissue prior to homogenization (n=5 for <sup>125</sup>I-Ang I, <sup>125</sup>I-Ang II, Ang I and Ang II). Recovery from tissue was 40-70% and from plasma 80-100%. There was no significant difference in recovery between Ang I and II or between radiolabeled and unlabeled peptides, which is in accordance with previous studies.<sup>48,75</sup> Routinely therefore, all angiotensin measurements were carried out with <sup>125</sup>I-Ang I as internal standard, and results were corrected for incomplete recovery.

*Renin and prorenin.* As assessed with the 'routine' enzyme-kinetic assay in acid-pretreated tissue extracts, practically all (>90%) porcine kidney renin that was added to frozen cardiac tissue before homogenization, was recovered from the acid-pretreated extracts (left ventricular tissue, sum of supernatants 1 and 2 and pellet, n=4), and the same was true for trypsin-activated recombinant human prorenin (n=6). The antibody-trapping assay also showed complete recovery of added porcine kidney renin and trypsin-activated recombinant human prorenin in the acid-pretreated extracts (n=4) as well as in the non-pretreated extracts (n=4). Porcine kidney renin (n=4) or trypsin-activated recombinant human prorenin (n=6) added to plasma were also fully recovered.

Less than 5% of non-activated recombinant human prorenin that was added to frozen left ventricular tissue was found to be activated after homogenization and extraction, as assessed by the antibody-trapping assay in the non-acidified extracts obtained from these tissues (n=4). The same was true for recombinant human prorenin added to plasma.

### *Angiotensin I and II content of cardiac tissue*

Cardiac tissue Ang I levels (expressed per g wet weight) were similar to the Ang I levels in blood plasma, whereas cardiac Ang II was higher than plasma Ang II (Table 1). The cardiac Ang II/I ratio was 2-3 times that in plasma. The plasma content of cardiac tissue amounts to 5-10% of wet weight.<sup>78</sup> The cardiac Ang I and II levels we measured are therefore too high to be explained by trapped blood

plasma. The high Ang II/I ratio, relative to plasma, in cardiac tissue was not caused by Ang I-to-II conversion after the tissue had been taken out, nor by degradation of Ang I into other peptides than Ang II. This was demonstrated by adding  $^{125}\text{I}$ -Ang I immediately before homogenization of the tissue in 0.1 mol/L HCl/80% ethanol. Addition of  $^{125}\text{I}$ -Ang I did not result in the appearance of any  $^{125}\text{I}$ -labeled metabolite of  $^{125}\text{I}$ -Ang I in the tissue extracts (Fig. 1).

**Table 1.** Angiotensin I and II content of cardiac tissue and plasma

Tissue	Ang I (fmol/g)	Ang II (fmol/g)	Ang II-to-I ratio
Plasma	16.6 (1.8-237)	9.8 (1.4-191)*	0.51 (0.26-1.33)*
Left atrium	15.1 (2.9-105)#	25.7 (11.6-244)#	1.96 (0.56-3.69)
Left ventricle	11.1 (2.0-61.0)	14.7 (2.4-217)	1.33 (0.62-12.3)
Right atrium	21.6 (8.1-56.9)#	21.1 (9.2-182)	0.98 (0.34-3.21)
Right ventricle	13.2 (6.4-31.8)	13.4 (2.5-128.8)	1.02 (0.39-9.75)

Data are geometric means and ranges (n=7).

\*p<0.05, cardiac tissue vs. plasma (Mann-Whitney U-test for paired observations).

# p<0.05, atrium vs. corresponding ventricle.

Keeping cardiac tissue for 30 min at room temperature before freezing did not significantly alter the results of the Ang I and II measurements, nor did it change the measured Ang II/I ratios (Table 2). This indicates that either no metabolic breakdown of angiotensins occurred as soon as the heart had been removed from the body, or that angiotensins were still produced at rates matching their breakdown.

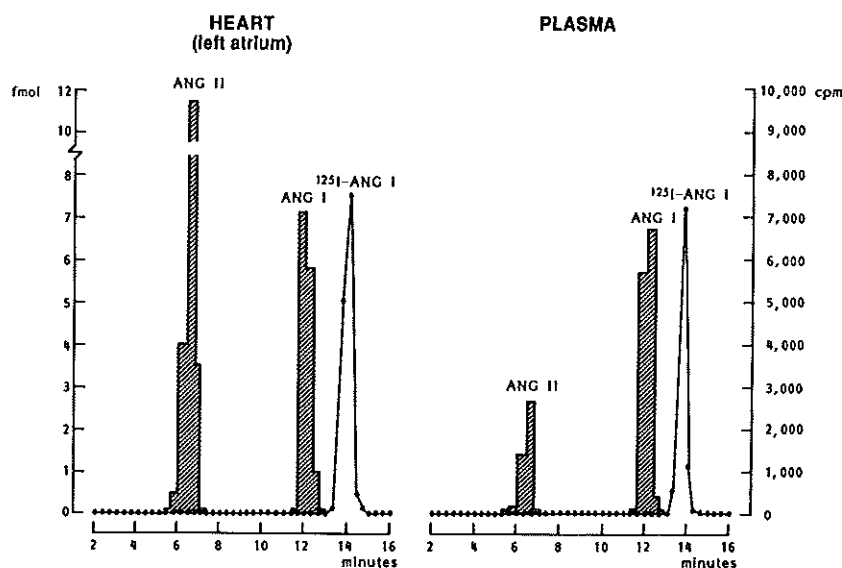
Tissue Ang I and II were higher in the atria than in the ventricles (Table 1). The Ang II/I ratios were higher in the heart than in plasma.

**Table 2.** Effect of delay in freezing and homogenization on the angiotensin I and II content of left ventricular tissue

Delay period	Ang I (fmol/g)	Ang II (fmol/g)	Ang II-to-I ratio
No Delay	21.3 ± 13.4	14.3 ± 6.5	0.83 ± 0.31
Delay 30 min	28.5 ± 29.0	17.3 ± 9.6	0.86 ± 0.34

Data are means and SD (n=7); Ang, angiotensin.

Cardiac tissue levels of Ang I and II were directly correlated with their levels in plasma (Fig. 2). Thirty hours after bilateral nephrectomy, Ang I and II were below the detection limit of the assay (approximately 1 fmol/g) both in cardiac tissue and in plasma (Fig. 3). The levels of Ang I and II were directly correlated both in cardiac tissue (left ventricle,  $r=0.70$ ,  $p<0.05$ ;  $n=7$ ) and in plasma ( $r=0.93$ ,  $p<0.05$ ;  $n=7$ ).



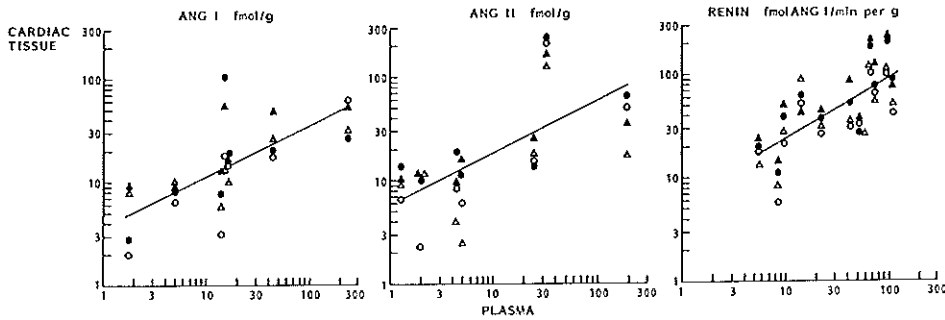
**Figure 1.** HPLC elution profile of endogenous (hatched area) and radiolabeled (closed circles) angiotensins in a porcine left atrial tissue extract (left panel) and plasma sample (right panel).  $^{125}\text{I}$ -Ang I was added as an internal standard during the purification procedure (see under 'Extraction, HPLC separation and measurement of angiotensins' for details).

#### *Renin content of cardiac tissue*

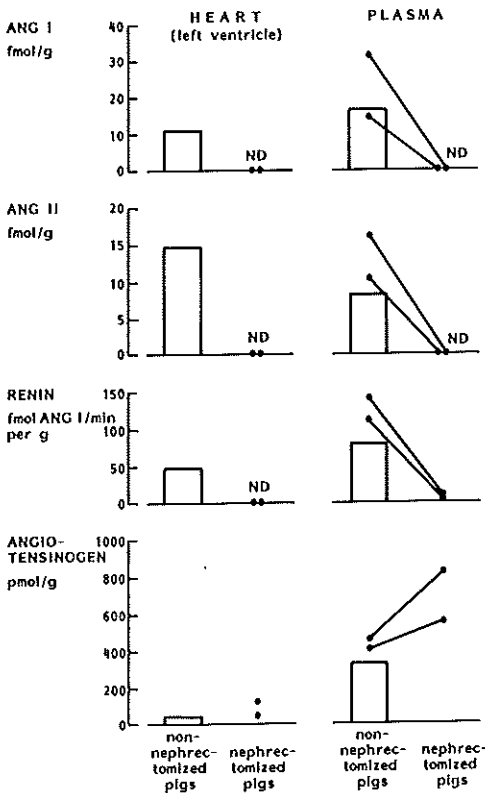
The cardiac renin levels are summarized in Table 3. They were measured at neutral pH by the 'routine' enzyme-kinetic assay, with acid-pretreatment of the samples. Renin could not be measured with this assay without acid-pretreatment because the tissue samples contained a high concentration of angiotensinase activity, which could not be inhibited by the enzyme inhibitors we used. We therefore tried the antibody-trapping enzyme-kinetic assay (see under 'Measurement of renin'), in the hope that the rapid breakdown of Ang I which occurs in the non-pretreated samples, might be prevented by binding Ang I to the Ang I antibody. With this assay, we found Ang I generation at  $37^{\circ}\text{C}$  in the non-pretreated samples to be linear for 30 min. After 30 min the rate of Ang I generation began to decline. In Fig. 4 the results obtained with the antibody-trapping assay in non-pretreated and acid-pretreated cardiac tissue extracts are compared with those obtained with the 'routine' assay in acid-pretreated samples. Results of the two assays were not different.

All measurements of cardiac renin were made both in the supernatants 1 and 2 and in the pellet fraction (see under 'Extraction of renin and prorenin'). The renin data presented in Table 3 were calculated by summation of the results obtained in these tissue fractions. In four crude homogenates of left ventricular tissue, renin was measured with the 'routine' enzyme-kinetic assay, with acid-pretreatment.

Similar measurements were made in the supernatants 1 and 2 and in the pellet fraction prepared from these crude homogenates. The results showed that the sum of the amounts of renin in the latter three fractions was equal to the amount of renin in the crude homogenate (Table 4).



**Figure 2.** Relationship between cardiac tissue levels and plasma levels of angiotensin I (left panel;  $r=0.70$ ,  $p<0.05$ ), angiotensin II (middle panel;  $r=0.64$ ,  $p<0.05$ ) and renin (right panel;  $r=0.72$ ,  $p<0.05$ ). Closed circles, left atrium; open circles, left ventricle; closed triangles, right atrium; open triangles, right ventricle.



**Figure 3.** Left ventricular and plasma levels of angiotensin I, angiotensin II, renin and angiotensinogen in non-nephrectomized pigs (bars;  $n=7$  for Ang I and II,  $n=11$  for renin and angiotensinogen) and in nephrectomized pigs (individual points). The plasma levels of angiotensin I and II, renin and angiotensinogen in the nephrectomized animals prior to nephrectomy are also given. ND, not detectable.

As shown in Table 3, the renin levels in cardiac tissue (expressed per g wet weight) were similar to those in blood plasma and, like the angiotensin levels, the renin levels are too high to be explained by trapped blood plasma. The tissue renin levels were higher in the atria than in the ventricles, which was also true for the angiotensins. The renin level in cardiac tissue was directly correlated with its level in plasma (Fig. 2). Thirty hours after bilateral nephrectomy renin was below or close to the detection limit of the assay (approximately 2 fmol Ang I/min per g) both in cardiac tissue and in plasma (Fig. 3).

**Table 3.** Renin and angiotensinogen content of cardiac tissue and plasma

Tissue	Renin (fmol Ang I/min per g)	Prorenin (fmol Ang I/min per g)	Angiotensinogen (pmol/g)
Plasma	46.0 (5.7-109)	27.7 (5.9-67.5)	340 (195-623)
Left atrium	74.5 (11.9-212)*	ND	71.8 (24.7-169)*
Left ventricle	46.6 (6.4-104)	ND	36.0 (4.1-64.8)
Right atrium	84.2 (15.1-215)*	ND	91.2 (28.8-176)
Right ventricle	52.3 (8.4-118)	ND	57.8 (29.1-107)

Data are means and ranges (n=11). Ang; angiotensin; ND, not detectable.

\* p<0.05, atrium vs. corresponding ventricle (Student's t-test for paired observations).

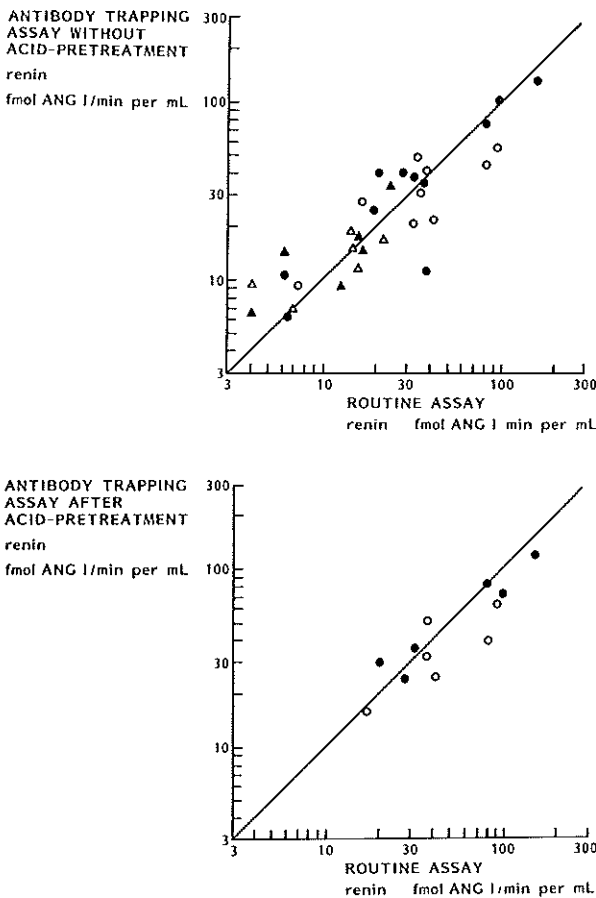
The data presented in Tables 3 and 4 and Figs. 2, 3 and 4 are corrected for Ang I-generating activity that was not due to renin. The correction factor was calculated from measurements of the percentage of Ang I-generating activity that could be inhibited by the specific renin inhibitor Ro 42,5892 ( $10^{-5}$  mol/L). The inhibition curves (percent inhibition plotted against inhibitor concentration) of acid-pretreated porcine cardiac tissue extracts were similar to those of porcine kidney renin or plasma renin (Fig. 5). The  $IC_{50}$  of Ro 42,5892 for porcine renin was approximately  $5 \times 10^{-8}$  mol/L, which is about two orders of magnitude higher than for human renin.<sup>61</sup> With the 'routine' enzyme-kinetic assay it appeared that  $91 \pm 12\%$  (mean and SD, n=16) of Ang I-generating activity was inhibited by the renin inhibitor in acid-pretreated cardiac tissue fractions. With the antibody-trapping enzyme-kinetic assay,  $88 \pm 2\%$  (n=12) of Ang I-generating activity was found to be inhibited by the renin inhibitor in acid-pretreated cardiac tissue fractions and  $74 \pm 19\%$  (n=32) in non-pretreated fractions. Thus, most of the Ang I-generating activity we measured in the cardiac tissue fractions, both with and without acid-pretreatment, was due to renin.

In non-pretreated plasma samples the Ang I generation was linear both in the 'routine' and the antibody-trapping enzyme-kinetic assays, and could be inhibited by more than 95% with the specific renin inhibitor Ro 42,5892. Ang I recovery was virtually complete, and the results were not different with the two assays. Thus, plasma renin could be reliably measured without acid-pretreatment.

**Table 4.** Renin and angiotensinogen content of various left ventricular tissue fractions

Tissue fraction	Renin (fmol Ang I/min per fraction)	Angiotensinogen (pmol per fraction)
Crude homogenate	65.3	157.4
Supernatant 1	35.1	96.1
Supernatant 2	26.9	33.6
Pellet	12.0	9.8
Supernatant 1 + 2 + pellet	75.0	139.6

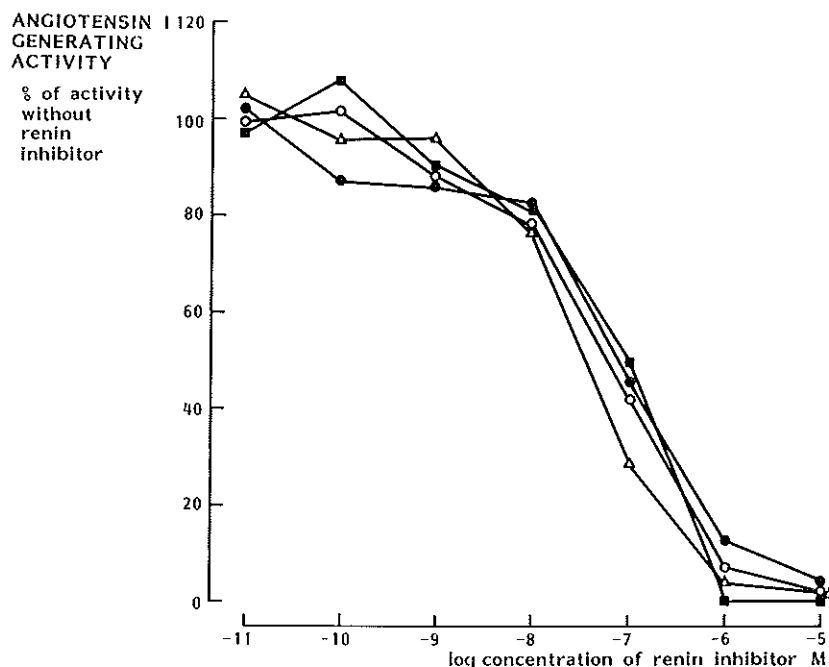
Data are means (n=4). Ang, angiotensin. Supernatant 1 and 2 and the pellet were prepared from the crude homogenate. The sum of the contents of supernatant 1 and 2 and pellet is not significantly different from the content of the crude homogenate, both for renin and angiotensinogen.



**Figure 4.** Comparison between the angiotensin I-generating activities measured in cardiac tissue by the antibody-trapping enzyme-kinetic assay and the 'routine' enzyme-kinetic assay. Upper panel, antibody-trapping assay in non-pretreated extracts. Lower panel, antibody-trapping assay in acid-pretreated extracts. The routine assay was performed in acid-pretreated extracts. The relationship between the two types of measurement is not different from the line of identity. closed circles, atria (supernatant 1); open circles, ventricles (supernatant 1); closed triangles, atria (supernatant 2); open triangles, ventricles (supernatant 2). See under 'Extraction of renin and prorenin' and 'Measurement of renin' for details.

By comparing the renin levels of the supernatants 1 and 2 and the pellet fraction, it became apparent that the pellet fraction contained a substantial amount

of renin, even though the pellet had been extensively washed. Table 4 gives the data for left ventricular tissue. It can be seen that the renin content of the pellet fraction was approximately 20% of the renin content in the supernatants 1 and 2. Similar results were obtained for right ventricular tissue and for right and left atrium.



**Figure 5.** Inhibition of angiotensin I-generating activity by increasing concentrations of the renin inhibitor Ro 42,5892. closed circles, acid-pretreated crude cardiac tissue homogenate ( $n=3$ ); closed squares, acid-pretreated purified cardiac membrane fraction ( $n=1$ ); open circles, kidney renin ( $n=2$ ); open triangles, plasma renin ( $n=3$ ).

These results prompted us to investigate the pellet fraction in more detail and to prepare a more purified cardiac membrane fraction. Despite repeated washings, the 'purified membrane fraction' contained a measurable amount of renin (Table 5); it contained  $12.2 \pm 2.9\%$  ( $n=6$ ) of the total amount of renin extracted from cardiac tissue (Fig. 6). Expressed per g protein, renin was enriched 2-3 fold in the purified membrane fraction (Table 5 and Fig. 6). Treatment with 1% Triton X-100 dissolved virtually all renin present in this fraction, thereby indicating that renin was bound to cell plasma-, sarcoplasmic- or lysosomal membranes. It is possible that some dissolution of membrane-bound renin has occurred during the washing and centrifugation procedures before treatment with Triton X-100. We may therefore have underestimated the quantity of membrane-bound renin.

**Table 5.** Enrichment of renin and ACE in a purified membrane fraction from left ventricular tissue

Tissue fraction	Renin (fmol Ang I/min per g protein)	Angiotensinogen (pmol per g protein)	ACE (units per g protein)
Plasma	591 (312-960)	11.6 (5.4-18.9)	1.4 (0.20-2.3)
Crude homogenate (H)	327 (60-523)	0.80 (0.55-1.5)	1.6 (1.1-3.0)
Purified membrane fraction (M)	798 (192-1044)	0.23 (0.04-0.53)	8.4 (3.8-15.0)
M/H ratio (relative specific activity)	2.7 (1.8-3.6)*	0.19 (0.06-0.65)*	5.4 (2.8-9.0)*

Data are means and ranges (n=6-8). ACE, angiotensin-converting enzyme; Ang, angiotensin. \* p<0.05, difference from 1.0 (Student's t-test for paired observations).

ACE was measured as a marker of membrane-bound enzymes. The purified membrane fraction contained  $20.3 \pm 5.0\%$  (mean and SD, n=5) of the total amount of ACE extracted from left ventricular tissue. ACE was enriched 5-6 fold in the purified membrane fraction (Table 5 and Fig. 6), and virtually all ACE activity in this fraction was dissolved by the addition of 1% Triton X-100, thereby confirming that ACE is a membrane-bound enzyme.

#### *Prorenin content of cardiac tissue*

Prorenin was measured with the 'routine' enzyme-kinetic assay at neutral pH, after it had been activated to renin. As described under 'Measurement of prorenin', three activation procedures were examined *i.e.*, 1) acid-treatment, 2) acid-treatment followed by treatment with plasmin at pH 7.4, and 3) treatment with trypsin-Sepharose at pH 7.4. Results are shown in Table 6.

After trypsin-Sepharose treatment of the cardiac tissue extracts, Ang I generation was not detectable during incubation with renin substrate. We were therefore unable to measure prorenin reliably in trypsin-treated tissue extracts.

After acid-treatment the Ang I generation during incubation with renin substrate was linear, and acid-treatment caused complete activation of added recombinant human prorenin without loss of prorenin (Table 6).

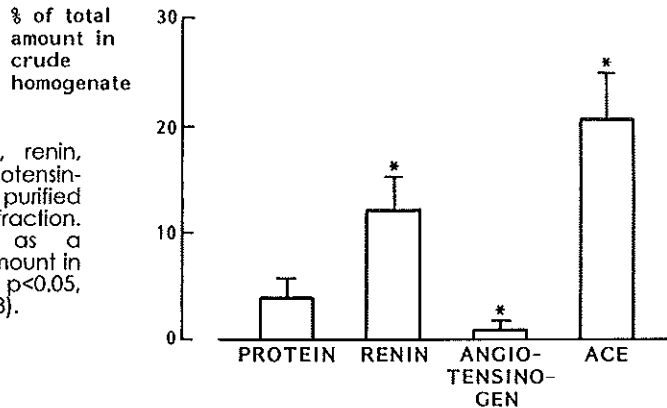
Acid-treatment followed by treatment with plasmin at pH 7.4 yielded an Ang I-generating activities that were  $19 \pm 7\%$  (mean and SD, n=12) lower than with acid-treatment alone (p<0.05; Table 6). Results for added recombinant human prorenin were  $14 \pm 6\%$  (n=4) lower with the combined acid- and plasmin-treatment than with acid-treatment alone. It appears therefore that plasmin had caused some destruction of prorenin or renin.

Both with the combined acid- and plasmin-treatment and the treatment with acid alone, more than 80% of the Ang I-generating activity was inhibited by the renin inhibitor Ro 42,5892 (Table 6).

As described in the previous section, renin in the acid-pretreated tissue extracts could be measured both with the antibody-trapping assay and the 'routine' enzyme-kinetic assay. Both methods gave similar results. In non-pretreated extracts reliable measurements of renin were only possible with the antibody-trapping assay but not



with the 'routine' assay. Results obtained with the antibody-trapping assay in non-pretreated extracts were not different from those in acid-pretreated extracts. It appears therefore that the cardiac tissue extracts contained predominantly renin and little or no prorenin (Table 3).



**Figure 6.** Total protein, renin, angiotensinogen and angiotensin-converting enzyme in a purified cardiac membrane fraction. Values are expressed as a percentage of the total amount in the crude homogenate. \*  $p < 0.05$ , compared to protein ( $n = 6-8$ ).

In porcine plasma, treatment with trypsin-Sepharose was the most effective way to activate prorenin (Table 6). This confirms earlier findings on prorenin activation in bovine and human plasma.<sup>38,43</sup> Acid-treatment and acid-treatment followed by treatment with plasmin at neutral pH did not lead to an increase in Ang I-generating activity of porcine plasma (Table 6), whereas these procedures are known to activate prorenin in bovine and human plasma.<sup>38,45</sup> Ang I-generating activity was linear over time both in the 'routine' and antibody-trapping enzyme-kinetic assays, and it was completely (>98%) inhibited with the renin-inhibitor Ro 42,5892 ( $10^{-5}$  mol/L). The concentration of prorenin in plasma was  $0.6 \pm 0.4$  times ( $n = 11$ ) the concentration of renin (Table 3).

#### *Angiotensinogen content of cardiac tissue*

The levels of angiotensinogen in cardiac tissue (expressed per g wet weight) were 10-26% of those in blood plasma (Table 3). Like the renin and angiotensin levels, the angiotensinogen levels are too high to be explained by trapped blood plasma (5-10% of wet weight).

All measurements of cardiac angiotensinogen were made both in the supernatants 1 and 2 and in the pellet fraction. The angiotensinogen data presented in Table 3 were calculated by summation of the results obtained in these tissue fractions. As with renin, the sum of the amounts of angiotensinogen in the three fractions was equal to the amount of angiotensinogen in the crude homogenate from which these fractions were prepared (Table 4).

**Table 6.** Effect of various procedures known to activate prorenin, on the angiotensin I-generating activity of left ventricular tissue fractions

Tissue fraction	No pretreatment			Acid-pretreatment			Plasmin-pretreatment			Trypsin-pretreatment		
	n	Activity (fmol Ang I/ min per mL)	Maximal inhibition by renin inhibitor (%)	Activity (fmol Ang I/ min per mL)	Maximal inhibition by renin inhibitor (%)	Activity (fmol Ang I/ min per mL)	Maximal inhibition by renin inhibitor (%)	Activity (fmol Ang I/ min per mL)	Maximal inhibition by renin inhibitor (%)	Activity (fmol Ang I/ min per mL)	Maximal inhibition by renin inhibitor (%)	
Plasma	3	54.0	98	42.4	85	30.8	92	90.0	99			
Human prorenin (hPR)	1	14.4	98	326	97	307	97	131	99			
Crude homogenate	4	ND		98.8	82	65.5	84	ND				
Crude homogenate + hPR	4	ND		491	89	387	91	ND				
Supernatant 1	4	ND		43.7	94	41.1	93	ND				
Supernatant 2	4	ND		14.1	92	10.3	91	ND				
Pellet	4	ND		8.9	83	8.6	92	ND				

Data are means. Ang, angiotensin; ND, not detectable due to high angiotensinase activity. Prorenin-activation procedures are described in more detail under 'Measurement of prorenin'.

Like renin and the angiotensins, angiotensinogen was higher in the atria than in the ventricles. Cardiac and plasma angiotensinogen levels did not correlate. Thirty hours after bilateral nephrectomy plasma angiotensinogen had increased (Fig. 3), and the left ventricular angiotensinogen levels in the nephrectomized animals were above the levels in non-nephrectomized animals.

The purified cardiac membrane fraction contained little or no angiotensinogen (Table 5 and Fig. 6), suggesting that angiotensinogen is not bound by cardiac membranes.

## DISCUSSION

In the present study renin, angiotensinogen, Ang I and Ang II were found to be present in cardiac tissue obtained from anesthetized, healthy pigs. Although the Ang I and II levels we measured in cardiac tissue were too high to be explained by trapped blood plasma, they were linearly correlated with the levels in the circulation. In fact, after bilateral nephrectomy, when Ang I and II in plasma were below the detection limit of the assay, their cardiac levels were also undetectable. It is not known whether the angiotensins in the heart are locally synthesized or derived from plasma. If one assumes that the angiotensins enter the tissue by simple diffusion from plasma, and that their localization in the tissue is restricted to the interstitial fluid compartment, their concentration in this compartment should not exceed that in blood plasma. In heart tissue the extracellular fluid compartment accounts for 20-25% of tissue weight. On the basis of the assumption that the angiotensins in the heart are restricted to this compartment it can be calculated from our results that the levels of Ang I and II would be 5-10 times higher in the extracellular fluid than in plasma. When the same assumption is applied to our results on angiotensinogen, the level of angiotensinogen in the extracellular fluid would be similar to that in plasma. Thus, it appears that the presence of angiotensins in the heart is not due to simple diffusion from plasma into the interstitium or, if it is due to diffusion, that their localization is not restricted to the extracellular fluid compartment.

With respect to angiotensinogen however, our results are compatible with the assumption that this component of the renin-angiotensin system enters the tissue by diffusion from plasma and is mainly present in extracellular fluid. The lack of a significant correlation between the angiotensinogen levels in cardiac tissue and in plasma, a finding that contrasts with the results on Ang I, Ang II and renin, may be attributed to the fact that the angiotensinogen results were within a narrow range as compared with the levels of the angiotensins and renin. Another explanation might be that cardiac angiotensinogen is, at least partly, locally produced in the heart. Angiotensinogen mRNA has been detected in cardiac tissue homogenates.<sup>106,142</sup>

Are the cardiac Ang I and II levels we measured the true *in-vivo* levels? Precautions were taken to prevent the *ex-vivo* generation and breakdown of angiotensins. Cardiac tissue samples were frozen in liquid nitrogen immediately after the heart had been taken out, and the frozen tissue pieces were homogenized in 0.1 mol/L HCl/80% ethanol. The possibility that some *ex-vivo* generation and breakdown of angiotensins had occurred in the short time that elapsed before the tissue was frozen cannot be excluded. Our observation however, that the Ang I and II levels were not significantly altered when cardiac tissue pieces were deliberately left for some time (30 min) at room temperature before they were frozen and extracted, indicate that, if there was *ex-vivo* generation of angiotensins, it could keep up, at least for some time, with their breakdown. Similar observations have been made on the *ex-vivo* generation of angiotensins in the rat kidney<sup>18</sup>. These observations lend support to the validity of our angiotensin measurements as a true measure of the *in-vivo* content of cardiac tissue.

The Ang II/I ratio in cardiac tissue was higher than in plasma. This finding is in agreement with a number of reports on the levels of Ang I and II in various tissues, including the heart.<sup>18,19,92,103</sup> We could not measure Ang II in cardiac tissue 30 hours after nephrectomy. This is somewhat at variance with observations in rats, where cardiac Ang II decreased by 50 percent in the first 24 hours and reached the detection limit after 48 hours.<sup>19</sup> Disappearance of cardiac angiotensins after nephrectomy may occur more slowly in the rat.

Reports on renin measurements in cardiac tissue are scarce, and it is not known whether it is really renin that was measured.<sup>70,74</sup> We used an enzyme-kinetic assay, which was based on the linear generation of Ang I at 37°C during incubation at neutral pH with an excess of renin substrate added to the tissue extracts. The specificity of the assay was assessed by adding a specific renin inhibitor in a high concentration to the incubates, and a correction was made for any Ang I-generating activity that could not be inhibited. Ang I breakdown during incubation was prevented by acid-pretreatment of the extracts or by rapid binding of the formed Ang I to Ang I antibody (antibody-trapping).<sup>46,129</sup>

Acid-pretreatment causes activation of prorenin, and this may have led to overestimation of the renin content of cardiac tissue and to underestimation of the prorenin content. Measurements in non-pretreated tissue extracts with the antibody-trapping assay however, yielded results that were not different from those obtained in the acid-pretreated extracts both with the antibody-trapping and 'routine' assays. Thus, the cardiac tissue extracts contained little or no prorenin. The porcine tissue homogenates contained trapped plasma, in which prorenin is known to be present. However, trapped plasma makes up for only 5-10% of cardiac tissue weight<sup>78</sup> and the prorenin concentration of plasma is too low to contribute significantly to our renin measurements in cardiac tissue. Activation of cardiac prorenin during the homogenization and extraction procedures should be considered. The results of the antibody-trapping assay in the non-acidified cardiac tissue extracts however, showed practically no activation of human prorenin that

was added to the tissue before homogenization and extraction. Also in rat adrenal tissue and in porcine reproductive organs renin and not prorenin could be detected.<sup>71,92</sup>

As with Ang I and II, the renin content of the heart was much too high to be explained by trapped blood plasma. Along the same lines of reasoning that were followed to explain the angiotensin results, it can be concluded from our renin measurements that the presence of renin in cardiac tissue is not due to simple diffusion from plasma or, if it is due to simple diffusion, that the localization of renin is not restricted to the extracellular fluid compartment. That renin in cardiac tissue is not restricted to extracellular fluid is indicated by our finding that at least 12% of the total amount of renin in left ventricular tissue could be recovered from a purified membrane fraction. ACE, which is known to be a membrane-bound enzyme, was enriched 5-6 fold in the membrane fraction, as compared to 2-3 fold enrichment of renin. The difference in enrichment between ACE and renin may be due, at least in part, to dissolution of renin during the centrifugation procedure. ACE has a carboxyl-terminal hydrophobic segment anchored to the cell membrane<sup>161</sup> and may be more tightly bound to the membrane than renin.

The direct and linear correlation of the renin levels in cardiac tissue with those in circulating plasma, together with the finding that the cardiac renin levels were practically zero 30 hours after bilateral nephrectomy, when renin in plasma is also practically zero, are strong evidence that by far the most of renin in cardiac tissue is derived from the circulation. Since most, if not all, renin in plasma is of renal origin, this interpretation of our results implies that cardiac renin is also of renal origin. This conclusion is supported by studies showing low or undetectable levels of renin mRNA in cardiac tissue from adult animals.<sup>54,85,108</sup> Uptake of renin in vascular tissue has been demonstrated in the early experiments by Loudon et al.<sup>109</sup> Studies in which bolus injections of radiolabeled renin were given to monkeys or rats showed that radioactivity accumulated not only in liver and kidney, but also, albeit in lower amounts, in heart and blood vessels.<sup>80,158</sup> Recently it was found that renin in human arteries was predominantly present in the endothelial layer, where it may have been taken up from the blood.<sup>121</sup> Using immunohistochemical techniques, it was shown that renin in the human left ventricle was only present in the endothelium of coronary blood vessels.<sup>55</sup> A renin-binding protein has been described in various organs, including the heart.<sup>168</sup> Recently it was proposed that prorenin becomes catalytically active after binding to a cell receptor.<sup>151</sup> Receptor binding of prorenin would unfold its prosegment and this renders the active site accessible to angiotensinogen. One may therefore speculate that membrane-bound renin is in fact 'activated' prorenin. However, there are no supporting data for this interesting proposal as yet.

We are aware of the fact that the present study is no proof of the cardiac production of Ang I at local tissue sites, although our results are certainly compatible with such local production. If local production is taking place, then it is clear from our data that most of it depends on kidney-derived renin that is taken

up from the circulation. The direct correlations we observed between the levels of renin and the angiotensins in the heart and their levels in plasma are in agreement with this conclusion.

The dependency of cardiac Ang I production on renin from plasma is also supported by previous work from our group, which showed that the overflow of intracardially produced Ang I into the coronary circulation is directly proportional to the level of circulating renin.<sup>31</sup> It is also in agreement with experiments in the isolated perfused rat Langendorff heart, where Ang I and II were only detected in the coronary effluent after renin had been added to the perfusion fluid.<sup>104</sup> In agreement with our observations in the pig, in a recent study in the rat the cardiac tissue levels of Ang I and II were also found to be reduced after nephrectomy.<sup>19</sup>

Taken together, the following sequence of events appears to emerge from our results. Renin, taken up by the heart from the circulation, acts in the tissue on angiotensinogen that is locally produced or derived from the circulation, to form Ang I. Ang I is then locally converted to Ang II by ACE. Our results suggest that plasma-derived angiotensinogen in the interstitial fluid is a source of cardiac angiotensins.

Enzymes other than ACE may be involved in Ang I-II conversion. In the human heart for instance a highly specific serine proteinase ("human heart chymase"), which converts Ang I to Ang II, has been demonstrated.<sup>177</sup> A similar enzyme may exist in porcine cardiac tissue.

The animals in our study were healthy 3-4 months old pigs. It is possible that during fetal development or under pathological conditions some of the genes of the renin-angiotensin system are switched on in the heart. Cultured myocytes and fibroblasts from neonatal rat hearts contain mRNA for renin and angiotensinogen and release both peptides into the medium.<sup>49</sup> Cardiac angiotensinogen mRNA is increased in post-infarction left ventricular remodeling,<sup>106</sup> and ACE gene expression is stimulated during pressure overload-induced ventricular hypertrophy.<sup>148</sup>

The Ang II levels (expressed per g tissue wet weight) we measured in cardiac tissue extracts were somewhat higher than the levels in plasma. In the *intact* tissue the levels might have been even higher in some localized compartment. High concentrations of Ang II are known to have chronotropic and inotropic effects<sup>99</sup> and may lead to arrhythmias<sup>181</sup> and myocyte necrosis.<sup>171</sup> A role of Ang II as a myocardial growth factor has also been suggested.<sup>145</sup> Finally, the therapeutic effect of ACE inhibitors in patients with congestive heart failure, an effect that may be independent, at least partly, of the effect of these compounds on blood pressure,<sup>107</sup> suggests that cardiac angiotensin production is involved in the development of heart failure.

*References are presented in the general reference list.*

## *Chapter 3*

**RENIN-ANGIOTENSIN SYSTEM  
COMPONENTS IN THE  
INTERSTITIAL FLUID OF THE  
ISOLATED PERFUSED RAT  
HEART.  
LOCAL PRODUCTION OF  
ANGIOTENSIN I.**

*Hypertension*. 1997; in press.

**SUMMARY**

*We used a modification of the isolated perfused rat heart, in which coronary effluent and interstitial transudate were separately collected, to investigate the uptake and clearance of exogenous renin, angiotensinogen and angiotensin I (Ang I) as well as the cardiac production of Ang I. The levels of these compounds in interstitial transudate were considered to be representative of the levels in the cardiac interstitial fluid. During perfusion with renin or angiotensinogen, the steady-state levels (mean  $\pm$  s.d.) in interstitial transudate were  $64 \pm 34\%$  ( $p < 0.05$  for difference from the arterial level,  $n=8$ ) and  $108 \pm 42\%$  ( $n=6$ ) of the arterial level, respectively, and the levels in coronary effluent were not significantly different from those in interstitial transudate. Ang I was not detectable in interstitial transudate during perfusion with Tyrode's buffer or angiotensinogen. It was very low in interstitial transudate during perfusion with renin, and rose to much higher levels during combined renin/angiotensinogen perfusion. The total production rate of Ang I 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 Ang I present in coronary effluent and the net ejection rate of Ang I via coronary effluent were, respectively,  $4.6 \pm 2.2$  and  $2.8 \pm 1.3$  ( $p < 0.01$  and  $p < 0.05$  for difference from 1.0,  $n=6$ ) times higher than could be explained by Ang I formation in the fluid phase of the intravascular compartment. Ang I from the interstitial fluid contributed little to the Ang I in the intravascular fluid, and vice versa. These data reveal two tissue sites of Ang I 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 Ang I into coronary effluent and interstitial transudate occurred independently of blood-derived renin or angiotensinogen.*

**ACKNOWLEDGEMENTS**

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## 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.<sup>138,146,181,199</sup> 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.<sup>19,32</sup> In addition, the tissue concentrations of Ang I and II are too high to be explained simply by passive diffusion out of the blood and distribution into the interstitial fluid.<sup>32</sup>

Perfusion of the isolated rat Langendorff heart with Ang I leads to the appearance of Ang II in the coronary effluent,<sup>104,149</sup> and perfusion with renin leads to the appearance of both Ang I and II.<sup>104</sup> Part of the Ang I in the coronary venous blood appears to originate from local production at cardiac tissue sites,<sup>31</sup> 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 Ang I into the coronary circulation is directly proportional to the level of renin activity in circulating plasma,<sup>31</sup> and that the cardiac tissue levels of renin, Ang I and Ang II are undetectably low 30 hours after bilateral nephrectomy, when plasma renin activity was practically zero.<sup>32</sup> In the rat, the cardiac tissue levels of Ang I and II were also lowered by nephrectomy, although small quantities of Ang II are still detectable both in cardiac tissue and in blood.<sup>19</sup> These observations indicate that the presence of Ang I 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 Ang I and II production are also unknown. A unique model to address these issues is a modified version of the Langendorff heart,<sup>35,86,162</sup> 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 Ang I.

## MATERIALS AND METHODS

### *Chemicals*

[Ile<sup>5</sup>]-Ang-(1-10) decapeptide (Ang I) was obtained from Bachem, <sup>51</sup>Cr-EDTA and <sup>125</sup>I-labeled human serum albumin (HSA) from Amersham, bovine serum albumin (BSA) from Sigma, 1,10-Phenanthroline from Merck, and sodium pentobarbital from Apharma. The angiotensin II type 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.

*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 were used for porcine renin measurements. Under the conditions of our experiments, sheep angiotensinogen yielded higher quantities of Ang I 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, wt/vol) with a Polytron (PT10/35, Kinematica) 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.<sup>32</sup> 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 20,000g for 20 min at 4°C. The supernatant (semipurified renin) was stored at -80°C. The renin concentration was 125 pmol Ang I/min per mL in the rat renin preparation, and 600 pmol Ang I/min per mL in the porcine renin preparation, as assessed by incubation with rat and porcine angiotensinogen, respectively.<sup>32</sup>

Angiotensinogen was prepared as described before.<sup>159</sup> 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.

*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' 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<sub>2</sub>, 20 mmol/L NaHCO<sub>3</sub>, 0.4 mmol/L NaH<sub>2</sub>PO<sub>4</sub>, 1.0 mmol/L MgCl<sub>2</sub>, and 10 mmol/L D-glucose, pH 7.4) until contractions stopped, and prepared for Langendorff perfusion. Continuously carbogen-gassed (95% O<sub>2</sub>/5% CO<sub>2</sub>) 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 min, the hearts were stabilized for 30 min.

With this modified Langendorff heart preparation it is possible to collect separately coronary effluent and interstitial transudate.<sup>35,86,162</sup> 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 greater than 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.

*Checking for leakage of perfusate into the interstitial transudate*

The hearts were perfused with red blood cells to check for leakage of perfusate into the 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  $5 \times 10^9$  cells/mL, and infused via a T-connection, into the cannulated aorta with a Harvard 22 pump 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.

*Measurements of intravascular and interstitial fluid volumes*

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

One-minute CE samples and individual IST drops (approximately 50  $\mu\text{L}$ ) were collected during the infusion period, and the hearts were removed immediately after the perfusion had been switched off. Radioactivity levels (cpm) of CE, IST and the whole heart were measured with a Minaxi 5000 multiple channel gamma-counter (Packard Instruments). Intravascular and extracellular fluid volumes (mL/g heart wet weight) were considered to be equal to the distribution volumes of  $^{125}\text{I}$ -HSA and  $^{51}\text{Cr}$ -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 that is present in the coronary vascular bed and the right ventricle. The interstitial fluid volume (mL/g) was calculated by subtracting the intravascular fluid volume from the extracellular fluid volume.

*Perfusions with renin-angiotensin system components and collection of coronary effluent and interstitial transudate*

The Langendorff hearts were perfused with Tyrode's buffer via the cannulated aorta. The buffer contained the angiotensin II type 1 receptor antagonist, losartan, in a concentration of  $10^{-6}$  mol/L. This concentration is sufficient to prevent Ang II-mediated vasoconstriction.<sup>14</sup> After a 30-minute 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-minute (4-8 mL) or 4- to 5-minute (16-40 mL) samples of CE were collected into BSA-coated 10- or 50-mL polystyrene tubes, and 1-minute (approximately 50  $\mu\text{L}$ ) or 9- to 10-minute (approximately 450-500  $\mu\text{L}$ ) samples of IST were collected into BSA-coated 1.5-mL Eppendorf cups. The Eppendorf cups and polystyrene tubes used to collect samples for Ang I measurement contained a mixture of inhibitors - 5 or 25  $\mu\text{L}$  in the Eppendorf cups (for the 1-minute and 9- to 10-minute IST samples) and 250 or 2500  $\mu\text{L}$  (for the 1-minute and 9- to 10-minute CE samples) in the polystyrene tubes - to prevent *ex-vivo* formation of Ang I, conversion of Ang I to Ang

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II and degradation of Ang I. 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.<sup>32</sup> Remikiren is an inhibitor of human renin ( $IC_{50}$   $7 \times 10^{-10}$  mol/L). It also inhibits porcine renin ( $IC_{50}$   $5 \times 10^{-8}$  mol/L).<sup>32</sup> 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 Ang I measurement were frozen at -80°C. Samples for the measurement of renin and angiotensinogen were frozen at -20°C.

#### *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 40 min. The 30-minute 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-minute collections of CE and IST were used for measurement of Ang I. Each 9-minute collection was followed by a 1-minute collection for measurement of renin and angiotensinogen.

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

Langendorff hearts were perfused with porcine renin, diluted 1:8 with Tyrode's buffer, with undiluted porcine angiotensinogen, or with Ang I, 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 (Ang I). After the infusion had been switched off, the hearts were either rapidly frozen in liquid nitrogen, or subjected to a 10-minute washout period.

One-minute samples of CE and individual drops of IST were collected during the infusion and washout periods for measurement of renin, angiotensinogen or Ang I. 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.

#### *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 Ang I 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 Ang I, 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 was infused into the perfusion system for 40 min, followed by a 10-minute washout. Nine-minute collections of CE and IST were used for measurement of Ang I. Each 9-minute collection was followed by a 1-minute 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 Ang I 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-minute washout. The renin and angiotensinogen solutions were kept at 0-4°C with ice until they reached the cannulated aorta. Ten-minute collections of CE and IST were used for measurement of Ang I. Each 10-minute collection period was followed by a 5-minute collection for measurement of renin and angiotensinogen.

#### *Biochemical measurements*

*Renin.* The concentration of renin in CE, IST or cardiac tissue homogenate was determined by measuring the rate of Ang I 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.<sup>32</sup> Renin concentration was defined as the maximal Ang I 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, wt/vol) in 0.01 mol/L phosphate buffer, pH 7.4, containing 0.15 mol/L NaCl, with a Polytron PT10/35. Homogenate that was 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.<sup>32,38</sup> 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 20,000g 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 of 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  $\mu$ L undiluted CE or 100  $\mu$ L IST diluted 1:3 in 0.01 mol/L phosphate buffer, pH 7.4, containing 0.15 mol/L NaCl, or 100  $\mu$ L acid-pretreated tissue homogenate, (2) 100  $\mu$ L of 0.01 mol/L phosphate buffer, pH 7.4, containing 0.15 mol/L NaCl, (3) 200  $\mu$ L angiotensinogen, and (4) 14  $\mu$ L 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 Ang I generation was linear during this period. Ang I was measured with a sensitive radioimmunoassay.<sup>1</sup>  $V_{\max}$  was calculated according to the equation:  $V_{\max} = V \times (K_m + [S])/[S]$ , in which  $V$  is the measured Ang I 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-minute incubations of renin with serial dilutions of angiotensinogen and by constructing Lineweaver-Burk plots of the measured Ang I 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.<sup>68,76</sup>  $K_m$  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 Ang I/min per mL in CE, 40 fmol/min per mL in IST and 40 fmol Ang I/min per g in cardiac tissue. For porcine renin (incubated with either porcine or sheep angiotensinogen), it was approximately 5 fmol Ang I/min per mL in CE, 25 fmol Ang I/min per mL in IST and 25 fmol Ang I/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 Ang I 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.<sup>32</sup> For measurement of angiotensinogen in cardiac tissue, the frozen hearts were rapidly minced and homogenized as described above under 'Renin', but the dialysis step 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 Ang I formation was completed within 1 hour.

The lowest levels 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 Ang I concentration of CE and IST, collected during Ang I perfusion, was measured directly with a sensitive Ang I radioimmunoassay.<sup>1</sup> Measurements were made in 50 µL undiluted CE and in 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 Ang I added to CE or IST was better than 95%. The lowest levels of Ang I that could be measured with the direct radioimmunoassay were 15 fmol/mL in CE and 40 fmol/mL in IST.

The Ang I 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.<sup>1,31,32</sup> For measurement of Ang I, the frozen hearts were minced and homogenized (1:10, wt/vol) in an iced solution of 0.1 mol/L HCl/80% ethanol. Homogenates were centrifuged at 20,000g 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 20,000g. 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 (C18, Waters), and the concentrated extracts were subjected to HPLC followed by radioimmunoassay. <sup>125</sup>I-labeled Ang I 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 Ang I results were corrected for incomplete recovery. The lowest levels of Ang I that could be measured with the Ang I 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.

*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 Ang I and *endogenous* Ang I formed in the Langendorff preparation.

The production of *endogenous* Ang I present in ISF (fmol per min) was calculated as follows:

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

in which  $[\text{Ang I}_{\text{IST}}]$  is the steady state concentration of *endogenous* Ang I (fmol/mL) in IST.

The clearance of Ang I<sub>ISF</sub> was calculated according to the equation:

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

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

The production rate of Ang I<sub>ISF</sub> (fmol per min), predicted on the basis of the renin-angiotensinogen reaction in the fluid phase of the interstitial compartment, was derived from the following equation:

$$\text{Ang I}_{\text{ISF}} \text{ production in the fluid phase} = \text{AGA}_{\text{IST}} \times \text{interstitial fluid volume} \quad (3)$$

in which AGA<sub>IST</sub> is the Ang I-generating activity (fmol Ang I/min per mL) of IST caused by the renin-angiotensinogen reaction in the fluid phase of the interstitial compartment. AGA<sub>IST</sub> was derived from the levels of renin and angiotensinogen in IST and from the Michaelis-Menten constant according to the equation:

$$\text{AGA}_{\text{IST}} = [\text{R}_{\text{IST}}] \times [\text{Aog}_{\text{IST}}] / (K_m + [\text{Aog}_{\text{IST}}]) \quad (4)$$

in which  $[\text{R}_{\text{IST}}]$  is the concentration of renin (fmol Ang I/min per mL) measured in IST,  $[\text{Aog}_{\text{IST}}]$  is the concentration of angiotensinogen (pmol/mL) in IST, and  $K_m$  (pmol/mL) is the Michaelis-Menten constant (see 'Biochemical Measurements' above).

Ang I<sub>ISF</sub> production calculated according to equation (1) is referred to as 'measured' Ang I<sub>ISF</sub> production, because the independent variables in this equation were measured experimentally. Ang I<sub>ISF</sub> production, predicted by equation (3), is referred to as 'predicted' Ang I<sub>ISF</sub> production.

The production rate of *endogenous* Ang I<sub>CE</sub> (fmol per min), was calculated as follows:

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

in which  $[\text{Ang I}_{\text{CE}}]$  is the steady state concentration of *endogenous* Ang I (fmol/mL) in CE, Q is the perfusate flow (mL per min), and ER is the extraction ratio of Ang I. ER was calculated as the fraction of *exogenous* Ang I 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 Ang I perfusion experiments. This equation may overestimate Ang I<sub>CE</sub> production, because it accounts for the extraction of arterially delivered Ang I, whereas *endogenous* Ang I is added to

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the perfusion fluid during its passage through the coronary vascular bed.

The net ejection rate of *endogenous* Ang I (fmol per min) via CE was calculated as follows:

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

The production rate of Ang I<sub>CE</sub> (fmol per min), predicted on the basis the renin-angiotensinogen reaction in the fluid phase of the intravascular compartment, was derived from the following equation:

$$\text{Ang I}_{\text{CE}} \text{ production in the fluid phase} = \text{AG}_{\text{ACE}} \times (\text{intravascular fluid volume} + 0.1 \text{ mL}) \quad (7)$$

in which  $\text{AG}_{\text{ACE}}$  is the Ang I-generating activity (fmol Ang I/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 <sup>125</sup>I-HSA, and 0.1 mL is the dead space of the pulmonary artery cannula.  $\text{AG}_{\text{ACE}}$  was derived from the levels of renin and angiotensinogen in CE and from the Michaelis-Menten constant according to the equation:

$$\text{AG}_{\text{ACE}} = [\text{R}_{\text{CE}}] \times [\text{A}_{\text{ogCE}}] / (K_m + [\text{A}_{\text{ogCE}}]) \quad (8)$$

in which  $[\text{R}_{\text{CE}}]$  is the concentration of renin (fmol Ang I/min per mL) measured in CE,  $[\text{A}_{\text{ogCE}}]$  is the concentration of angiotensinogen (pmol/mL) in CE, and  $K_m$  (pmol/mL) is the Michaelis-Menten constant (see 'Biochemical Measurements' above).

Ang I<sub>CE</sub> production calculated according to equation (5) is referred to as 'measured' Ang I<sub>CE</sub> production, because the independent variables in this equation were measured experimentally. Ang I<sub>CE</sub> production, predicted by equation (7), is referred to as 'predicted' Ang I<sub>CE</sub> production.

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

In assumptions (1) and (2) above, the ISF 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<sup>194</sup> and by studies of the release of cardiac enzymes and metabolites into IST<sup>60,86,162,194</sup> 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 Langendorff model as we used here.<sup>35</sup> 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 'Results').

Assumption (3) implies that in the ISF the half life of *endogenous* Ang I is the same as the half life of *exogenous* Ang I. The half life of Ang I in ISF 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 ISF these mechanisms act on *endogenous* Ang I in the same way as on *exogenous* Ang I.



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

*Statistical analysis*

Data are expressed as means ± SD, 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 Ang I 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.

**RESULTS**

*Leakage of perfusate into the interstitial transudate*

During 10-minute and 20-minute 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)
CE (red cells), 10 <sup>12</sup> /L	0.11 ± 0.5	0.17 ± 0.08
IST (red cells), 10 <sup>12</sup> /L	0.001 ± 0.001	0.002 ± 0.004
[IST/CE]x100%	0.9 ± 0.8	1.1 ± 1.6

Values are means ± SD.

*Intravascular and interstitial fluid volumes*

<sup>125</sup>I-HSA, when infused into the rat Langendorff heart, entered the IST and reached levels comparable to those in CE after approximately 10 min. After 1-minute perfusion with <sup>125</sup>I-HSA the level in IST was approximately 10% of the

level in CE. We used the  $^{125}\text{I}$ -HSA level in CE, collected during the first min 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.<sup>6</sup>

$^{51}\text{Cr}$ -EDTA, when infused into the perfusion system, reached levels in IST comparable to those in CE within 5 min of infusion. No further increase was observed when the infusion was prolonged. We used  $^{51}\text{Cr}$ -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.

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

Cardiac fluid or cardiac tissue	$^{125}\text{I}$ -HSA ( $n=4$ )	$^{51}\text{Cr}$ -EDTA ( $n=6$ )
CE, cpm/mL	14600 $\pm$ 5490	7650 $\pm$ 1790
IST, cpm/mL	1790 $\pm$ 898	6720 $\pm$ 2360
Cardiac tissue, cpm/g	5500 $\pm$ 2150	5090 $\pm$ 1110
Volume of distribution, mL/g cardiac wet weight	0.38 $\pm$ 0.08	0.61 $\pm$ 0.06

Values are means  $\pm$  SD.  $^{125}\text{I}$ -HSA was infused into the perfusion system for 1 min, and  $^{51}\text{Cr}$ -EDTA for 10 min. The intravascular space consists of the volume of fluid in the coronary vascular bed and the right ventricle.

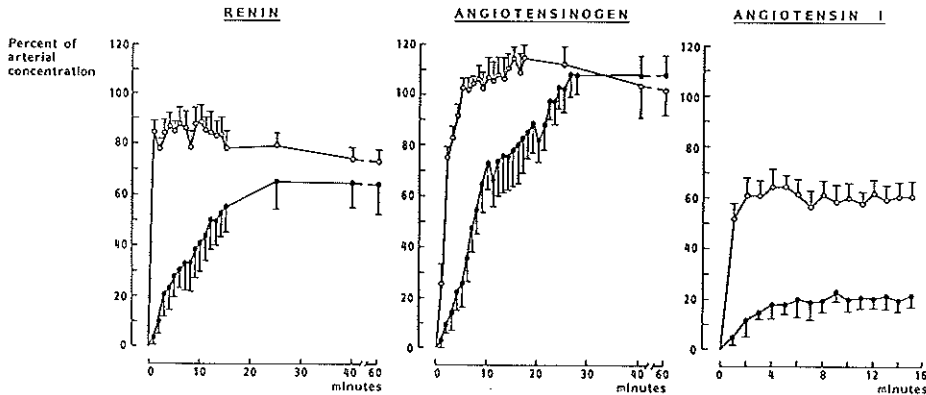
#### *Release of endogenous renin, angiotensinogen and angiotensin I*

During 40-minute perfusion with Tyrode's buffer immediately after preparation of the Langendorff hearts ( $n=4$ ), renin and Ang I 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  $4 \pm 1$  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.

#### *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. 1), and it was not significantly different from that in CE (Table 3, Fig. 1). After discontinuation of the renin perfusion, renin disappeared from CE in a biphasic pattern (Fig. 2). The rapid first phase had a  $t_{1/2}$  of  $0.42 \pm 0.03$  min, and the slow second phase had a  $t_{1/2}$  of  $3.3 \pm 0.8$  min. Renin disappeared from IST in a

monophasic way with a  $t_{1/2}$  of  $3.9 \pm 1.4$  min.



**Figure 1.** 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 (solid circles) during perfusion of the modified Langendorff heart with these renin-angiotensin system components. Values (means and SEM) are expressed as percentage of the arterial level.

The cardiac tissue level of renin (per g tissue), immediately after the renin perfusion had been switched off, was  $56 \pm 9\%$  ( $n=5$ ) 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  $0.61 \times 78 = 48\%$  (mean) of the arterial level, assuming that renin in the heart is located in the extracellular fluid compartment and that 1 g tissue corresponds with a volume of 1 mL.<sup>32,91</sup> 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 ( $n=4$ ).

**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. 1). After discontinuation of the angiotensinogen perfusion, angiotensinogen disappeared rapidly ( $t_{1/2}$   $0.54 \pm 0.32$  min) from CE in a monophasic way (Fig. 2), 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  $2.9 \pm 1.0$  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

Cardiac fluid	Renin (n=8)	Angiotensinogen (n=6)	Angiotensin I (n=5)
CE	78.3 ± 14.6	102.9 ± 17.3	59.9 ± 10.3
IST	63.9 ± 34.4	107.8 ± 41.7	16.8 ± 4.6

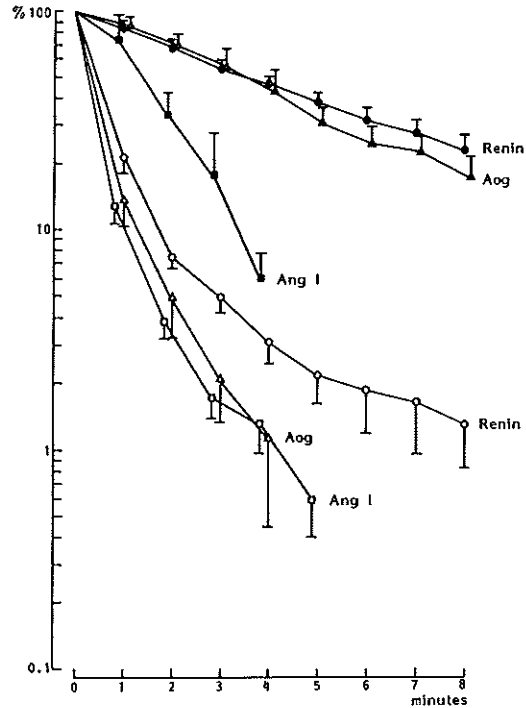
Values are means ± SD. Renin, angiotensinogen and angiotensin I (Ang I) levels in coronary effluent (CE) and interstitial transudate (IST) are expressed as a percentage of the arterial level, which was calculated as the rate of renin, angiotensinogen and angiotensin I infusion into the perfusion system divided by the perfusate flow. Arterial renin, angiotensinogen and Ang I were  $1.1 \pm 0.2$  pmol Ang I/min per mL,  $11.4 \pm 5.3$  pmol/mL and  $6.4 \pm 2.6$  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 Ang I were averages of the levels after 13, 14 and 15 min of Ang I perfusion (see Fig. 1). The renin and angiotensinogen levels were not significantly different between IST and CE. Ang I 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  $53 \pm 18\%$  ( $n=5$ ) 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  $0.61 \times 100 = 61\%$  (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.<sup>32,91</sup> 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 Ang I ( $n=5$ ), the steady-state Ang I 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 Ang I was removed from the perfusate by the heart. A steady-state level of Ang I in IST was reached within 5 min (Fig. 1). It was 17% of the arterial level, which was significantly lower than the level in CE ( $p < 0.01$ ). After discontinuation of the Ang I perfusion, Ang I disappeared rapidly from CE in a monophasic way (Fig. 2), with a  $t_{1/2} < 0.5$  min. Ang I disappeared from IST, also in a monophasic way, with a  $t_{1/2}$  of  $0.9 \pm 0.6$  min. Ang I 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 Ang I from IST that was observed in the Ang I perfusion experiments, was not caused by the presence of peptidases in IST, but by the rapid removal of Ang I from the cardiac interstitial fluid.

**Figure 2.** Washout of porcine renin (n=8), porcine angiotensinogen (Aog, n=6) and angiotensin I (n=5) from coronary effluent (open symbols) and interstitial transudate (solid symbols) of the modified Langendorff heart after its perfusion with these renin-angiotensin system components. Values (means and SEM) are expressed as a percentage of the level immediately before discontinuation of infusion of the renin-angiotensin system components into the perfusion system.

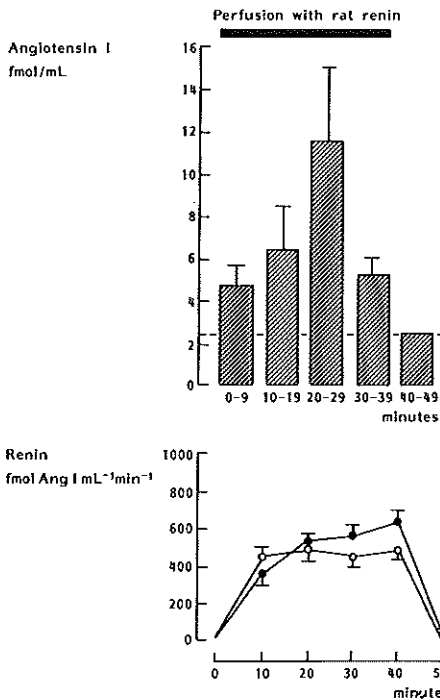


The cardiac tissue level of Ang I (per g tissue), immediately after the Ang I perfusion had been switched off, was less than 5% (n=3) of the arterial level (per mL perfusate). The Ang I levels in CE and IST were 60% and 17% of the arterial level, respectively, whereas in a different series of experiments the intravascular and interstitial fluid spaces were 0.38 mL/g and 0.23 mL/g, respectively (see Table 2). Thus, the cardiac tissue level of Ang I is expected to be  $0.38 \times 60 + 0.23 \times 17 = 27\%$  of the arterial level, assuming that Ang I is located in the extracellular fluid compartment and that 1 g of tissue corresponds with a volume of 1 mL.<sup>32,91</sup> The difference from the measured result might be related to the rapid degradation of Ang I in the vascular compartment. It is possible that the short period between the moment the Ang I 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 Ang I. Ang I was not detectable in control hearts perfused with Tyrode's buffer (n=3).

*Cardiac production of angiotensin I*

Results obtained during perfusion with rat renin ( $n=4$ ), in the absence of exogenous angiotensinogen, are shown in Fig. 3. Ang I was not detectable in CE and IST samples collected before renin was infused into the perfusion system. In samples collected during renin perfusion, Ang I remained undetectable in CE, whereas in IST Ang I rose to levels above the detection limit of the assay. After an initial increase, Ang I 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.

Ang I 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 Ang I production by endogenous (rat) renin or renin-like enzymes (cathepsins).



**Figure 3.** Top, Angiotensin I levels in interstitial transudate during 40-minute perfusion of the modified Langendorff heart with rat renin followed by a 10-minute washout ( $n=4$ ). Samples were collected over 9 min. Angiotensin I in simultaneously collected samples of coronary effluent was below the detection limit of the assay. Values are means and SEM.

Ang I was easily detectable in samples of CE and IST collected during perfusion with porcine renin combined with porcine or sheep angiotensinogen (Fig. 4 and Tables 4 and 5). During combined renin/angiotensinogen perfusion, the level of Ang I in CE had reached its steady state maximum in the 30-40-minute and 45-55-minute samples, but not in the 0-10-minute and 15-25-minute samples. This

contrasts with the level of renin and angiotensinogen, which had reached their steady-state maximum in CE within less than 5 min (see Fig. 1). The slow increase of Ang I 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 Ang I in CE. Also in IST, Ang I had reached its steady-state maximum in the 30-40-minute and 45-55-minute samples, but not in the 0-10-minute and 15-25-minute samples; this corresponds well with the time required for renin and angiotensinogen to reach their steady state maximum in IST (see Fig. 1).

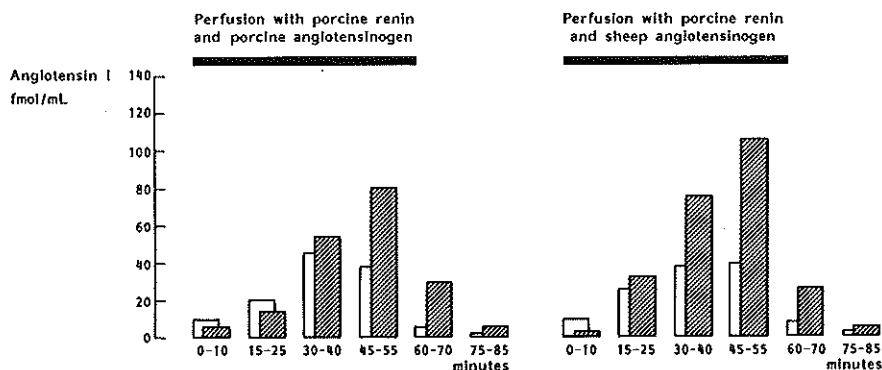
**Table 4.** Levels of renin, angiotensinogen and angiotensin I in coronary effluent and interstitial transudate during perfusion of the modified Langendorff heart with porcine renin and porcine angiotensinogen

RAS component	Sample collection period	
	30-40 min (n=6)	45-55 min (n=6)
Renin, (fmol Ang I/min)/mL		
CE	3150 ± 540	3100 ± 560
IST	2090 ± 360	2160 ± 370
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 ± SD.

\* Significantly different from 1.00 ( $p < 0.05$ ).

Figs. 5 and 6 give the measured  $\text{Ang I}_{\text{ISF}}$  and  $\text{Ang I}_{\text{CE}}$  production rates as derived from equations (1) and (5) (see 'Calculations' above), for the 30-40-minute and 45-55-minute 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.



**Figure 4.** Angiotensin I levels in coronary effluent (open bars) and interstitial transudate (hatched bars) during 60-minute perfusion of the modified Langendorff heart with porcine renin, combined with porcine angiotensinogen (left panel) or sheep angiotensinogen (right panel), followed by a 30-minute washout. Results of two individual experiments are shown.

**Table 5.** Renin, angiotensinogen and angiotensin I levels 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 Ang I/min)/mL		
CE	1875 (1441-2444)	1948 (1618-2251)
IST	2225 (1032-4151)	2075 (1096-3512)
IST/CE	1.11 (0.59-1.70)	1.04 (0.59-1.56)
Angiotensinogen, pmol/mL		
CE	7.0 (5.7-9.3)	6.5 (5.9-7.7)
IST	6.1 (5.7-6.8)	6.5 (5.9-7.5)
IST/CE	0.91 (0.73-1.00)	0.99 (0.97-1.00)
Angiotensin I, fmol/mL		
CE	90 (38-150)	100 (38-160)
IST	186 (77-370)	236 (126-380)
IST/CE	1.96 (1.38-2.45)*	2.56 (2.00-3.29)*

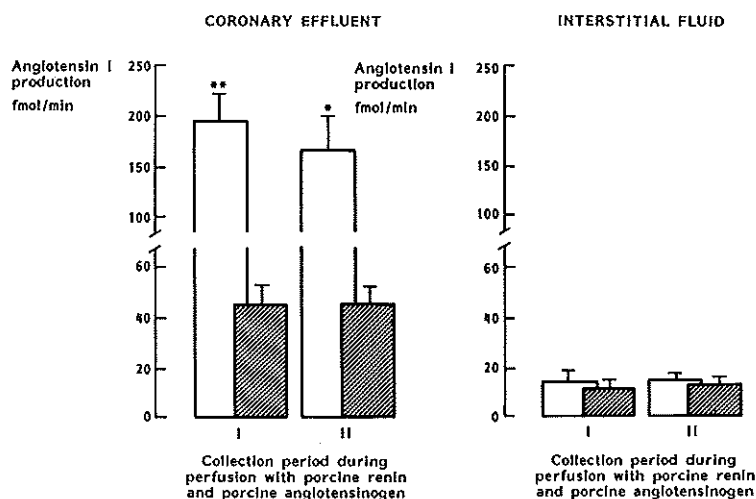
Values are means (range).

\* Significantly different from 1.00 ( $p < 0.05$ ).

As shown in Figs. 5 and 6, the production of Ang I<sub>ISF</sub> could be accounted for by the renin-angiotensinogen reaction in the fluid phase of the interstitial compartment. The production of Ang I<sub>CE</sub>, 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 Ang I<sub>CE</sub> production rate was  $4.6 \pm 2.2$  times (average of the two samples collected at 30-40 min and 45-55-minute 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.

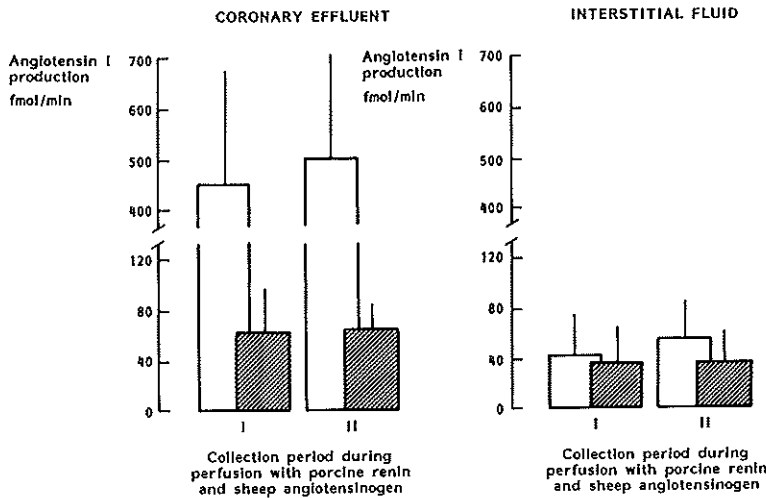


**Figure 5.** Left, Measured total production rate (open bars) vs predicted production rate (hatched bars) of angiotensin I present in coronary effluent during perfusion of the modified Langendorff heart with porcine renin combined with porcine angiotensinogen (means and SEM, n=6). Predicted production 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 (\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ).

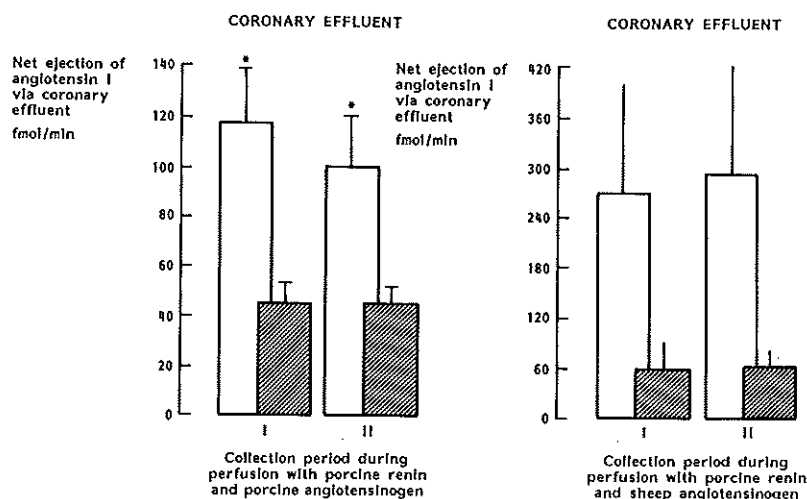
Right, Measured total production rate (open bars) vs predicted production rate (hatched bars) of angiotensin I in interstitial fluid during perfusion of the modified Langendorff heart with porcine renin combined with porcine angiotensinogen (means and SEM, n=6). Predicted production rate was calculated on the basis of the renin-angiotensinogen reaction in the fluid phase of the interstitial compartment. Measured and predicted production rates were not different.

The measured production rate of Ang I<sub>CE</sub> may be somewhat higher than the true production rate, because the measured production rate accounts for the extraction of arterially delivered Ang I, whereas, during combined renin/angiotensinogen perfusion, Ang I is formed in the perfusate during its passage through the coronary vascular bed. Fig. 7, therefore, gives the net ejection rate of Ang I via CE, as derived from equation (6), which is less than the total production of Ang I<sub>CE</sub>. It can be seen, that even with this underestimation of the true Ang I<sub>CE</sub> 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 Ang I was  $2.8 \pm 1.3$

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. Therefore, it appears that part of the Ang I in CE is produced at tissue sites. The Ang I<sub>ISF</sub> production rate was much lower than the Ang I<sub>CE</sub> production rate. The contribution of Ang I from the interstitial fluid to the Ang I level in CE was therefore minimal.



**Figure 6.** Left, 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 plus 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, 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 plus 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. The measured and predicted production rates were similar.



**Figure 7.** Left, 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 SEM,  $n=6$ ). 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, 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 plus 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 the predicted production in all three experiments.

## 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 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 IST, keeps dripping from the apex, and we assumed the levels of renin, angiotensinogen and Ang I 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.<sup>35</sup> Creatine kinase, lactate dehydrogenase and malate dehydrogenase activity levels are about 100 times higher in IST than in CE.<sup>194</sup> 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.<sup>35</sup> 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, Wiene and Kammermeier<sup>194</sup> found that, during perfusion with dextran T-70 (molecular weight (MW) 70 kD), the dextran level in IST was equal to that in CE, and the same observation was made for albumin (MW 70 kD) during perfusion with 0.01% bovine serum albumin. In the experimental setup Wiene and Kammermeier<sup>194</sup> used, 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 Ang I 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 Ang I from the IST, while it was still on the cardiac surface (as observed after the discontinuation of Ang I perfusion), reflects the rapid disappearance of Ang I 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 Ang I are probably similar in the two fluid compartments, as are the levels of *endogenous* Ang I formed in the Langendorff preparation. This is indicated by our finding that the Ang I level measured in IST in the combined renin/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 (MW 48 kD) and angiotensinogen (MW 65 kD) into the IST was slow as compared with the release of Ang I into the IST during Ang I perfusion. This is probably related to the much smaller molecular size of Ang I (MW 1.297 kD). Similar observations have been published with respect to dextran-T70 (MW 70 kD) and disulfin blue (MW 0.566 kD).<sup>194</sup> It should be noted that, in order to obtain sufficient amounts of IST for Ang I 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.<sup>147</sup> 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,<sup>32</sup> and binding of renin to vascular membranes has been reported.<sup>21</sup> 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.

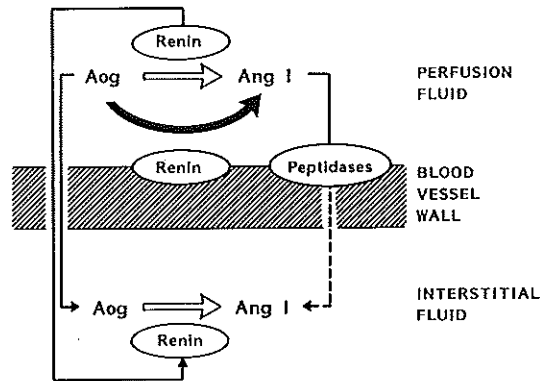
In our perfusion experiments, approximately 40% of the infused Ang I was removed from the perfusate by the heart. This is in accordance with studies in intact pigs, which demonstrated that, in the coronary vascular bed, 45-50% of the arterially delivered Ang I was removed from the circulation,<sup>30</sup> most likely by peptidases on the endothelial surface.

An important aspect of the present study is the evidence it provides for local Ang I formation by the heart, outside the perfusate compartment. According to our calculations, the level of Ang I in IST during combined renin/angiotensinogen perfusion can be explained by the renin-angiotensinogen reaction in the fluid phase of the interstitial compartment. In contrast, most of the Ang I in CE was not formed by the renin-angiotensinogen reaction in the fluid phase of the intravascular compartment. Ang I from the intravascular compartment contributed little to the Ang I level in IST, and Ang I from the cardiac interstitial fluid contributed little to the Ang I level in CE. Most of the Ang I in CE appears to be formed at tissue sites, and the direction of Ang I 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.<sup>167</sup> The finding that the Ang I level in CE was not higher than in IST does not argue against this conclusion, because Ang I, 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 Ang I concentration in coronary venous plasma was too high to be explained by the plasma renin activity.<sup>31</sup> With the use of biochemical and immunohistochemical methods, renin has been demonstrated in the endothelial cells of normal human gastroepiploic arteries.<sup>121</sup> 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. 8 presents a hypothetical scheme that is compatible with our observations. It shows two sites of Ang I formation outside the perfusion fluid, namely the vascular surface and the interstitial fluid. Some renin and also the peptidases involved in Ang I metabolism are bound to the vascular endothelial cells. Angiotensinogen and Ang I are present in the fluid phase. Most of the Ang I

present in coronary effluent is produced by endothelium-bound renin. Most of the Ang I present in the interstitial fluid compartment is produced by renin present in the fluid phase of this compartment.

**Figure 8.** 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 local formation of Ang I at cardiac tissue sites depends on arterially delivered renin. In our perfusion experiments, Ang I 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 Ang I, unless renin had been added to the perfusion fluid.<sup>104</sup> Conclusive evidence that cardiac Ang I production depends on blood-derived renin comes from our studies of the effect of nephrectomy on the cardiac tissue levels of Ang I and II in pigs.<sup>32</sup> Both peptides became undetectable in cardiac tissue after bilateral nephrectomy. In the present study, Ang I levels in IST and CE were much higher during combined renin/angiotensinogen perfusion than during perfusion with renin alone. It is, therefore, reasonable to conclude that Ang I production by the heart not only depends on arterially delivered renin but also on arterially delivered angiotensinogen.

The production of Ang I at cardiac tissue sites may, via conversion of Ang I to Ang II, lead to local concentrations of Ang II that are higher than can be obtained with arterially delivered Ang II. That the local formation of Ang I 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.<sup>182</sup> 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 Ang I and II. In addition to its short-term effect on cardiac contractility, Ang II also has long-term effects; it promotes left ventricular hypertrophy and the remodeling that occurs after myocardial infarction.<sup>138,146,199</sup> Ang I produced locally in the heart may, after its conversion to Ang II, participate in these processes. This

### *Local production of angiotensin I*

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 Ang II but also by a decrease in the conversion of locally formed Ang I.

*References are presented in the general reference list.*





## *Chapter 4*

**ANGIOTENSIN II TYPE 1 (AT<sub>1</sub>)  
RECEPTOR-MEDIATED  
ACCUMULATION OF  
ANGIOTENSIN II IN TISSUES  
AND ITS INTRACELLULAR HALF  
LIFE IN VIVO.**

*Hypertension*. 1997; in press.

## SUMMARY

*Angiotensin (Ang) II 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  $^{125}\text{I}$ -Ang II and  $^{125}\text{I}$ -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  $^{125}\text{I}$ -Ang II in skeletal muscle, heart, kidney and adrenal were 8-41%, 64-150%, 340-550% and 680-2100% of the  $^{125}\text{I}$ -Ang II concentration in arterial blood plasma, respectively (ranges of 6 experiments). The tissue concentrations of  $^{125}\text{I}$ -Ang I were <5% of the concentration in arterial plasma. The accumulation of  $^{125}\text{I}$ -Ang II, as seen in heart, kidney and adrenal, was almost completely blocked by a specific  $\text{AT}_1$  receptor antagonist. Steady-state concentrations of  $^{125}\text{I}$ -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  $^{125}\text{I}$ -Ang II in heart, kidney and adrenal was approximately 15 min, as compared with 0.5 min in the circulation. Thus, Ang II from the circulation, but not Ang I, is accumulated by some tissues, and this is mediated by  $\text{AT}_1$  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  $\text{AT}_1$  receptor, so that it is protected from rapid degradation by endothelial peptidases. The results of this study are also in agreement with the growing evidence for an important physiological role of internalized Ang II.*

## ACKNOWLEDGEMENTS

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## 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<sub>1</sub> and AT<sub>2</sub>).<sup>37</sup> Most of the classical physiological actions of the renin-angiotensin system appear to be mediated by the AT<sub>1</sub> receptors.

Many cell surface receptors are internalized following binding to their agonists. Endocytosis of the complex of the AT<sub>1</sub> receptor with its agonist Ang II has been demonstrated in a number of target cells, *i.e.* vascular smooth muscle cells,<sup>4,175</sup> renal tubule cells<sup>144</sup> and cells from the adrenal cortex and medulla.<sup>9,10,28,188</sup> 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.<sup>4,28</sup>

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.<sup>67</sup> Receptor-mediated endocytosis of Ang II may also be important for the inositol 1,4,5-triphosphate (IP<sub>3</sub>) generation and for the sodium transport in response to stimulation of the apical AT<sub>1</sub> receptors of proximal renal tubule cells.<sup>144</sup> It has been reported that, in adrenal glomerulosa cells, the inhibition of Ang II-induced internalization reduces the sustained phase of IP<sub>3</sub> generation and abolishes the second phase of the cytoplasmic calcium response.<sup>82,185</sup> It is known that stimulated steroidogenesis by these cells closely follows the changes in intracellular calcium.<sup>130</sup> Others found that, in adrenal glomerulosa cells, internalization of the AT<sub>1</sub> receptor is required for protein kinase C activation but not for IP<sub>3</sub> release and steroidogenesis.<sup>89</sup>

There is also evidence to suggest 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.<sup>96,153</sup> Ang II is rapidly accumulated in vascular and cardiac muscle cell nuclei,<sup>136</sup> and AT<sub>1</sub>-type Ang II-binding sites have been identified in liver cell nuclei.<sup>88,172</sup> It has been reported that Ang II binds to chromatin and may influence transcriptional processes.<sup>52,132,133</sup> Ang II induces the expression of proto-oncogenes and has growth-promoting effects in various cells.<sup>145</sup> An intracellular action would require a sufficiently high 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 were

carried out *in vitro*. The *in vitro* studies made use of  $^{125}\text{I}$ -labeled Ang II, but did not discriminate between *intact*  $^{125}\text{I}$ -Ang II and  $^{125}\text{I}$ -labeled peptide fragments. In the course of studies in our laboratory, aimed at quantifying Ang II production in different regional vascular beds,  $^{125}\text{I}$ -labeled Ang I or II were infused into pigs.<sup>30,31</sup> Here we report on the *in vivo* accumulation of *intact*  $^{125}\text{I}$ -Ang II in cardiac, renal, and adrenal tissues during these infusions, and on the effect of a specific  $\text{AT}_1$  receptor antagonist on the Ang II uptake by these tissues. The data on the tissue accumulation and the half life of  $^{125}\text{I}$ -Ang II were compared with data obtained for  $^{125}\text{I}$ -Ang I. Unlike Ang II, Ang I is not biologically active and is probably not subjected to receptor-mediated endocytosis.

## MATERIALS AND METHODS

### Chemicals

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

### Antisera

Ang I and Ang II antisera prepared in New Zealand White rabbits, were used to identify the peptides in the 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 patterns of crossreactivity show that the antibodies in both antisera were directed against the C-terminal sequences of Ang I and Ang II.<sup>31</sup>

### 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' approved by the American Physiological Society. Twenty-three female pigs (crossbred Yorkshire x Landrace, Hedelse Varkens Combinatie) 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<sup>32</sup> and which will be reported in a separate paper, the release of renin from the kidney was stimulated with the diuretic furosemide, 40 mg twice daily for 2 days prior to 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  $^{125}\text{I}$ -Ang I and  $^{125}\text{I}$ -Ang II.

### *Instrumentation of the animals*

Animals were sedated with an intramuscular injection of 20 mg.kg<sup>-1</sup> ketamine (AUV), and anesthetized with 20 mg.kg<sup>-1</sup> sodium pentobarbitone (Apharma) 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. To maintain an adequate anesthesia, a 7 French (Fr) catheter was placed in the superior caval vein for administration of 8.5-10 mg.kg<sup>-1</sup>.h<sup>-1</sup> sodium pentobarbitone. Another 7Fr catheter was placed in the superior caval vein for infusion of saline to correct for fluid losses and to administer the AT<sub>1</sub> receptor antagonist L-158,809<sup>23,156</sup> (a gift of Dr. R. D. Smith, Du Pont Merck Pharmaceutical Company, Wilmington, Del., USA). A 7Fr catheter was inserted, via the left carotid artery, into the left ventricle under X-ray control to infuse <sup>125</sup>I-Ang I or <sup>125</sup>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) a midline thoracotomy was performed, and the heart was suspended in a pericardial cradle. An electromagnetic flow probe (Skalar) 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 <sup>125</sup>I-Ang I or <sup>125</sup>I-Ang II.

### *Infusions of <sup>125</sup>I-angiotensin I or <sup>125</sup>I-angiotensin II*

<sup>125</sup>I-Ang I was infused into the left cardiac ventricle at a constant rate of approximately 5 x 10<sup>6</sup> cpm/min. Steady-state plasma levels of <sup>125</sup>I-Ang I and <sup>125</sup>I-Ang II were reached within 10 min.<sup>30</sup> In order to determine the time required for the tissue levels to reach steady state, heart, kidney and adrenals were removed after various time periods of <sup>125</sup>I-Ang I infusion, *i.e.* after 15, 60 or 120 min of <sup>125</sup>I-Ang I infusion. Blood samples were taken from the aorta at 10 min and 60 min of <sup>125</sup>I-Ang I infusion.

In some experiments, in which <sup>125</sup>I-Ang I had been infused for 15 min, 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 <sup>125</sup>I-Ang I and II. The *in-vivo* half-life of <sup>125</sup>I-Ang I and <sup>125</sup>I-Ang II in the circulation was determined by measuring the plasma levels of <sup>125</sup>I-Ang I and <sup>125</sup>I-Ang II in blood samples taken from the aorta at 0.25, 0.5, 1, 1.5 and 2 min after the <sup>125</sup>I-Ang I infusion had been stopped.

The effect of blockade of the AT<sub>1</sub>-type Ang II receptor on the tissue levels of <sup>125</sup>I-Ang I and <sup>125</sup>I-Ang II during <sup>125</sup>I-Ang I infusion, was studied by administration of the AT<sub>1</sub>-receptor antagonist L-158,809, 1 mg/min *i.v.* for 10 min, 30 min before the start of the <sup>125</sup>I-Ang I infusion. At this dose of the AT<sub>1</sub>-receptor antagonist, the pressor effect of systemically administered Ang II (0.1-1.0 µg.kg<sup>-1</sup>) is completely blocked.<sup>182</sup> After 10 min of <sup>125</sup>I-Ang I infusion an arterial blood sample was taken, and heart, kidney and adrenals were removed at 15 min of infusion.

<sup>125</sup>I-Ang II was infused into the left cardiac ventricle at a constant rate of approximately 3 x 10<sup>6</sup> cpm/min. Heart, kidney and adrenal were removed at 15, 60 or 120 min of <sup>125</sup>I-Ang II infusion. Blood samples were taken from the aorta at 10 min and 60 min of <sup>125</sup>I-Ang II infusion. A steady-state plasma level of <sup>125</sup>I-Ang II had been reached at that time.<sup>2</sup>

*Blood and tissue sampling*

During the infusions of  $^{125}\text{I}$ -Ang I or  $^{125}\text{I}$ -Ang II, blood samples (10 mL) were taken from the aorta to measure the plasma levels of  $^{125}\text{I}$ -Ang I and  $^{125}\text{I}$ -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) (final concentrations in blood), and immediately transferred into prechilled polystyrene tubes and centrifuged at 3,000g 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  $^{125}\text{I}$ -Ang I or  $^{125}\text{I}$ -Ang II had been stopped. Prior to 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 the 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.

To study the *ex-vivo* degradation of  $^{125}\text{I}$ -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.

*Measurements of  $^{125}\text{I}$ -angiotensin I and  $^{125}\text{I}$ -angiotensin II in tissue and plasma*

Frozen tissue samples were homogenized with a Polytron (PT10/35, Kinematica) in 20 mL ice-cold ethanol/0.1 mol/L HCl 4:1 (vol/vol).<sup>32</sup> Homogenates were centrifuged at 20,000g 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 20,000g. 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). 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 90% methanol / 10% water (vol/vol) into polypropylene tubes, and the eluted samples were vacuum dried.

Separations were performed by reversed-phase high-performance chromatography (HPLC) using a Nucleosil C18 steel column of 250x4.6 mm and 10  $\mu\text{m}$  particle size (Alltech) as previously described.<sup>1</sup> Water for HPLC was prepared with a Milli-Q system (Waters). 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  $\mu\text{L}$  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). The concentrations of <sup>125</sup>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 <sup>125</sup>I-Ang I as an internal standard prior to the extraction procedure. The recovery of <sup>125</sup>I-Ang I after HPLC separation was then used to correct for losses (maximally 20-30%) occurring during extraction and separation.<sup>32</sup> In the present study, we did not add <sup>125</sup>I-Ang I as an internal standard since the tissue already contained <sup>125</sup>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.

## RESULTS

### *Hemodynamic effects of <sup>125</sup>I-angiotensin I and <sup>125</sup>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<sub>1</sub> receptor blocker L-158,809 (Table 1). Infusions of <sup>125</sup>I-Ang I or <sup>125</sup>I-Ang II did not affect any of these parameters (data not shown), which is in agreement with previous studies.<sup>2,30</sup>

### *Identification of <sup>125</sup>I-angiotensin I, <sup>125</sup>I-angiotensin II and their <sup>125</sup>I-labeled metabolites in blood plasma and in tissue by HPLC*

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

In plasma, >90% of the radioactivity in the peak with the same retention time as <sup>125</sup>I-Ang I was bound by Ang I antiserum, as compared with <5% by Ang II antiserum. This peak was therefore identified as <sup>125</sup>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 <sup>125</sup>I-Ang I, but <10% of the radioactivity in this peak was bound to Ang I antiserum. This peak therefore was not <sup>125</sup>I-Ang I.

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

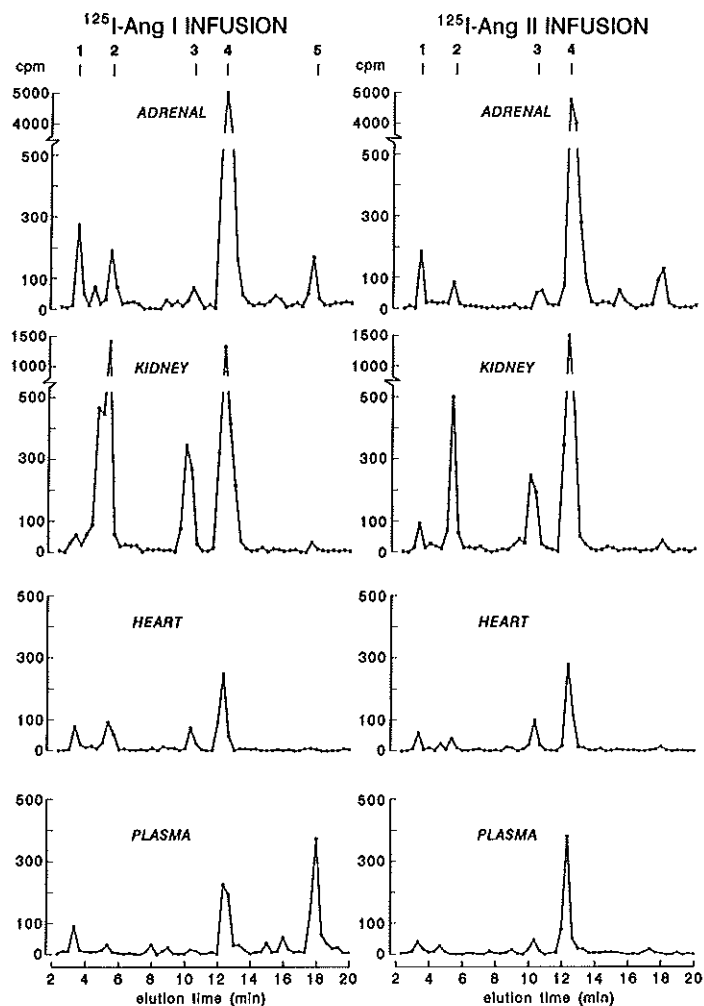
Treatment	Infusion	n	Mean Arterial Pressure (mm Hg)	Heart Rate (beats/min)	Cardiac Output (L/min)	$^{125}\text{I}$ -Ang I Conc. (cpm/mL)	$^{125}\text{I}$ -Ang II Conc. (cpm/mL)	$^{125}\text{I}$ -Ang II-to-I Conc. Ratio
Furosemide	$^{125}\text{I}$ -Ang I	11	81±4	105±18	2.4±0.5	1470±350	1000±350	0.67±0.14
Furosemide & AT <sub>1</sub> receptor blockade	$^{125}\text{I}$ -Ang I	2	82-83	107-135	3.0-5.2	1150-1220	660-970	0.57-0.80
Captopril	$^{125}\text{I}$ -Ang I	6	85±4	114±20	2.8±0.4	2440±410*	370±350*	0.15±0.08*
Furosemide	$^{125}\text{I}$ -Ang II	4	78±13	110±15	2.3±0.7	-	1960±530	-

Data are means ± SD or individual values (AT<sub>1</sub> receptor blockade). Ang, angiotensin; cpm, counts per min.

\* p < 0.002 for difference from results in furosemide-pretreated pigs receiving  $^{125}\text{I}$ -angiotensin I infusion (unpaired Student's t test).



*AT<sub>1</sub> receptor-mediated accumulation of angiotensin II in tissues*



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

Both in plasma and in the tissues, >90% of the radioactivity in the peak with the same retention time as  $^{125}\text{I}$ -Ang II was bound by Ang II antiserum, as compared with <3% by Ang I antiserum. This peak was therefore identified as  $^{125}\text{I}$ -Ang II.

In addition to the  $^{125}\text{I}$ -Ang I and  $^{125}\text{I}$ -Ang II peaks, separate peaks with retention times corresponding with  $^{125}\text{I}$ -tyrosine,  $^{125}\text{I}$ -Ang-(1-7) and  $^{125}\text{I}$ -Ang-(4-8) were observed in the tissues. More than 80% of the radioactivity in the peak with the retention time of  $^{125}\text{I}$ -Ang-(4-8) was bound by Ang II antiserum, whereas <10% was bound by Ang I antiserum. We concluded therefore that this peak was indeed

$^{125}\text{I}$ -Ang-(4-8). The radioactivity in the peaks with the retention times of  $^{125}\text{I}$ -tyrosine and  $^{125}\text{I}$ -Ang-(1-7) was not bound by these antisera. The conclusion that these peaks were indeed  $^{125}\text{I}$ -tyrosine and  $^{125}\text{I}$ -Ang-(1-7) needs further confirmation.

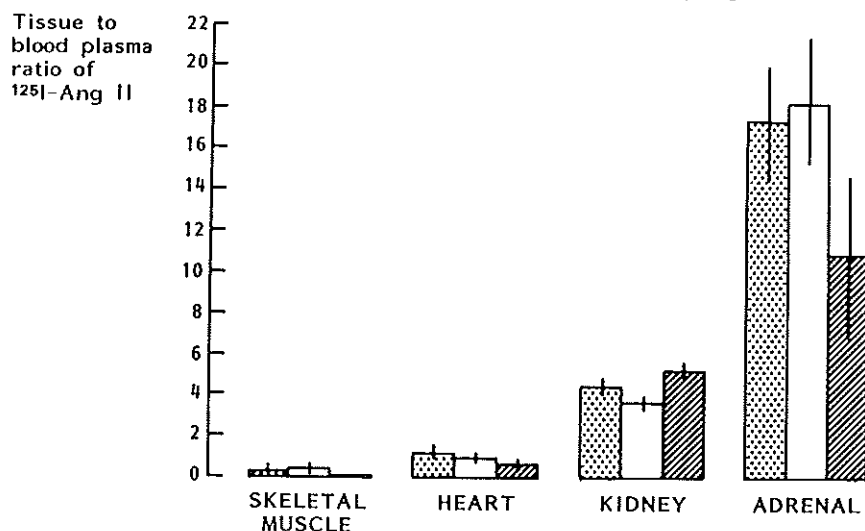
*Accumulation of  $^{125}\text{I}$ -angiotensin II in tissue and the effect of  $\text{AT}_1$  receptor blockade*

Figure 2 shows the tissue levels of  $^{125}\text{I}$ -Ang II (expressed relative to the  $^{125}\text{I}$ -Ang II levels in blood plasma) after 60 min infusions of  $^{125}\text{I}$ -Ang I or  $^{125}\text{I}$ -Ang II. The  $^{125}\text{I}$ -Ang II levels in the kidney and the adrenal (expressed per g tissue) were 340-550% and 680-2100% of the  $^{125}\text{I}$ -Ang II level in plasma (expressed per mL plasma), respectively (ranges of 6 experiments). In the heart, the  $^{125}\text{I}$ -Ang II level was 64-150% of the level in plasma. The  $^{125}\text{I}$ -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  $^{125}\text{I}$ -Ang I infusion were similar to those after  $^{125}\text{I}$ -Ang II infusion. In the kidney and the adrenal, and also in the heart, the tissue levels of  $^{125}\text{I}$ -Ang II were too high to be explained by the presence of  $^{125}\text{I}$ -Ang II in the extracellular fluid. The tissue levels of  $^{125}\text{I}$ -Ang I were < 5% of the level in plasma.

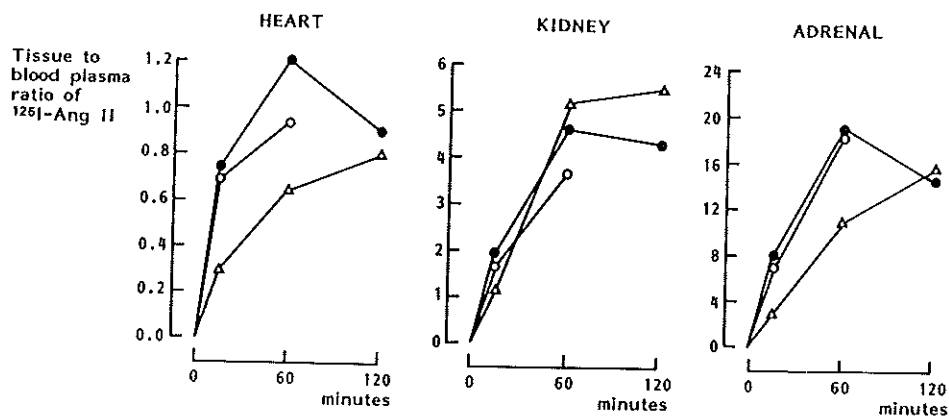
These results demonstrate that Ang II from the circulation is accumulated by the heart, kidney and adrenal and that, in the case of the kidney and the adrenal, the tissue concentration of the accumulated Ang II is several times the plasma concentration. Ang I from the circulation is not accumulated by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

In Figure 3, the  $^{125}\text{I}$ -Ang II tissue-to-blood plasma concentration ratios that were reached in heart, kidney and adrenal after 60-min infusions of  $^{125}\text{I}$ -Ang I or  $^{125}\text{I}$ -Ang II are compared with the ratios after 15- or 120-min infusions. It took between 30 and 60 min for  $^{125}\text{I}$ -Ang II to reach steady-state levels in the tissues. This is much longer than in plasma, where it takes <10 min to reach steady state.<sup>1,2,30</sup>

The effect of the  $\text{AT}_1$  receptor antagonist L-158,809 on the accumulation of  $^{125}\text{I}$ -Ang II in heart, kidney and adrenal was studied during a 15-min infusion of  $^{125}\text{I}$ -Ang I. Skeletal muscle was not studied because of the apparent lack of  $^{125}\text{I}$ -Ang II accumulation by this tissue (see above). As shown in Figure 4, L-158,809 caused nearly complete blockade of  $^{125}\text{I}$ -Ang II accumulation. L-158,809 had no effect on the plasma levels of  $^{125}\text{I}$ -Ang I and  $^{125}\text{I}$ -Ang II (Table 1). Thus, the accumulation of Ang II from the circulation by heart, kidney and adrenal appears to depend on  $\text{AT}_1$  receptors.



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

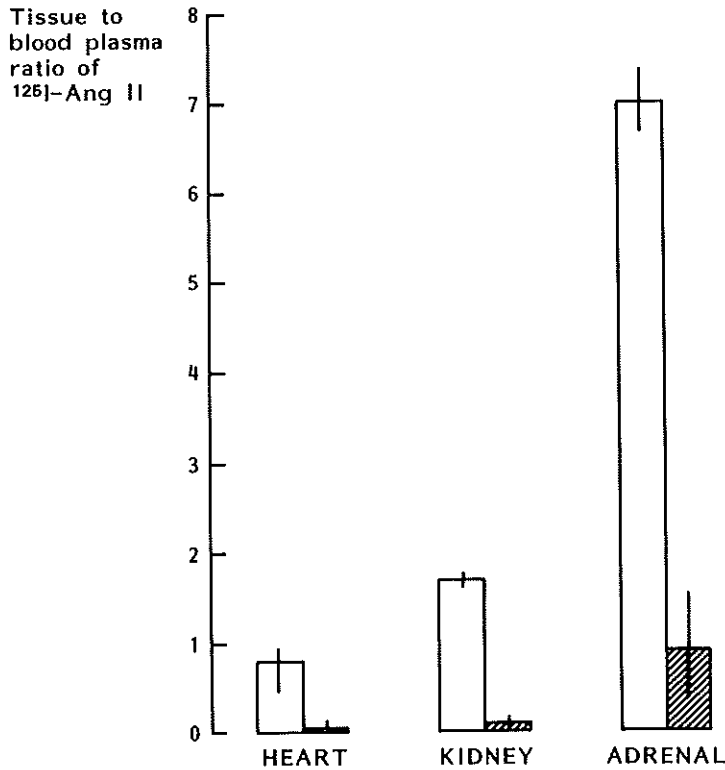


**Figure 3.** Tissue-to-blood plasma <sup>125</sup>I-angiotensin II concentration ratios in heart, kidney and adrenal, during infusion of <sup>125</sup>I-angiotensin I in furosemide- (closed circles) or captopril-pretreated (open circles) pigs or during infusion of <sup>125</sup>I-angiotensin II in furosemide-treated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

*Ex-vivo and in-vivo half life of <sup>125</sup>I-angiotensin II in tissue*

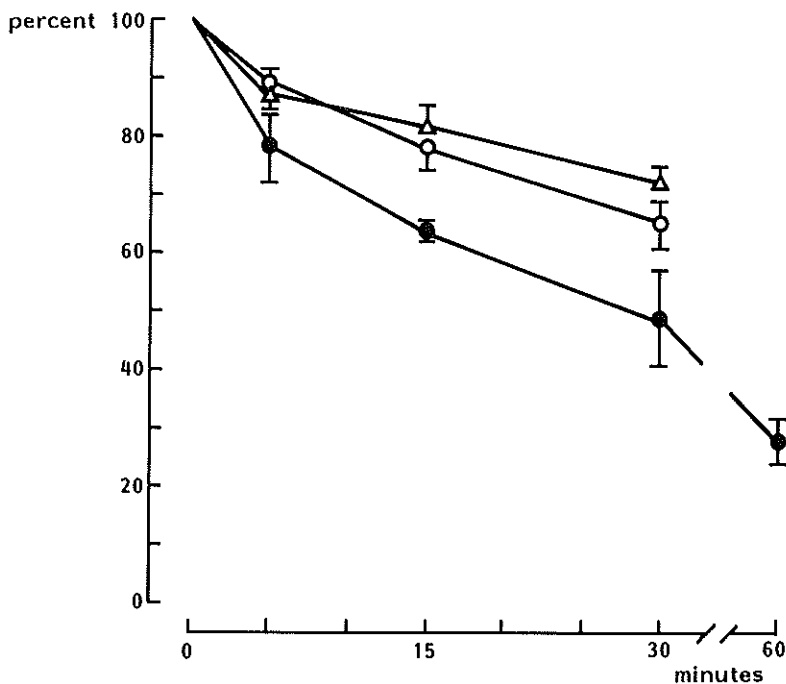
The process of tissue removal, cutting and transfer into liquid nitrogen took <1 min. Still, the metabolism of <sup>125</sup>I-Ang II might be so rapid that the measured levels of this peptide are substantially below the levels *in vivo*. We, therefore, investigated the decrease in the tissue levels of <sup>125</sup>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  $^{125}\text{I}$ -Ang II accumulation in this tissue (see above).



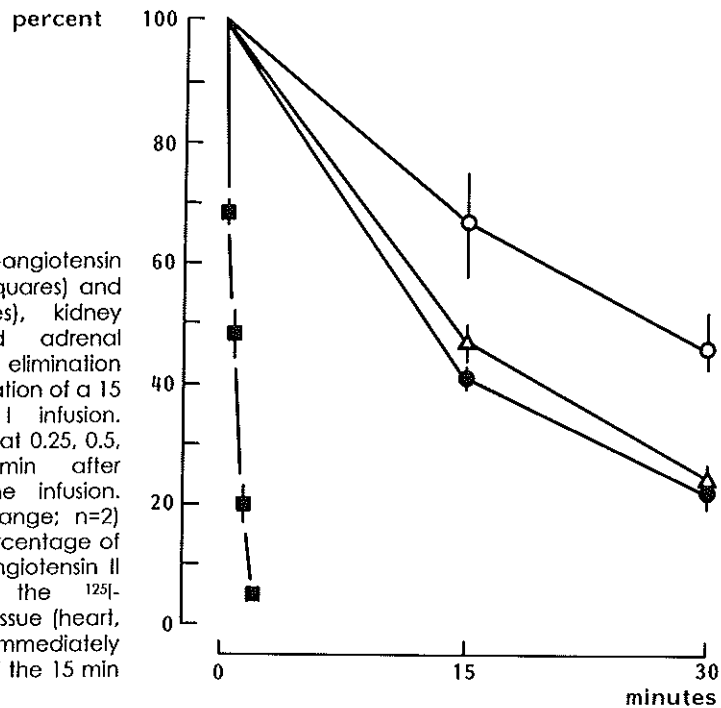
**Figure 4.** Tissue-to-blood plasma  $^{125}\text{I}$ -angiotensin II concentration ratios in heart, kidney and adrenal, at 15 min of infusion of  $^{125}\text{I}$ -angiotensin I in furosemide-pretreated pigs either with (hatched bars) or without (open bars) AT<sub>1</sub> receptor blockade by L-158,809. Values are means and range (n=2).

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



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

In order to get some information on the *in-vivo* half life of  $^{125}\text{I}$ -Ang II in tissue, the  $^{125}\text{I}$ -Ang II levels were measured in heart, kidney and adrenal after these organs had been kept in the body for 15 or 30 min after the  $^{125}\text{I}$ -Ang I infusion had been stopped. As shown in Figure 6, the tissue levels of  $^{125}\text{I}$ -Ang II, 15 and 30 min after discontinuation of the  $^{125}\text{I}$ -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  $^{125}\text{I}$ -Ang II in the circulation was approximately 0.5 min, which is in agreement with earlier studies.<sup>30</sup> 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  $^{125}\text{I}$ -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  $^{125}\text{I}$ -angiotensin I infusion. Plasma was collected at 0.25, 0.5, 1, 1.5 and 2 min after discontinuation of the infusion. Values (means and range;  $n=2$ ) are expressed as a percentage of the steady-state  $^{125}\text{I}$ -angiotensin II level (plasma) or the  $^{125}\text{I}$ -angiotensin II level in tissue (heart, kidney and adrenal) immediately after discontinuation of the 15 min of infusion.

## DISCUSSION

In the experiments reported in this paper,  $^{125}\text{I}$ -Ang I or  $^{125}\text{I}$ -Ang II was infused at a constant rate into the left cardiac ventricle of pigs, in order 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 the labeled and unlabeled peptides. In a previous study, also in pigs, the arterial and venous plasma levels of  $^{125}\text{I}$ -Ang I and  $^{125}\text{I}$ -Ang II and unlabeled Ang I and II were measured in a number of regional vascular beds in animals receiving infusions of  $^{125}\text{I}$ -Ang I combined with unlabeled Ang I, to compare the regional extraction rates of the arterially delivered labeled and unlabeled angiotensins.<sup>30,31</sup> 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  $^{125}\text{I}$ -Ang I-to-II conversion rate being two times the Ang I-to-II conversion rate, but the degradation of  $^{125}\text{I}$ -Ang I into peptides other than  $^{125}\text{I}$ -Ang II occurred at the same rate as the degradation of Ang I. The

<sup>125</sup>I-Ang II degradation rate was also not different from the Ang II degradation rate.<sup>2</sup>

Another important methodological aspect of our study is the possibility that the measured tissue levels of <sup>125</sup>I-Ang I and II differed from the levels *in vivo*, because of rapid degradation of these peptides after the <sup>125</sup>I-Ang I or <sup>125</sup>I-Ang II infusions had been stopped. This issue was addressed by investigating the *ex-vivo* degradation of <sup>125</sup>I-Ang I and <sup>125</sup>I-Ang II when the tissues were kept at 37°C. On the basis of the results of these experiments, it may be concluded that the measured tissue levels of <sup>125</sup>I-Ang II are probably representative of the levels present *in vivo*. The measured tissue level of <sup>125</sup>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<sub>1</sub> 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 <sup>125</sup>I-Ang II we measured were about half maximal after 15-30 min of <sup>125</sup>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<sub>1</sub> receptor-dependent accumulation of radioactivity in isolated bovine adrenocortical and chromaffin cells, when incubated with <sup>125</sup>I-Ang II.<sup>28,188</sup> In these *in-vitro* studies, 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 <sup>125</sup>I-Ang II. In addition, the increase in the *total* specific (AT<sub>1</sub> receptor-related) radioactivity of the cells during the first 30 min of incubation with <sup>125</sup>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<sup>4,175</sup> and in COS-7 cells expressing the rat AT<sub>1</sub> receptor.<sup>83</sup> Also in our *in-vivo* experiments, the progressive accumulation of <sup>125</sup>I-Ang II in heart, kidney and adrenal appeared to be mediated by AT<sub>1</sub> receptors, since it was inhibited by the AT<sub>1</sub> receptor antagonist L-158,809. Because of this and because of the similarities between the kinetics of the <sup>125</sup>I-Ang II accumulation process in the *intact* animal and the kinetics of <sup>125</sup>I-Ang II internalization by cells in culture, we conclude that the increase in the tissue levels of <sup>125</sup>I-Ang II in heart, kidney and adrenal that we observed during the infusion of <sup>125</sup>I-Ang I or <sup>125</sup>I-Ang II, is mainly determined by AT<sub>1</sub> receptor-mediated internalization and, therefore, reflects the increase in intracellular <sup>125</sup>I-Ang II.

The steady-state tissue level of <sup>125</sup>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 <sup>125</sup>I-Ang II were several times those in plasma. In contrast, the tissue levels of <sup>125</sup>I-Ang I were < 5 % of the level 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-to-blood plasma  $^{125}\text{I}$ -Ang II concentration ratios, *i.e.* adrenal > kidney > heart, is in agreement with the Ang II receptor densities in these organs.<sup>118,134,135,193,201</sup> Since most of the  $^{125}\text{I}$ -Ang II in tissue seems to be localized in the cells, following its internalization through  $\text{AT}_1$  receptors, it is indeed logical to assume that the tissue-to-plasma  $^{125}\text{I}$ -Ang II concentration ratio is proportional to the  $\text{AT}_1$  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  $^{125}\text{I}$ -Ang II concentration ratios after captopril-treatment were similar to those after furosemide.

Recently Zou *et al.*<sup>202</sup> reported that [Val<sup>5</sup>]-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<sup>5</sup>]-Ang II). These authors suggested that renal accumulation of circulating Ang II might be caused by  $\text{AT}_1$  receptor-mediated endocytosis. In a subsequent study from the same laboratory,<sup>203</sup> also in uninephrectomized rats, it was found that chronic low-dose infusion of [Ile<sup>5</sup>]-Ang II increased the level of this peptide in the kidney, and that this increase was prevented by the  $\text{AT}_1$  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.*<sup>202,203</sup> are indeed explained, at least in part, by  $\text{AT}_1$  receptor-mediated endocytosis.

There was little difference in the tissue-to-blood plasma concentration ratios of  $^{125}\text{I}$ -Ang II between the animals subjected to  $^{125}\text{I}$ -Ang I infusions after treatment with furosemide and the animals subjected to  $^{125}\text{I}$ -Ang I infusions after treatment with captopril. There was also little difference between the results obtained after  $^{125}\text{I}$ -Ang I infusion and those after  $^{125}\text{I}$ -Ang II infusion. This indicates that most of the  $^{125}\text{I}$ -Ang II in the tissues was derived from arterially delivered  $^{125}\text{I}$ -Ang II and not, via conversion, from arterially delivered  $^{125}\text{I}$ -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*  $^{125}\text{I}$ -Ang II rather than *total* tissue radioactivity. This enabled us to provide an estimate of the *in-vivo* intracellular half life of *intact*  $^{125}\text{I}$ -Ang II, by measuring its levels in tissue at different time intervals after the discontinuation of  $^{125}\text{I}$ -Ang I infusion. The intracellular half life of  $^{125}\text{I}$ -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.  $\text{AT}_1$  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



*AT<sub>1</sub> receptor-mediated accumulation of angiotensin II in tissues*

interfere with the receptor-mediated endocytosis of Ang II, and, as our study demonstrates, the AT<sub>1</sub> receptor antagonist L-158,809 reduces the tissue concentrations of blood-derived Ang II to very low levels. There is growing evidence that AT<sub>1</sub> receptor-mediated endocytosis of Ang II is important for some physiological responses to Ang II.<sup>67,82,144,185</sup> 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<sub>1</sub> receptor antagonists.

*References are presented in the general reference list.*



*Chapter 5*

**UPTAKE AND PRODUCTION  
OF ANGIOTENSINS BY THE  
HEART.**

**A QUANTITATIVE STUDY IN  
PIGS WITH THE USE OF  
RADIOLABELED  
ANGIOTENSIN INFUSIONS.**

Submitted

## SUMMARY

*Local angiotensin (Ang) production may be involved in post-infarction remodeling and failure. To determine whether cardiac Ang I and II are synthesized in situ or derived from the circulation, we infused  $^{125}\text{I}$ -Ang I or II into pigs and measured  $^{125}\text{I}$ -Ang I and II as well as endogenous Ang I and II in cardiac tissue and blood plasma. In untreated pigs the tissue Ang II concentration (per g wet weight) in different parts of the heart was 5 times the concentration (per mL) in plasma, and the tissue Ang I concentration was 70% of the plasma Ang I concentration. Tissue  $^{125}\text{I}$ -Ang II during  $^{125}\text{I}$ -Ang II infusion was 75% of  $^{125}\text{I}$ -Ang II in arterial plasma, whereas tissue  $^{125}\text{I}$ -Ang I during  $^{125}\text{I}$ -Ang I infusion was less than 4% of  $^{125}\text{I}$ -Ang I in arterial plasma. Following treatment with the ACE inhibitor captopril, Ang II fell in plasma but not in tissue, and Ang I and renin rose both in plasma and tissue, whereas angiotensinogen did not change in plasma and fell in tissue. Tissue  $^{125}\text{I}$ -Ang II derived, by conversion, from arterially delivered  $^{125}\text{I}$ -Ang I fell from 25% to less than 2% of  $^{125}\text{I}$ -Ang I in arterial plasma. Most of the cardiac Ang II appears to be produced at tissue sites by conversion of in-situ synthesized, rather than blood-derived, Ang I. The heart can maintain its Ang II production when the production of plasma Ang II is effectively suppressed.*

## ACKNOWLEDGEMENTS

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## INTRODUCTION

From clinical studies there is evidence to suggest that the long-term beneficial effects of angiotensin-converting enzyme (ACE) inhibitor drugs on post-infarction cardiac failure and remodeling as well as on left ventricular hypertrophy are, at least partly, independent of their systemic effect on blood pressure.<sup>29,107</sup> A reduction of angiotensin (Ang) II production locally in the heart may explain the blood pressure-independent effects of ACE inhibition in cardiac patients.

Indeed, the heart contains all components required for Ang I and Ang II production, *i.e.* renin, angiotensinogen and ACE.<sup>32</sup> Renin mRNA may be present in the heart in low concentrations,<sup>54,85,108,124</sup> but observations on the effect of bilateral nephrectomy in pigs demonstrated that most, if not all, renin in the heart is derived from the kidney, at least under normal conditions.<sup>32</sup> There is also convincing evidence for angiotensinogen gene expression in cardiac tissue.<sup>106</sup> ACE is synthesized in the heart by the vascular endothelium, the endocardium and possibly other cardiac cells.<sup>58,81,148,164</sup> Ang II from the circulation is taken up in cardiac tissue by a type 1 Ang II (AT<sub>1</sub>) receptor-mediated process.<sup>183</sup>

The study reported here addresses the following questions: 1) how much of the Ang I and II in cardiac tissue is derived from the circulation, 2) how much of the cardiac Ang II is locally synthesized by the conversion of blood-derived Ang I and how much by the conversion of Ang I that is formed locally in the heart, and 3) what is the effect of ACE inhibitor treatment on cardiac angiotensin production.

## MATERIALS AND METHODS

### *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' approved by the American Physiological Society. Twenty-four female pigs (crossbred Yorkshire x Landrace, Hedelse Varkens Combinatie) with a body weight of 25-30 kg were included in the study. Ten animals were pretreated with the ACE inhibitor captopril, 25 mg twice daily for 3 days, in order to study the effect of ACE inhibition on cardiac Ang I and II production.

### *Instrumentation of the animals*

The animals were prepared for hemodynamic measurements, for administration of anesthetic and <sup>125</sup>I-labeled angiotensins, and for blood and tissue sampling as described previously.<sup>32,182,183</sup> After a stabilization period of 30-45 minutes 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 <sup>125</sup>I-Ang I or <sup>125</sup>I-Ang II.

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### *Preparation of <sup>125</sup>I-labeled angiotensins*

[Ile<sup>5</sup>]-Ang-(1-10) decapeptide (Ang I) and [Ile<sup>5</sup>]-Ang-(1-8) octapeptide (Ang II) (Bachem) were used to prepare mono-iodinated <sup>125</sup>I-Ang I and II. The chloramine-T method was used for <sup>125</sup>I-labeling and the radiolabeled peptides were purified as described previously.<sup>1</sup> The specific radioactivity of the <sup>125</sup>I-Ang I and <sup>125</sup>I-Ang II preparations was ~3.6x10<sup>6</sup> cpm/pmol.

### *Infusion of <sup>125</sup>I-labeled angiotensins*

<sup>125</sup>I-Ang I or <sup>125</sup>I-Ang II were administered via constant infusion into the left cardiac ventricle for 15, 60 or 120 minutes. The infusion rate was approximately 4x10<sup>6</sup> cpm/ minute for <sup>125</sup>I-Ang I and 3x10<sup>6</sup> cpm/ minute for <sup>125</sup>I-Ang II. The levels of <sup>125</sup>I-Ang I and <sup>125</sup>I-Ang II reached their steady-state maximum within 10 minutes in plasma<sup>30</sup> and within 60 minutes in cardiac tissue.<sup>183</sup> <sup>125</sup>I-Ang I was infused for 15 minutes into 7 untreated and 7 captopril-treated pigs. <sup>125</sup>I-Ang I was infused for 60 minutes into 3 untreated and 3 captopril-treated pigs. <sup>125</sup>I-Ang II was infused for 60 minutes into 4 untreated pigs. In order to reduce the total quantities of radiolabeled angiotensins required for the infusions, the number of 60-minute infusion experiments was kept at a minimum. Measurements of the steady-state plasma levels of <sup>125</sup>I-Ang I and II were made both in the 15-minute and 60-minute experiments. Measurements of the steady-state tissue levels of <sup>125</sup>I-Ang I and II were made in the 60-minute experiments.

### *Collection of blood and cardiac tissue samples*

Blood samples (10 mL) were taken from the aorta and great cardiac vein during infusion of <sup>125</sup>I-Ang I or II to measure the plasma levels of <sup>125</sup>I-Ang I and II and endogenous Ang I and II. The blood was rapidly drawn with a plastic syringe containing the following inhibitors (0.5 mL inhibitor solution in 10 mL blood), 0.01 mmol/L of the renin inhibitor remikiren (a kind 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) (final concentrations in blood). The blood was immediately transferred into prechilled polystyrene tubes and centrifuged at 3,000g for 10 minutes at 4°C. Plasma was stored at -70°C and assayed within 3 days.

Aortic blood samples (5 mL) for measurements of renin and angiotensinogen were collected in polystyrene tubes containing disodium citrate (0.1 mL in 5 mL blood; final concentration 13 mmol/L). The samples were centrifuged at 1,000g for 10 minutes at room temperature and plasma was stored at -70°C.

Cardiac tissue was collected as follows. The heart was stopped by fibrillation, while the <sup>125</sup>I-Ang I or II infusion was still running. The heart was quickly removed from the body and pieces of 1-2 g were quickly excised from the left and right atrium and from the left and right ventricular free wall. The tissue pieces were immediately transferred into liquid nitrogen. The tissue was frozen within 15 seconds after the heart had been stopped.

To study the *ex-vivo* metabolism of endogenous angiotensins in cardiac tissue, the remaining part of the left ventricular free wall was kept at 37°C. Pieces of left ventricular tissue were then cut off and frozen in liquid nitrogen at various time points after the heart had been stopped. The frozen tissues were stored at -70°C and assayed within 3 days.

### *Measurements of <sup>125</sup>I-labeled and endogenous angiotensins in cardiac tissue and blood plasma*

Frozen tissue samples were homogenized in 20 mL ice-cold 0.1 mol/L HCl / 80% ethanol as previously described.<sup>32,183</sup> The homogenate was centrifuged at 20,000g for 25 minutes at 4°C.

Ethanol in the supernatant was evaporated under constant air flow and the remainder of the supernatant was diluted in 20 mL 1% ortho-phosphoric acid and centrifuged again at 20,000g. The supernatant was diluted with an equal volume of 1% ortho-phosphoric acid and then concentrated on Sep-Pak cartridges. Plasma was directly applied to the Sep-Pak cartridges.

The preparation of the Sep-Pak extracts for high-performance liquid chromatographic (HPLC) separation of the angiotensins and the HPLC procedure have been described elsewhere.<sup>183</sup> The concentrations of *intact* <sup>125</sup>I-Ang I and <sup>125</sup>I-Ang II and the concentrations of *intact* Ang I and Ang II in the HPLC eluate fractions were measured by gamma-counting and radioimmunoassay respectively.<sup>1</sup> Data were not corrected for losses occurring during extraction and separation. These losses were less than 10% in plasma and maximally 20-30% in tissue extracts.<sup>183</sup> In some plasma samples the angiotensin levels were below the limit of detection. These were taken to be equal to the lower limit of detection (1.0 fmol/mL for Ang I and 0.5 fmol/mL for Ang II) to allow calculation of mean values.

#### *Measurements of renin and angiotensinogen in cardiac tissue and blood plasma*

Frozen tissue samples were homogenized (1:3, wt:vol) in 0.01 mol/L phosphate buffer, pH 7.4, containing 0.15 mol/L NaCl as previously described.<sup>32</sup> Homogenates used for renin measurements were pretreated with acid to remove angiotensinase activity.<sup>32</sup> In short, one mL of homogenate was dialyzed for 48 hours at 4°C against 0.05 mol/L glycine buffer, pH 3.3, containing 0.095 mol/L NaCl.<sup>32</sup> This was followed by dialysis at 4°C for 24 hours 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, and the volume was adjusted to 1 mL with phosphate buffer.

The concentration of renin in acid-pretreated cardiac tissue extract and in non-acid-pretreated plasma was determined by the enzyme-kinetic assay, which measures the rate of Ang I generation at pH 7.4 during incubation at 37°C with a saturating amount of porcine renin substrate, in the presence of inhibitors of angiotensinases, ACE and serine proteases.<sup>32</sup> The Ang I-generating activity of cardiac tissue extracts measured in the absence of the renin inhibitor remikiren, minus the Ang I-generating activity in the presence of remikiren (final concentration 10<sup>-5</sup> mol/L) was taken as a measure of the concentration of renin. Inhibition of porcine renin is virtually complete at this concentration of remikiren.<sup>32</sup> Any remaining Ang I-generating activity was assumed to be caused by enzymes other than renin. Plasma had no Ang I-generating activity in the presence of 10<sup>-5</sup> mol/L remikiren.

The concentration of angiotensinogen was determined in non-acid-pretreated cardiac tissue extracts and plasma. It was measured as the maximum quantity of Ang I that was generated during incubation at 37°C with a high concentration of porcine kidney renin in the presence of inhibitors of angiotensinases and ACE.<sup>32</sup>

#### *Calculations*

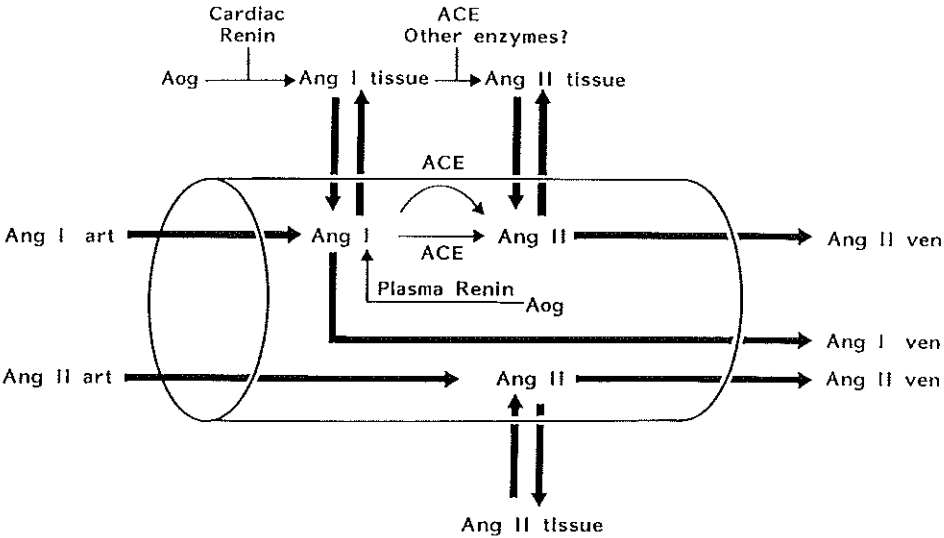
The possible sources of Ang I and II in cardiac tissue are shown in Figure 1 and summarized in Table 1. The level of arterially delivered Ang II in cardiac tissue was calculated as follows:

$$[\text{Ang II}_{\text{tissue}} \text{ from Ang II}_{\text{art}}] = R_1 \times [\text{Ang II}_{\text{art}}], \quad (1)$$

in which  $[\text{Ang II}_{\text{tissue}} \text{ from Ang II}_{\text{art}}]$  is the Ang II concentration in cardiac tissue (fmol/g) derived from arterially delivered Ang II,  $[\text{Ang II}_{\text{art}}]$  is the Ang II concentration in aortic blood plasma (fmol/mL), and  $R_1$  is defined by the equation:

$$R_1 = \frac{[^{125}\text{I-Ang II}_{\text{tissue}}]}{[^{125}\text{I-Ang II}_{\text{art}}]}, \tag{2}$$

in which  $[^{125}\text{I-Ang II}_{\text{tissue}}]$  and  $[^{125}\text{I-Ang II}_{\text{art}}]$  are the steady-state concentrations of  $^{125}\text{I-Ang II}$  in cardiac tissue (cpm/g) and aortic blood plasma (cpm/mL) respectively, both during the constant infusion of  $^{125}\text{I-Ang II}$ .



**Figure 1.** Schematic representation of the sources of angiotensin I and II in cardiac tissue and coronary venous plasma. The angiotensin II-to-I conversion by plasma ACE and endothelial ACE are separately indicated. Ang, angiotensin; Aog, angiotensinogen; ACE, angiotensin-converting enzyme; art, arterial; ven, venous.

**Table 1.** Sources of angiotensin I and II in cardiac tissue

Angiotensin I	Angiotensin II
- Ang I in coronary artery	- Ang II in coronary artery
- Ang I produced by the fluid-phase reaction of renin with angiotensinogen in the coronary circulation	- Ang I in coronary artery
- Ang I synthesized <i>in situ</i> in cardiac tissue	- Ang I produced by the fluid-phase reaction of renin with angiotensinogen in the coronary circulation
	- Ang I synthesized <i>in situ</i> in cardiac tissue

The level of arterially delivered Ang I in cardiac tissue was calculated as follows:

$$[\text{Ang I}_{\text{tissue}} \text{ from Ang I}_{\text{art}}] = R_2 \times [\text{Ang I}_{\text{art}}], \tag{3}$$

in which  $[\text{Ang I}_{\text{tissue}} \text{ from Ang I}_{\text{art}}]$  is the Ang I concentration in cardiac tissue (fmol/g) derived from arterially delivered Ang I,  $[\text{Ang I}_{\text{art}}]$  is the Ang I concentration in aortic blood



*Uptake and production of angiotensins by the heart*

plasma (fmol/mL), and  $R_2$  is defined by the equation:

$$R_2 = [^{125}\text{I-Ang I}_{\text{tissue}}] / [^{125}\text{I-Ang I}_{\text{art}}], \quad (4)$$

in which  $[^{125}\text{I-Ang I}_{\text{tissue}}]$  and  $[^{125}\text{I-Ang I}_{\text{art}}]$  are the steady-state concentrations of  $^{125}\text{I-Ang I}$  in cardiac tissue (cpm/g) and aortic blood plasma (cpm/mL) respectively, both during the constant infusion of  $^{125}\text{I-Ang I}$ .

The cardiac tissue level of Ang II that is derived, by conversion, from arterially delivered Ang I, was calculated as follows:

$$[\text{Ang II}_{\text{tissue from Ang I}_{\text{art}}}] = R_3 \times [\text{Ang I}_{\text{art}}], \quad (5)$$

in which  $[\text{Ang II}_{\text{tissue from Ang I}_{\text{art}}}]$  is the Ang II concentration in cardiac tissue (fmol/g) derived from arterially delivered Ang I,  $[\text{Ang I}_{\text{art}}]$  is the Ang I concentration in aortic blood plasma (fmol/mL), and  $R_3$  is defined by the equation:

$$R_3 = ([^{125}\text{I-Ang II}_{\text{tissue}}] / [^{125}\text{I-Ang I}_{\text{art}}]) - (R_1 \times [^{125}\text{I-Ang II}_{\text{art}}] / [^{125}\text{I-Ang I}_{\text{art}}]), \quad (6)$$

in which  $[^{125}\text{I-Ang II}_{\text{tissue}}]$  and  $[^{125}\text{I-Ang II}_{\text{art}}]$  are the steady-state concentrations of  $^{125}\text{I-Ang II}$  in cardiac tissue (cpm/g) and aortic blood plasma (cpm/mL) respectively, and  $[^{125}\text{I-Ang I}_{\text{art}}]$  is the steady-state concentration of  $^{125}\text{I-Ang I}$  in aortic blood plasma (cpm/mL), all during the constant infusion of  $^{125}\text{I-Ang I}$ .

Some of the Ang I and II in cardiac tissue may originate from Ang I and II that is generated by the action of circulating renin with circulating angiotensinogen during the passage of blood from the arterial to the venous end of the coronary circulation. This Ang I-generating capacity was calculated as follows:

$$\text{AGC}_{\text{plasma}} = [\text{Ren}] \times [\text{Aog}] / (K_M + [\text{Aog}]), \quad (7)$$

in which  $\text{AGC}_{\text{plasma}}$  is the Ang I-generating capacity of plasma (fmol Ang I/mL per minute),  $[\text{Ren}]$  is the renin concentration of plasma (fmol Ang I/mL per minute),  $[\text{Aog}]$  is the angiotensinogen concentration of plasma (pmol/mL) and  $K_M$  is the Michaelis-Menten constant for the reaction of porcine renin with porcine angiotensinogen, which equals 420 pmol/mL.<sup>40</sup> The cardiac tissue level of Ang I that originates from the Ang I generating capacity of plasma as calculated as follows:

$$[\text{Ang I}_{\text{tissue from AGC}_{\text{plasma}}}] = R_2 / R'_2 \times \text{AGC}_{\text{plasma}} \times t, \quad (8)$$

in which  $[\text{Ang I}_{\text{tissue from AGC}_{\text{plasma}}}]$  is the Ang I concentration in cardiac tissue (fmol/g) originating from the Ang I-generating capacity of plasma,  $t$  is the coronary blood transit time, which equals 0.1 minute,<sup>154</sup>  $R_2$  is given by equation (4), and  $R'_2$  is defined as follows:

$$R'_2 = [^{125}\text{I-Ang I}_{\text{ven}}] / [^{125}\text{I-Ang I}_{\text{art}}], \quad (9)$$

in which  $[^{125}\text{I-Ang I}_{\text{ven}}]$  and  $[^{125}\text{I-Ang I}_{\text{art}}]$  are the steady-state concentrations (cpm/mL) of

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$^{125}\text{I}$ -Ang I in coronary venous and aortic blood plasma respectively, both during the constant infusion of  $^{125}\text{I}$ -Ang I.  $R'_2$  represents the fraction of arterial Ang I that reaches the coronary vein, and corresponds with  $R_2$  in equation (3). Equation (8) does not account for the conversion and degradation of Ang I during the passage of blood from the arterial to the venous end of the coronary vascular bed. If, however, the Ang I that is produced by the Ang I-generating capacity of plasma in the coronary circulation is exposed to ACE and the degrading enzymes during the full period of blood transit, in the same way as arterially delivered Ang I, the factor  $R_2/R'_2$  in equation (8) has to be reduced to  $R_2$ . In reality, the fractional conversion and degradation of plasma Ang I generated in the coronary circulation are probably lower than the fractional conversion and degradation of arterially delivered Ang I. By taking the  $R_2$ -to- $R'_2$  ratio, equation (8) gives a maximum value of [Ang I<sub>tissue</sub> from AGC<sub>plasma</sub>].

The cardiac tissue level of Ang II that originates from the Ang I-generating capacity of plasma was calculated as follows:

$$[\text{Ang II}_{\text{tissue}} \text{ from AGC}_{\text{plasma}}] = (R'_3 \times R_2/R'_2) \times \text{AGC}_{\text{plasma}} \times t, \quad (10)$$

in which  $R'_3$  corresponds with  $R_3$  in equation (5) and is defined as follows:

$$R'_3 = ([^{125}\text{I}\text{-Ang II}_{\text{ven}}]/[^{125}\text{I}\text{-Ang I}_{\text{art}}]) - (R'_1 \times [^{125}\text{I}\text{-Ang II}_{\text{art}}]/[^{125}\text{I}\text{-Ang I}_{\text{art}}]). \quad (11)$$

In equation (11),  $[^{125}\text{I}\text{-Ang II}_{\text{ven}}]$  and  $[^{125}\text{I}\text{-Ang II}_{\text{art}}]$  are the steady-state concentrations (cpm/mL) of  $^{125}\text{I}$ -Ang II in coronary venous and aortic blood plasma respectively, and  $[^{125}\text{I}\text{-Ang I}_{\text{art}}]$  is the steady-state concentration (cpm/mL) of  $^{125}\text{I}$ -Ang I in aortic blood plasma, all during the constant infusion of  $^{125}\text{I}$ -Ang I.  $R'_1$  is defined as follows:

$$R'_1 = [^{125}\text{I}\text{-Ang II}_{\text{ven}}]/[^{125}\text{I}\text{-Ang II}_{\text{art}}], \quad (12)$$

in which  $[^{125}\text{I}\text{-Ang II}_{\text{ven}}]$  and  $[^{125}\text{I}\text{-Ang II}_{\text{art}}]$  are the steady-state concentrations (cpm/mL) of  $^{125}\text{I}$ -Ang II in coronary venous and aortic blood plasma respectively, both during the constant infusion of  $^{125}\text{I}$ -Ang II.  $R'_1$  represents the fraction of arterial Ang II that reaches the coronary vein, and corresponds with  $R_1$  in equation (1). Equation (10) gives a maximum value of [Ang II<sub>tissue</sub> from AGC<sub>plasma</sub>], for the same reasons as discussed with regard to equation (8).

### Statistical analysis

Data are expressed as means  $\pm$  SD, except when indicated otherwise. Differences in plasma levels of renin-angiotensin system components between untreated and captopril-treated pigs were tested by Student's *t* test. Differences in cardiac tissue levels of renin-angiotensin system components between untreated and captopril-treated pigs were tested by multiple analysis of variance (MANOVA). Statistical significance was accepted for  $p < 0.05$ .

## RESULTS

### Hemodynamic effects of $^{125}\text{I}$ -labeled angiotensins

Baseline heart rate, cardiac output and mean arterial pressure did not differ

between untreated and captopril-treated pigs (Table 2). Infusions of either  $^{125}\text{I}$ -Ang I or  $^{125}\text{I}$ -Ang II did not affect any of these parameters (data not shown), which is in agreement with previous studies.<sup>1,30,183</sup>

**Table 2.** Baseline hemodynamic parameters and steady-state arterial plasma levels of  $^{125}\text{I}$ -labeled angiotensin I and angiotensin II during infusion of  $^{125}\text{I}$ -angiotensin I or II in untreated and captopril-treated pigs

Treatment Group	Mean Arterial Pressure (mm Hg)	Heart Rate (beats/min)	Cardiac Output (L/min)	$^{125}\text{I}$ -Ang I Conc. (cpm/mL)	$^{125}\text{I}$ -Ang II Conc. (cpm/mL)	$^{125}\text{I}$ -Ang II-to-I Ratio
<i><math>^{125}\text{I}</math>-Ang I infusion</i>						
Control (n=10)	86±12	123±12	2.5±0.5	1350±380	960±390	0.71±0.22
Captopril (n=10)	85±4	112±15	2.7±0.6	1960±720*	240±210*	0.11±0.04*
<i><math>^{125}\text{I}</math>-Ang II infusion</i>						
Control (n=4)	78±13	110±15	2.3±0.7	-	1960±530	-

The plasma levels of  $^{125}\text{I}$ -Ang I and II presented here were measured in blood samples taken at the end of a 10-minute infusion period. Data are means ± SD. Ang, angiotensin; cpm, counts per minute. \*  $p < 0.01$ , for difference from results in control animals receiving  $^{125}\text{I}$ -Ang I infusion (unpaired Student's *t* test).

*$^{125}\text{I}$ -labeled angiotensins in cardiac tissue and blood plasma*

The steady-state levels of  $^{125}\text{I}$ -Ang I and II in aortic plasma, during constant infusion of  $^{125}\text{I}$ -Ang I or II, are shown in Table 2.  $^{125}\text{I}$ -Ang II in plasma was lower and  $^{125}\text{I}$ -Ang I was higher in the captopril-treated pigs than in the controls. The  $^{125}\text{I}$ -Ang II to I ratio in plasma, which is a measure of the degree of ACE inhibition, fell from 0.71 to 0.11 after captopril.

Table 3 gives the R-values as defined by equations (2), (4), (6), (9), (11) and (12) and calculated from the steady-state  $^{125}\text{I}$ -Ang I and II levels in cardiac tissue (cpm/g), coronary venous plasma (cpm/mL) and aortic plasma (cpm/mL), during  $^{125}\text{I}$ -Ang I or II infusions.

Following captopril treatment, the coronary venous plasma concentration of  $^{125}\text{I}$ -Ang II that is derived, by conversion, from arterially delivered  $^{125}\text{I}$ -Ang I fell from 33% to 4% of the  $^{125}\text{I}$ -Ang I concentration in arterial plasma. This demonstrates effective ACE inhibition in the coronary vascular bed.

In the control group the  $^{125}\text{I}$ -Ang II concentration in cardiac tissue during  $^{125}\text{I}$ -Ang II infusion, was 75% of its concentration in arterial plasma. In contrast, the cardiac tissue concentration of  $^{125}\text{I}$ -Ang I during  $^{125}\text{I}$ -Ang I infusion was less than 4% of its concentration in arterial plasma. The tissue concentration of  $^{125}\text{I}$ -Ang II that was derived, by conversion, from arterially delivered  $^{125}\text{I}$ -Ang I was 25% of the  $^{125}\text{I}$ -Ang I concentration in arterial plasma. This percentage was much lower, less than 2%, in the captopril-treated group, which is again an indication of effective blockade of Ang I-to-II conversion.

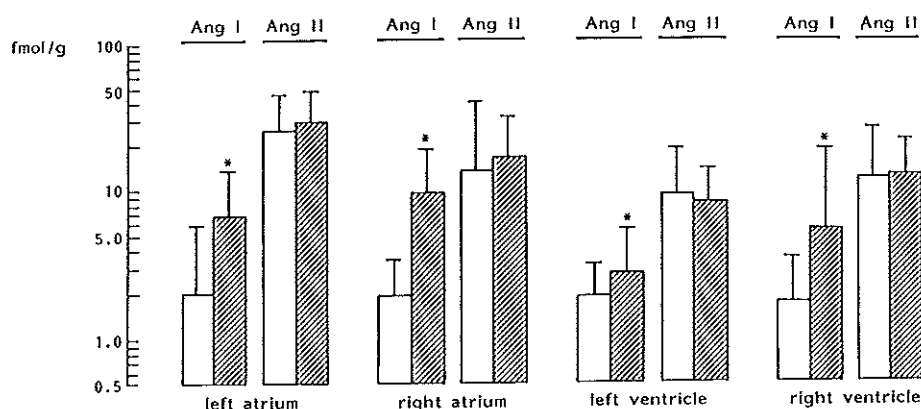
**Table 3.** R-values defined by equations (2), (4), (6), (9), (11) and (12) and calculated from the  $^{125}\text{I}$ -angiotensin I and II levels in cardiac tissue, coronary venous blood plasma and arterial blood plasma during  $^{125}\text{I}$ -angiotensin I or II infusions

	CARDIAC TISSUE			CORONARY VENOUS PLASMA		
	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	R' <sub>1</sub>	R' <sub>2</sub>	R' <sub>3</sub>
Control	0.75 (0.71-0.81) (n=3)	<0.04 (n=3)	0.23 (0.12-0.37) (n=3)	0.52±0.17 (n=4)	0.41±0.14 (n=10)	0.32±0.22 (n=10)
Captopril	ND	<0.02 (n=3)	<0.02 (n=3)	ND	0.58±0.15* (n=10)	0.04±0.05* (n=10)

The calculations were made from the tissue and plasma levels measured in tissue and blood samples taken at the end of a 60-minute infusion period. R<sub>1</sub> and R'<sub>1</sub> represent the concentrations of angiotensin II in cardiac left ventricular tissue and coronary venous plasma respectively, derived from arterially delivered angiotensin II and expressed as a fraction of the concentration of angiotensin II in aortic plasma. R<sub>2</sub>, R<sub>3</sub>, R'<sub>2</sub> and R'<sub>3</sub> represent the concentrations of angiotensin I in tissue, angiotensin II in tissue, angiotensin I in coronary venous plasma and angiotensin II in coronary venous plasma, respectively, all derived from arterially delivered angiotensin I and expressed as a fraction of the concentration of angiotensin I in aortic plasma. Data are means ± SD or means and ranges. ND, not done. \* p < 0.01 for difference from results in control animals (unpaired Student's t test).

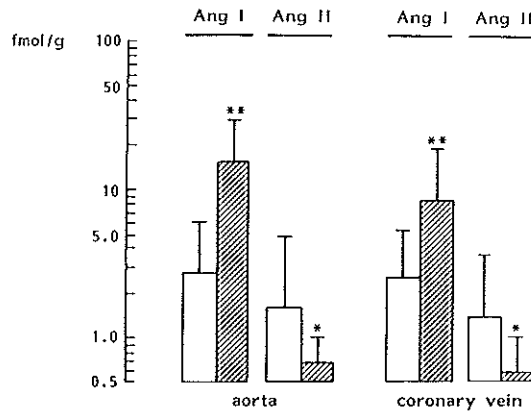
#### *Endogenous angiotensins in cardiac tissue and plasma*

The levels of endogenous Ang I and II in cardiac tissue and blood plasma are shown in Figures 2 and 3. Plasma Ang I and II in untreated pigs were within the normal range.<sup>31</sup> No significant differences were observed between aortic and coronary venous plasma.



**Figure 2.** Angiotensin I and II levels in cardiac tissue of untreated (open bars, n=10) and captopril-treated (hatched bars, n=10) pigs. Values are expressed as geometric means and SD. \* p < 0.01 for difference from control animals (MANOVA).

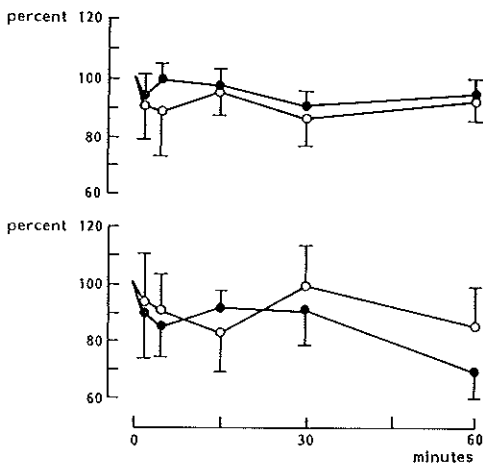
**Figure 3.** Angiotensin I and II levels in blood plasma of untreated (open bars, n=14) and captopril-treated (hatched bars, n=10) pigs. Values are expressed as geometric means and SD. \*  $p < 0.05$ , \*\*  $p < 0.01$  for difference from control animals (unpaired Student's *t* test).



The tissue levels in the various parts of the heart were not significantly different both in the captopril-treated and untreated pigs, but there were marked differences between tissue and plasma. The tissue concentration of Ang II in the untreated group was about 5 times the plasma concentration of Ang II. In addition, the tissue concentration of Ang II was more than 5 times the concentration of Ang I, whereas in plasma Ang II was lower than Ang I.

Ang I rose after captopril both in tissue and in plasma. Ang II in tissue did not change after captopril, whereas in plasma Ang II fell to values close to the detection limit of the assay.

Routinely, as described in the Methods section, Ang I and II were measured in cardiac tissue that was frozen within 15 seconds after the heart had been stopped and removed from the body. When cardiac tissue was kept at 37°C for 1 hour after the heart had been removed from the body, the tissue levels of Ang I and II were virtually constant during this period and they were similar to the routinely measured levels (Figure 4).



**Figure 4.** Ex-vivo changes of angiotensin I (closed circles) and II (open circles) at 37°C in cardiac tissue of untreated (upper panel) and captopril-treated (lower panel) pigs. Values (means and SD of 4 experiments) are expressed as a percentage of the level in tissue samples frozen within 15 sec after removal of the heart from the body.

*Renin and angiotensinogen in cardiac tissue and blood plasma*

The tissue levels of renin and angiotensinogen in the various parts of the heart were not significantly different both in the captopril-treated animals and in controls (Table 4). The tissue concentration of renin (expressed per g tissue) was similar to the plasma concentration (expressed per mL). This suggests that the presence of renin in tissue is not restricted to the extracellular fluid compartment. In contrast, the tissue concentration of angiotensinogen (expressed per g tissue) was 10-30% of the plasma concentration (expressed per mL).

**Table 4.** Renin, angiotensinogen, and angiotensin II-to-I ratio in cardiac tissue and arterial blood plasma in untreated (n=14) and captopril-treated (n=10) pigs

Treatment Group	Aortic Plasma	Left Atrium	Right Atrium	Left Ventricle	Right Ventricle
Renin, (fmol Ang I/mL)/minute or (fmol Ang I/g)/minute					
Control	26±34	38±25	40±17	31±16	25±13
Captopril	78±39*	114±61†	101±43†	107±52†	85±39†
Angiotensinogen, pmol/mL or pmol/g					
Control	340±72	62±47	47±17	98±39	73±45
Captopril	339±103	41±25†	30±6†	60±42†	44±27†
Ang I-generating capacity, (fmol Ang I/minute)/mL					
Control	12±16				
Captopril	29±14*				
Angiotensin II-to-I ratio					
Control	0.67±0.35	15.9±6.4	8.4±5.0	5.2±2.2	9.4±9.3
Captopril	0.06±0.07**	6.2±5.2†	2.3±2.1†	2.8±1.8†	5.0±7.7†

Data are means ± SD. Ang, angiotensin; g, gram tissue wet weight. \* p<0.05,

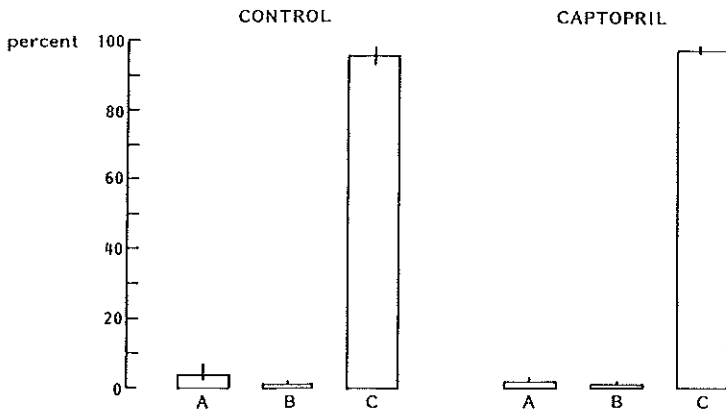
\*\* p<0.01 for difference from control animals (unpaired Student's *t* test). † p<0.01 for difference from control animals (MANOVA).

As expected, the plasma and tissue levels of renin were higher in the captopril-group than in controls. The plasma levels of angiotensinogen were not different between the two groups but the tissue levels of angiotensinogen were lower in the captopril-group, which is an indication of increased substrate consumption due to elevated renin.

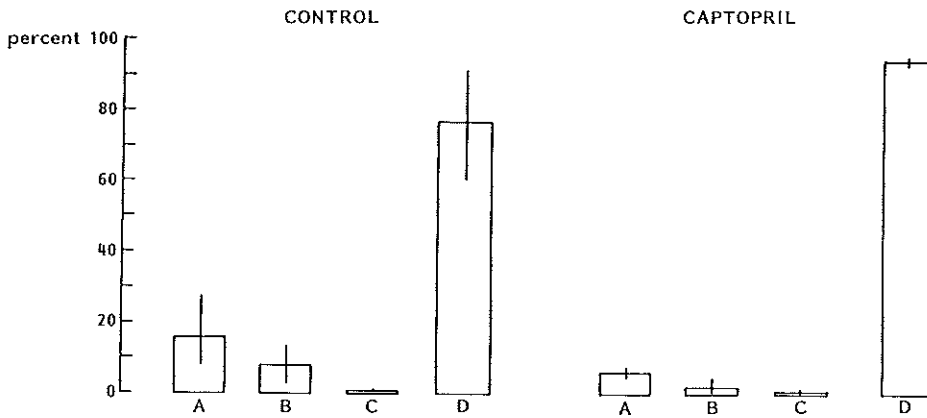
*Contributions of blood-derived angiotensins to the angiotensin levels in cardiac tissue*

Figures 5 and 6 show the results for Ang I and II in left ventricular free wall tissue both in the captopril-treated pigs and in controls. It appears that more than 90% of the Ang I in tissue is synthesized in the tissue itself and is not derived from the circulation. Most of the Ang II in tissue is also synthesized in the tissue and its source is *in-situ* synthesized Ang I rather than Ang I from the circulation. The

contribution of Ang I from the circulation to the cardiac tissue level of Ang II was small in the control group and fell to nearly zero after captopril, due to the blockade of Ang I-to-II conversion.



**Figure 5.** Contribution of the various sources of angiotensin I to the cardiac left ventricular tissue level of this peptide in untreated and captopril-treated pigs. Values (means and ranges of 3 experiments) are expressed as a percentage of the total tissue level of angiotensin I. A, arterially delivered angiotensin I; B, angiotensin I produced by the angiotensin I-generating capacity of plasma in the coronary circulation; C, angiotensin I synthesized *in situ* in cardiac tissue.



**Figure 6.** Contribution of the various sources of angiotensin II to the cardiac left ventricular tissue level of this peptide in untreated and captopril-treated pigs. Values (means and ranges of 3 experiments) are expressed as a percentage of the total tissue level of angiotensin II. A, arterially delivered angiotensin II; B, angiotensin II derived, by conversion, from arterially delivered angiotensin I; C, angiotensin II derived, by conversion, from angiotensin I produced by the angiotensin I-generating capacity of plasma in the coronary circulation; D, angiotensin II derived, by conversion, from angiotensin I that is synthesized *in situ* in cardiac tissue.

## DISCUSSION

In the present study  $^{125}\text{I}$ -Ang I and  $^{125}\text{I}$ -Ang II were infused into the left cardiac ventricle to determine how much of the Ang I and II in cardiac tissue is derived from the circulation, and how much is synthesized *in situ*. It appears that 1) most, if not all, cardiac Ang I is not derived from the circulation, and 2) approximately 75-90% of cardiac Ang II is generated at cardiac tissue sites by conversion of this *in-situ* synthesized Ang I.

In our calculations it was assumed that the fate of the radiolabeled angiotensins in cardiac tissue and the coronary circulation is comparable to that of arterially delivered non-labeled angiotensins. Previous studies in pigs<sup>30</sup> and humans<sup>2</sup> demonstrated that the  $^{125}\text{I}$ -Ang I-to-II conversion rate, both *in vitro* and *in vivo*, is about two times the Ang I-to-II conversion rate, and that the rates at which  $^{125}\text{I}$ -Ang I and Ang I are degraded into peptides other than  $^{125}\text{I}$ -Ang II and Ang II are not different. The  $^{125}\text{I}$ -Ang II and Ang II degradation rates are also not different.<sup>2</sup> The fact that the  $^{125}\text{I}$ -Ang I-to-II conversion rate is somewhat higher than the Ang I-to-II conversion rate may have led us to overestimate the amount of Ang II in cardiac tissue that is derived from Ang I in the circulation. This, therefore, does not invalidate our conclusion that most of the Ang II in cardiac tissue is generated from *in-situ* synthesized, rather than blood-derived, Ang I.

An important methodological aspect of this study relates to the question of whether the radiolabeled and endogenous angiotensin levels we measured in cardiac tissue are representative for the levels *in vivo*. The finding that the *ex-vivo* cardiac tissue levels of Ang I and II remained practically constant at 37°C lends support to the assumption that the measured levels are close to the *in-vivo* levels. The *ex-vivo* half life of  $^{125}\text{I}$ -Ang II in cardiac tissue at 37°C is 30-40 minutes.<sup>183</sup> Thus, the  $^{125}\text{I}$ -Ang II level we measured in cardiac tissue that was frozen within 15 seconds after the  $^{125}\text{I}$ -Ang I and II infusions had been stopped and the heart had been removed from the body, is probably also close to the  $^{125}\text{I}$ -Ang II level *in vivo*. *Ex-vivo* production of Ang II may explain, at least partly, why endogenous cardiac Ang II as opposed to radiolabeled Ang II, remained constant when cardiac tissue was kept at 37°C.

The tissue level of  $^{125}\text{I}$ -Ang I was too low to determine the *ex-vivo* half life of  $^{125}\text{I}$ -Ang I. The  $^{125}\text{I}$ -Ang I level we measured in cardiac tissue that was frozen within 15 seconds after  $^{125}\text{I}$ -Ang I infusion had been stopped and the heart had been removed from the body, was less than 4% of the plasma level in the aorta. That, also *in vivo*, the tissue level of  $^{125}\text{I}$ -Ang I is probably only a small fraction of the level in arterial plasma is supported by the following considerations: 1) the rate of Ang I production in tissue *ex vivo* is probably not higher than *in vivo*, 2) the tissue concentration of Ang I we measured *ex vivo* is close to the level *in vivo*, and 3) Ang I delivery by the aorta contributes to the tissue level of Ang I *in vivo* but not *ex vivo*. The third consideration implies that, if the two other considerations are correct,



arterially delivered Ang I will contribute little to its level in tissue.

Our finding that the tissue-to-plasma ratio of  $^{125}\text{I}$ -Ang II during  $^{125}\text{I}$ -Ang II infusion was much higher than the tissue-to-plasma ratio of  $^{125}\text{I}$ -Ang I during  $^{125}\text{I}$ -Ang I infusion, may suggest that the two peptides are located in different tissue compartments. An earlier study, in which we infused  $^{125}\text{I}$ -Ang I and  $^{125}\text{I}$ -Ang II into the left cardiac ventricle of pigs, provided evidence that most of the  $^{125}\text{I}$ -Ang II in cardiac tissue had been accumulated by the cells via an angiotensin  $\text{AT}_1$ -type receptor-mediated process.<sup>183</sup>  $^{125}\text{I}$ -Ang I does not bind to the  $\text{AT}_1$  receptor and does not enter the cells via this receptor, so that the location of  $^{125}\text{I}$ -Ang I in the tissue may be restricted to the extracellular compartment.

Studies with a modified rat Langendorff heart model, which allowed us to collect interstitial fluid transudate separately from the coronary effluent, showed that the Ang I concentration in interstitial fluid, during perfusion of the heart with Ang I, was only 10-20% of the Ang I concentration of the inflowing perfusion fluid.<sup>40</sup> When this also holds for the  $^{125}\text{I}$ -Ang I we infused into the pigs in the present experiments, and when, as discussed above,  $^{125}\text{I}$ -Ang I in cardiac tissue is restricted to the extracellular compartment, it is easy to understand why the cardiac tissue level of  $^{125}\text{I}$ -Ang I we measured in the present study was so low.

The conclusion that  $^{125}\text{I}$ -Ang I, and therefore also the arterially delivered non-labeled Ang I, are localized in the extracellular compartment and that  $^{125}\text{I}$ -Ang II and the arterially delivered non-labeled Ang II are accumulated in the cells via binding to  $\text{AT}_1$  receptors, may also hold for Ang I that is synthesized at tissue sites and for Ang II that originates from this *in-situ* synthesized Ang I. Further studies are needed to settle this issue.

Our observations in nephrectomized pigs demonstrated that, at least in the healthy heart, most if not all of the cardiac Ang I and II is generated by blood-derived renin.<sup>32</sup> The captopril-induced parallel increments in plasma renin and cardiac Ang I, as shown in the present study, support the assumption that, also during captopril-treatment, the cardiac production of Ang I and II depends on blood-derived renin. This, together with the evidence that most of the Ang I and II in cardiac tissue does not originate from the circulation but from local production, suggests a new concept of the renin-angiotensin system. According to this concept, the renin-angiotensin system is not only an endocrine system, with angiotensinogen as the circulating prohormone and Ang II as the circulating hormone. The system also serves to generate Ang II locally, via Ang I, from angiotensinogen in the tissues. Local production of Ang II is in fact the main source of Ang II in cardiac tissue. The studies in the isolated rat heart referred to above, demonstrated that the cardiac production of Ang I depended on the presence of both renin and angiotensinogen in the inflowing perfusion fluid.<sup>40</sup> Thus, the local production of Ang I and II in cardiac tissues, as demonstrated in the present study, probably does not only depend on blood-derived renin but also on blood-derived angiotensinogen.

Recent evidence seems to indicate that high-affinity binding sites for renin are present in the cell membrane fractions of rat cells from different organs including the heart, blood vessels and kidney.<sup>21,33,116,152</sup> Also in the porcine heart, renin was found to be membrane-bound.<sup>32</sup> Cell membrane-binding would be a mechanism by which renin from the circulation is accumulated at certain tissue sites resulting in higher renin concentrations at these sites than in the circulating blood.

Ang II that is generated in cardiac tissue by the conversion of *in-situ* synthesized Ang I may serve important functions. Intracoronary infusion of a specific renin inhibitor in pigs led to reduced cardiac contractility.<sup>182</sup> This was not observed after systemic administration of the inhibitor, despite the fact that both the systemic and intracoronary infusions had caused virtually complete suppression of circulating Ang II.

In view of the possibility that the beneficial effects of ACE inhibitor drugs on cardiac function and structure depend on their effect on cardiac Ang II production, rather than on a decrease in circulating Ang II, it is of interest to note that in our experiments the cardiac tissue level of Ang II, as opposed to its level in plasma, did not fall following ACE inhibition by captopril. Experiments in rats have also shown that ACE inhibitor treatment has a greater effect on circulating Ang II than on cardiac Ang II.<sup>20</sup> Several explanations can be proposed. Firstly, the dose of captopril may not have been high enough to obtain complete blockade of cardiac ACE. In this case a compensatory increase in Ang I production might overcome the blockade of ACE. The increased tissue concentration of Ang I together with the decreased tissue concentration of angiotensinogen, as observed in the present study, are indeed indications that the cardiac production of Ang I was increased. Secondly, captopril may not reach the tissue sites of Ang II production. Ang II may be formed within the cells.<sup>42</sup> Thirdly, enzymes other than ACE may be involved in the Ang I-to-II conversion in cardiac tissue.<sup>177</sup>

The results of the present study, together with our recent observations on the AT<sub>1</sub> receptor-mediated cardiac uptake of Ang II and its intracellular half life<sup>183</sup> also raise the interesting possibility that AT<sub>1</sub> receptor antagonist drug treatment may potentiate the therapeutic response to ACE inhibition. Both treatment modalities tend to reduce the formation of complexes of the AT<sub>1</sub> receptor with its agonist Ang II and in both cases this is counteracted by a compensatory response of stimulated renin and Ang I production. However, AT<sub>1</sub> receptor-mediated endocytosis of Ang II may protect this peptide from rapid degradation to which extracellular Ang II is subjected, so that AT<sub>1</sub> receptor blockade may result in increased degradation of Ang II in the tissues. This is also supported by the finding that the cardiac Ang II-to-I ratio was decreased in rats by treatment with the AT<sub>1</sub> receptor antagonist losartan, which had no effect on cardiac ACE.<sup>22</sup> It is therefore possible that, for a given increment of Ang I, the formation of complexes between the AT<sub>1</sub> receptor and Ang II is more reduced by AT<sub>1</sub> receptor antagonist drugs than by ACE inhibitors and that combined use of the two classes of drugs may

### *Uptake and production of angiotensins by the heart*

result in decreased tissue levels of Ang II, an effect we did not see with ACE inhibitor alone. This could conceivably have important implications in the clinical use of these classes of drugs in combination.

Further studies on the effects of ACE inhibitors, AT<sub>1</sub> receptor antagonists and renin inhibitors on the local production of Ang I and II in cardiac and vascular tissues along the lines of the present study will clarify the pathophysiological significance of Ang II production in cardiovascular tissues and may help to better define the place of these drugs in the management of heart failure and hypertension.

*References are presented in the general reference list.*



## *Chapter 6*

**ASSESSMENT OF THE ROLE OF  
THE RENIN-ANGIOTENSIN  
SYSTEM IN CARDIAC  
CONTRACTILITY UTILIZING THE  
RENIN INHIBITOR REMIKIREN.**

*British Journal of Pharmacology.*

1996; 117: 891-901.

## SUMMARY

*The role of the renin-angiotensin system in the regulation of myocardial contractility is still debated. In order to investigate whether renin inhibition affects myocardial contractility and whether this action depends on intracardiac rather than circulating angiotensin II, the regional myocardial effects of systemic (i.v.) and intracoronary (i.c.) infusions of the renin inhibitor remikiren were compared and related to the effects on systemic haemodynamics and circulating angiotensin II in open-chest anaesthetized pigs (25-30 kg). The specificity of the remikiren-induced effects was tested 1) by studying its i.c. effects after administration of the AT<sub>1</sub> receptor antagonist L-158,809 and 2) by measuring its effects on contractile force of isolated porcine cardiac trabeculae. Consecutive 10 min i.v. infusions of remikiren were given at 2, 5, 10 and 20 mg.min<sup>-1</sup>. Mean arterial pressure (MAP), cardiac output (CO), heart rate (HR), systemic vascular resistance (SVR), myocardial oxygen consumption (MVO<sub>2</sub>) and left ventricular (LV) dP/dt<sub>max</sub> were not affected by remikiren at 2 and 5 mg.min<sup>-1</sup>, and were lowered at higher doses. At the highest dose, MAP decreased by 48%, CO by 13%, HR by 14%, SVR by 40%, MVO<sub>2</sub> by 28% and LV dP/dt<sub>max</sub> by 52% (mean values; P<0.05 for difference from baseline, n=5). The decrease in MVO<sub>2</sub> was accompanied by a decrease in myocardial work (MAP × CO), but the larger decline in work (55% vs. 28%; P<0.05) implies a reduced myocardial efficiency (MAP × CO)/MVO<sub>2</sub>. Consecutive 10 min i.c. infusions of remikiren were given at 0.2, 0.5, 1, 2, 5 and 10 mg.min<sup>-1</sup>. MAP, CO, MVO<sub>2</sub> and LV dP/dt<sub>max</sub> were not affected by remikiren at 0.2, 0.5 and 1 mg.min<sup>-1</sup>, and were lowered at higher doses. At the highest dose, MAP decreased by 31%, CO by 26%, MVO<sub>2</sub> by 46%, and LV dP/dt<sub>max</sub> by 43% (mean values; P<0.05 for difference from baseline, n=6). HR and SVR did not change at any dose. Thirty minutes after a 10 min i.v. infusion of the AT<sub>1</sub> receptor antagonist L-158,809 at 1 mg.min<sup>-1</sup>, consecutive 10 min i.c. infusions (n=5) of remikiren at 2, 5 and 10 mg.min<sup>-1</sup> did no longer affect CO and MVO<sub>2</sub>, and decreased LV dP/dt<sub>max</sub> by maximally 27% (P<0.05) and MAP by 14% (P<0.05), which was less than without AT<sub>1</sub> receptor blockade (P<0.05). HR and SVR remained unaffected. Plasma renin activity and angiotensin I and II were reduced to levels at or below the detection limit at doses of remikiren that were not high enough to affect systemic haemodynamics or regional myocardial function, both after i.v. and i.c. infusion. Remikiren (10<sup>-10</sup> to 10<sup>-4</sup> M) did not affect contractile force of isolated porcine cardiac trabeculae precontracted with noradrenaline. In trabeculae that were not precontracted no decrease in baseline contractility was observed with remikiren in concentrations up to 10<sup>-5</sup> M, whereas at 10<sup>-4</sup> M baseline contractility decreased by 19% (P<0.05). Results show that with remikiren i.v., at the doses we used, blood pressure was lowered primarily by vasodilatation and with remikiren i.c. by cardiac depression. The blood levels of remikiren required for its vasodilator action are lower than the levels affecting cardiac contractile function. A decrease in circulating angiotensin II does not appear to be the sole explanation for these haemodynamic responses. Data support the contention that myocardial contractility is increased by renin-dependent angiotensin II formation in the heart.*

## ACKNOWLEDGEMENTS

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## INTRODUCTION

The renin-angiotensin system (RAS) plays an important role in the regulation of blood pressure and body fluid volumes. An early and rate-limiting step in the synthesis of angiotensin (Ang) II, the active end-product of the RAS, is the cleavage of angiotensinogen by renin. This results in the formation of the inactive decapeptide Ang I. Angiotensin-converting enzyme (ACE) catalyzes the conversion of Ang I to Ang II. It is now generally believed that these reactions occur both in the circulating blood and at tissue sites.<sup>1,17,31</sup>

Blockers of the formation of Ang II, the ACE inhibitors in particular, are at present widely used for the treatment of hypertension and congestive heart failure. Renin inhibitors are also effective blood pressure lowering agents both in humans<sup>87,95,178,179,189</sup> and in experimental animals.<sup>27,61,62,98,143,174,191,192,196,197</sup> However, due to the low oral bioavailability of the renin inhibitors studied so far, these agents are not widely used.

ACE inhibitors lower arterial blood pressure through a reduction in peripheral vascular resistance, most likely mediated by inhibition of Ang II synthesis. The favourable effects of ACE inhibition in heart failure are not yet fully understood. It has been suggested that they are unrelated, at least in part, to the reduction in systemic vascular resistance.<sup>107</sup> A local cardiac RAS has been postulated,<sup>32,103</sup> and ACE inhibitors therefore may exert their effect through interference with cardiac Ang II production. Ang II is known to have chronotropic and inotropic effects<sup>99</sup> and may have effects on myocardial hypertrophy and remodelling.<sup>145</sup>

ACE also acts on substrates other than Ang I, including bradykinin and substance P.<sup>56</sup> On the basis of experiments with the bradykinin antagonist Hoe 140, it has been claimed that some of the beneficial effects of ACE inhibitors in heart failure are mediated through bradykinin accumulation.<sup>73</sup>

Little information is available on the cardiac effects of renin inhibitors. In early studies on the vasodilator activity of peptidic renin inhibitors, both increases and decreases in cardiac output were observed.<sup>69,111,143,204</sup> There is, however, some doubt as to whether these effects were caused by RAS blockade. More recent studies using non-peptidic renin inhibitors have focused mainly on their ability to lower blood pressure and do not address cardiac effects. The blood pressure lowering effects of these inhibitors appear to be caused by inhibition of the RAS.<sup>27,61,63,174,196,197</sup> It is still a matter of debate whether this involves RAS inhibition in the circulation or at tissue sites.<sup>61,62,178</sup>

The aim of the present study was to investigate whether renin inhibition affects myocardial contractility and whether this action depends on intracardiac rather than circulating Ang II. We therefore compared the myocardial effects of systemic and intracoronary infusions of remikiren, and related these regional effects with systemic haemodynamic responses and the effects on the circulating RAS. In order to determine whether remikiren's effect on cardiac contractility is indeed mediated

via Ang II, the intracoronary effects of the inhibitor were also evaluated after systemic administration of the highly potent and selective AT<sub>1</sub> receptor antagonist L-158,809.<sup>23,156</sup> The haemodynamic responses to Ang II are known to be AT<sub>1</sub> receptor-dependent.<sup>156</sup> All studies were performed in open-chest anaesthetized pigs; remikiren is a potent inhibitor of human renin, but it also inhibits porcine renin.<sup>30</sup>

## MATERIALS AND METHODS

### *General*

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

Cross-bred Landrace x Yorkshire pigs (Hedelse Varkens Combinatie; wt 25-30 kg) of either sex were used. The effects of incremental intravenous (i.v.) and intracoronary (i.c.) doses of remikiren (Ro 42,5892) were studied in 5 and 6 animals, respectively. The effects of incremental i.c. doses of remikiren after AT<sub>1</sub> receptor blockade with L-158,809 were studied in 5 animals.

### *Instrumentation*

Animals were sedated with an intramuscular injection of 20 mg.kg<sup>-1</sup> ketamine (AUV), and anaesthetized with 20 mg.kg<sup>-1</sup> sodium pentobarbitone (Apharma) 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 (1:2). Respiratory rate and tidal volume were adjusted to keep arterial blood gases (ABL3, Radiometer) within the physiological range: 7.35 < pH < 7.45; 35 mmHg < PCO<sub>2</sub> < 45 mmHg and 100 mmHg < PO<sub>2</sub> < 160 mmHg.

A 7 French (Fr) catheter was placed in the superior caval vein for infusion of 10-15 mg kg<sup>-1</sup>.h<sup>-1</sup> sodium pentobarbitone to maintain a constant depth of anaesthesia. Catheters were also placed in the superior caval vein for administration of haemaccel (Behringwerke A.G) to replace blood withdrawn during sampling and for intravenous infusion of remikiren, L-158,809 or Ang II. The femoral arteries were cannulated with 8Fr catheters, which were advanced into the descending aorta to measure central aortic blood pressure and to withdraw blood samples for blood gas analysis and measurements of renin and angiotensins. A 7Fr Sensodyn micromanometer-tipped catheter (B. Braun Medical B.V), inserted via the left carotid artery, was used to measure left ventricular pressure and its first derivative (*LV dP/dt*). Rectal temperature was monitored throughout the experiment and maintained between 37 °C and 38 °C with external heating pads and appropriate coverage of the animal with blankets.

After administration of 4 mg pancuronium bromide (Organon Teknika B.V.) and a midline thoracotomy, the heart was suspended in a pericardial cradle, while the left mammary vessels were ligated and the second left rib was removed to allow further instrumentation. The adventitia surrounding the aorta was dissected free and an electromagnetic flow probe (Skalar) was positioned around the artery for measurement of ascending aortic blood flow (cardiac output, CO). A small segment of the left anterior descending coronary artery



(LADCA) was dissected free for positioning of an electromagnetic flow probe. For intracoronary infusion of remikiren or its vehicle a small canula was placed in the proximal LADCA, just distal of the flow probe. The cardiac vein accompanying the LADCA was cannulated to withdraw blood samples for blood gas analysis.

Regional myocardial segment length changes were measured by sonomicrometry (Triton Technology Inc.) using two pairs of ultrasound crystals (Sonotek Corporation). One pair was positioned in the distribution area of the LADCA, and the other pair in the distribution area of the left circumflex coronary artery (LCXCA).

#### *Infusion experiments*

After a 30-45 min stabilization period following completion of the surgical procedures, baseline measurements of systemic haemodynamic variables, myocardial blood flow and regional segment shortening were made, and blood samples were collected for determination of the arterial and coronary venous oxygen content and the arterial levels of plasma renin activity (PRA) and angiotensin (Ang) I and II. The animals were then subjected to one of the following protocols.

*Protocol 1.* I.v. infusion of remikiren (as the methane sulphonate;  $n = 5$ ) was started at a rate of  $2 \text{ mg}\cdot\text{min}^{-1}$ . At 10 min intervals the infusion rate was increased stepwisely to 5, 10 and  $20 \text{ mg}\cdot\text{min}^{-1}$ , respectively. These infusion rates corresponded to a dosage regimen ranging from  $74 \pm 3$  (mean  $\pm$  s.e. mean) to  $744 \pm 26 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ . After the highest dose had been infused for 10 min, the infusion was stopped, and the study was continued for a 60 min recovery period.

Systemic haemodynamic and regional myocardial variables were measured and blood samples were collected for the determination of PRA, Ang I and Ang II at the end of each infusion step, when a steady-state situation had been reached, and during the recovery period.

*Protocol 2.* In this series of i.c. experiments, remikiren (as the methane sulphonate;  $n = 6$ ) or vehicle, *i.e.* equivalent amounts of methane sulphonic acid ( $n = 4$ ), were infused via the LADCA. Remikiren was infused in incremental doses of 0.2, 0.5, 1, 2, 5 and  $10 \text{ mg}\cdot\text{min}^{-1}$  (10 min). The remikiren infusion rates corresponded to a dosage regimen ranging from  $8.0 \pm 0.1$  to  $379 \pm 6 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ . After the last dose step, the infusion was stopped and the study was continued for a 120 min recovery period. Recordings of systemic haemodynamic and regional myocardial variables were made and blood samples were collected at the end of each infusion step and during the recovery period.

*Protocol 3.* In a second series of i.c. experiments, a 10 min i.v. infusion of the  $\text{AT}_1$  receptor antagonist L-158,809 was given at  $1 \text{ mg}\cdot\text{min}^{-1}$  prior to remikiren administration ( $n=5$ ). To test  $\text{AT}_1$  receptor blockade, 3 i.v. bolus injections of Ang II ( $0.1$ ,  $0.3$  and  $1.0 \mu\text{g}\cdot\text{kg}^{-1}$ ) were given immediately before and after L-158,809 infusion.

Thirty min after L-158,809 administration, remikiren (as the methane sulphonate) was infused in incremental doses of 2, 5 and  $10 \text{ mg}\cdot\text{min}^{-1}$  (10 min). The remikiren infusions corresponded to a dosage regimen ranging from  $74 \pm 4$  to  $368 \pm 20 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ . After the last dose step, the infusion was stopped, and the study was continued for a 60 min recovery period. Then  $\text{AT}_1$  receptor blockade was tested once more by i.v. bolus injections of Ang II. Recordings of systemic haemodynamic and regional myocardial variables were made and blood samples were collected before and after L-158,809 infusion, at the end of each

## Chapter 6

remikiren infusion step, and finally during the recovery period. Recordings of haemodynamic variables were also made during the i.v. Ang II bolus injections.

The i.v. and i.c. doses of remikiren we used were based on a previous study in humans, in whom dose-dependent hypotensive effects of remikiren were observed after 10 min i.v. infusions of 10 and 100 mg.kg<sup>-1</sup>.min<sup>-1</sup>. In the present study in pigs we choose to give higher doses because the IC<sub>50</sub> of remikiren for porcine renin is higher (approximately 80 times) than for human renin.<sup>30</sup>

### *Blood sampling*

Blood samples for measurement of Ang I and Ang II were rapidly drawn (5-10 sec) with a plastic syringe containing the following inhibitors (0.25 ml inhibitor solution in 5 ml blood) 6.25 mM disodium EDTA, 1.25 mM 1,10-phenanthroline and 10<sup>-5</sup> M remikiren (final concentrations in blood).<sup>30</sup> The blood samples were immediately transferred to polystyrene tubes and centrifuged at 3,000g for 10 min at 4 °C. Plasma was stored at -70 °C and extracted within 2 days after collection.

Blood samples for measurement of PRA were collected in polystyrene tubes containing disodium citrate (0.1 ml in 5 ml blood; final concentration 13 mM). The samples were centrifuged at 1,000g for 10 min at room temperature and plasma was stored at -70 °C.

### *Measurement of angiotensin I and II*

Ang I, Ang II and their metabolites were extracted from plasma by reversible adsorption to octadecylsilyl-silica (Sep-Pak C18, Waters) and separated by high-performance liquid chromatography (HPLC), according to the method described by Nussberger *et al.*,<sup>119</sup> with some modifications<sup>1</sup>. Prior to extraction <sup>125</sup>I-Ang I was added to 2 ml plasma as internal standard. Separations were performed on a reversed-phase Nucleosil C18 steel column of 250 x 4.6 mm and 10 µm particle size (Alltech). Mobile phase A was 25% methanol (vol/vol) in 0.085% ortho-phosphoric acid, containing 0.02% sodium azide. Mobile phase B was 75% methanol (vol/vol) in 0.085% orthophosphoric acid, containing 0.02% sodium azide. The flow was 1.5 ml.min<sup>-1</sup> and the working temperature was 45 °C. Vacuum dried SepPak plasma extracts were dissolved in 100 µL mobile phase A 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 16 min. The eluate was collected in 20-sec fractions into polystyrene tubes coated with bovine serum albumin. The fractions containing Ang I and Ang II were neutralized with 0.17 M sodium hydroxide and vacuum dried.

The concentration of <sup>125</sup>I-Ang I in the HPLC fractions was measured in a gamma counter. The concentrations of Ang I and Ang II in the vacuum dried HPLC fractions were measured by radioimmunoassay<sup>1</sup>. The detection limit for Ang I and II were 1 and 0.5 fmol.ml<sup>-1</sup> respectively. The Ang I antiserum crossreacted with Ang-(2-10) nonapeptide (100%), but not (< 0.1%) with Ang II, Ang III, Ang-(3-8) hexapeptide, Ang-(4-8) pentapeptide, Ang-(1-7) heptapeptide, or Ang-(1-4) tetrapeptide. The Ang II antiserum crossreacted with Ang III (55%), Ang-(3-8) hexapeptide (73%) and Ang-(4-8) pentapeptide (100%), but virtually not (< 0.2%) with Ang I, Ang-(2-10) nonapeptide, Ang-(1-7) heptapeptide, or Ang-(1-4) tetrapeptide.

### *Measurement of plasma renin activity*

PRA was determined by measuring the rate of Ang I generation at pH = 7.4 during incubation at 37 °C in the presence of excess Ang I antibody (antibody trapping assay) according to Poulsen and Jorgensen.<sup>129</sup> The incubation mixture consisted of 500 µL sample, 10 µL Ang I antiserum (final dilution 1:10,000) and 10 µL disodium EDTA solution (final concentration 10 mM). Incubation time was 30 min. The trapped Ang I was quantitated by radioimmunoassay, after dilution of the samples in cold 0.15 M Tris buffer, pH=7.4, containing <sup>125</sup>I-Ang I, to give a final antiserum dilution of 1:120,000. The diluted samples were kept for 18 h at 4 °C. The detection limit of the antibody trapping assay was 1 fmol Ang I.ml<sup>-1</sup>.min<sup>-1</sup>.

#### *Experiments with isolated papillary muscle*

Left ventricular cardiac tissue was obtained from four pigs. The pigs had served as controls for a series of acute pharmacological experiments, using sodium pentobarbitone anaesthesia (20 mg.kg<sup>-1</sup>.h<sup>-1</sup>). Tissues pieces of 1 g were placed in ice-chilled Krebs buffer (composition in mM: NaCl 118, KCl 4.7, CaCl<sub>2</sub> 2.5, MgSO<sub>4</sub> 1.2, NaHCO<sub>3</sub> 25, KH<sub>2</sub>PO<sub>4</sub> 1.2 and glucose 8.3), which was gassed with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. Trabeculae of 1 mm thickness were carefully dissected free from surrounding tissue. The trabeculae were mounted in organ baths, kept at 37 °C and containing Krebs buffer gassed with 95% O<sub>2</sub> and 5% CO<sub>2</sub>, and attached to Harvard isometric transducers. Resting tension was set to approximately 20 mN to provide optimal loading conditions. Trabeculae were paced at 1.5 Hz, using field stimulation (3 msec, voltage 20% above threshold).

After a stabilization period of at least 60 min, baseline contractile force was recorded and a concentration-response curve for noradrenaline (10<sup>-10</sup> to 10<sup>-5</sup> M) was obtained to check the viability of the tissue. After washing and restabilization, a dose-response for remikiren (as the methane sulphonate; 10<sup>-10</sup> to 10<sup>-4</sup> M or 0.0729 ng.ml<sup>-1</sup> to 72.9 mg.ml<sup>-1</sup>) was constructed either directly, or after the tissues had been precontracted with 10<sup>-5</sup> M noradrenaline. Dose-response curves with equivalent amounts of methane sulphonic acid were also constructed. Responses are expressed as percentage change from baseline (direct dose-response curve) or as a percentage of the response to 10<sup>-5</sup> M noradrenaline (dose-response curve after precontraction with noradrenaline).

#### *Chemicals*

[Ile<sup>5</sup>]-Ang-(1-10) decapeptide (Ang I), [Ile<sup>5</sup>]-Ang-(1-8) octapeptide (Ang II), and [Ile<sup>5</sup>]-Ang-(2-8) heptapeptide (Ang III) were obtained from Bachem, [Ile<sup>5</sup>]-Ang-(2-10) nonapeptide (Ang-(2-10)) from Senn Chemicals, and [Ile<sup>5</sup>]-Ang-(3-8) hexapeptide (Ang-(3-8)), [Ile<sup>5</sup>]-Ang-(4-8) pentapeptide (Ang-(4-8)), [Ile<sup>5</sup>]-Ang-(1-7) heptapeptide (Ang-(1-7)) and tyrosine from Peninsula Laboratories. Methanol, ortho-phosphoric acid (both analytical grade) and 1,10-phenanthroline were purchased from Merck, methane sulphonic acid from Sigma. Water for high performance liquid chromatography (HPLC) was prepared with a Milli-Q system from Waters. The specific renin inhibitor remikiren (as the methane sulphonate; molecular weight 729) was a gift of dr. W. Fischli (Hoffmann-LaRoche, Basel, Switzerland). Its IC<sub>50</sub> for human renin is 0.7 nM and for porcine renin 50 nM.<sup>30</sup> The AT<sub>1</sub> receptor antagonist L-158,809 (as the tetrazolic acid monohydrate; molecular weight 427.5) was a gift of dr. R.D. Smith (Du Pont Merck Pharmaceutical Company, Wilmington, Del., USA). This antagonist is 50-200 times more potent than losartan (IC<sub>50</sub> = 0.2-0.8 nM).<sup>23</sup>

### Data analysis

Systemic and coronary vascular resistance were calculated as the ratios between mean arterial blood pressure (MAP) and CO and between MAP and coronary blood flow (CBF) respectively, while myocardial work was calculated as the product of MAP and CO. Segment length was measured at end-systole (ESL) and at end-diastole (EDL) in order to calculate systolic segment shortening as  $SS = (EDL - ESL)/EDL \times 100\%$ . Myocardial oxygen consumption ( $MVO_2$ ) was calculated as the product of CBF and the difference in the arterial and coronary venous  $O_2$  contents.<sup>11</sup>

All data are presented as mean and s.e. mean. A one way analysis of variance for repeated measurements was performed to determine the statistical significance of the remikiren- or vehicle-induced changes. Statistical significance was accepted for  $P < 0.05$ .

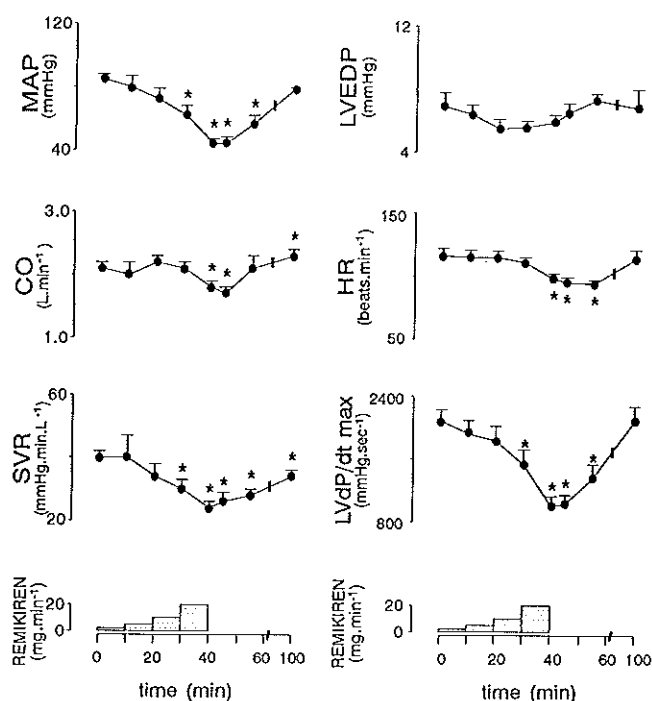
## RESULTS

### Intravenous infusions

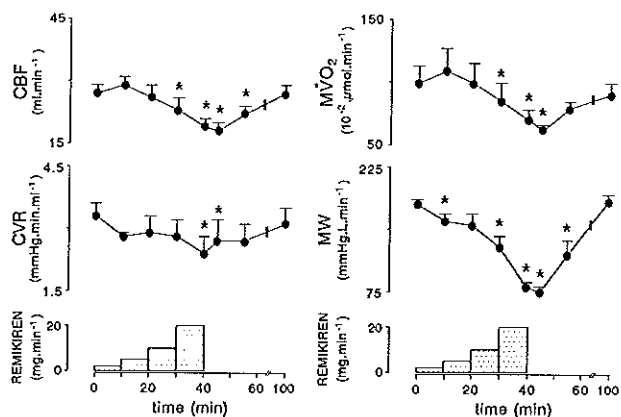
*Systemic haemodynamics, myocardial function and oxygen consumption.* MAP, CO, heart rate (HR), systemic vascular resistance (SVR), LV  $dP/dt_{max}$ , CBF and  $MVO_2$  were not affected by remikiren at 2 and 5  $mg \cdot min^{-1}$  (Figs. 1 and 2), and were lowered at higher doses. At the highest dose, MAP decreased by  $48 \pm 4\%$  ( $P < 0.05$ ) and CO by only  $13 \pm 2\%$  ( $P < 0.05$ ). As a result, SVR decreased by  $40 \pm 4\%$ , suggesting that the decrease in arterial blood pressure was caused predominantly by systemic vasodilation. Arterial pulse pressure (*i.e.*, systolic arterial blood pressure - diastolic arterial blood pressure) and left ventricular end-diastolic pressure (LVEDP) were not affected. Stroke volume also did not change, because the decrease in HR ( $14 \pm 4\%$  at the highest dose,  $P < 0.05$ ) corresponded very closely with the decrease in CO. LV  $dP/dt_{max}$  decreased by maximally  $52 \pm 6\%$  ( $P < 0.05$ ).

CBF and coronary vascular resistance (CVR) decreased by maximally  $34 \pm 8\%$  and  $28 \pm 5\%$  respectively (Fig. 2). Myocardial oxygen extraction did not change; coronary venous oxygen saturation was  $23 \pm 2\%$  at baseline and  $23 \pm 1\%$  after infusion of the highest dose. Consequently, the changes in  $MVO_2$  paralleled the changes in CBF (Fig. 2). The decrease in  $MVO_2$  was accompanied by a decrease in myocardial work, but the larger decline in myocardial work ( $55 \pm 4\%$  vs.  $28 \pm 3\%$ ;  $P < 0.05$ ) implies a reduced myocardial efficiency. EDL, ESL and SS in the distribution areas of the LADCA and the LCXCA were not significantly affected (Table 2).

After the infusions had been stopped all variables started to return to their baseline values. During the 60 min recovery period, systemic vascular resistance increased from  $60 \pm 4\%$  to  $86 \pm 3\%$  of baseline ( $P < 0.05$ ), which was still lower than baseline ( $P < 0.05$ ).



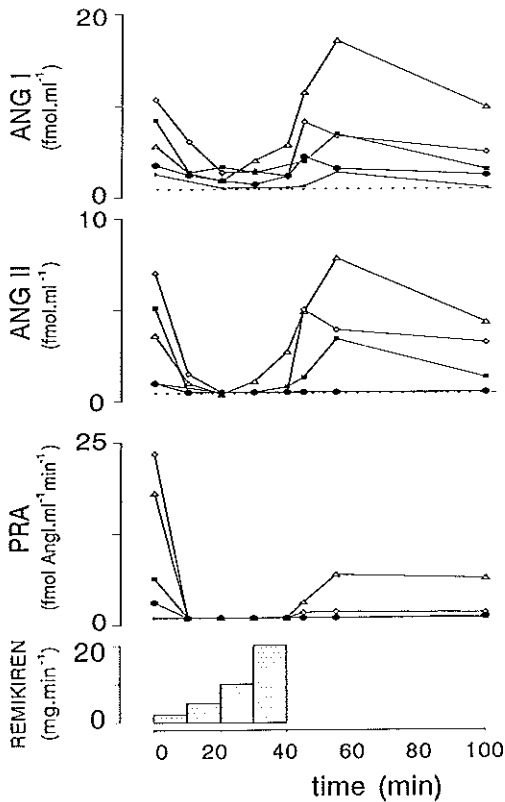
**Figure 1.** Effects of consecutive 10 min intravenous infusions of remikiren (2, 5, 10 and 20 mg.min<sup>-1</sup>) on mean arterial pressure (MAP), cardiac output (CO), systemic vascular resistance (SVR), left ventricular end-diastolic pressure (LVEDP), heart rate (HR) and LV dP/dt<sub>max</sub> in open-chest anaesthetized pigs. The recovery of the various parameters at 15 and 60 min after discontinuation of the infusions is also shown. Data are presented as mean and s.e. mean (n=5). \* P<0.05 vs. baseline.



**Figure 2.** Effects of consecutive 10 min intravenous infusions of remikiren (2, 5, 10 and 20 mg.min<sup>-1</sup>) on coronary blood flow (CBF), coronary vascular resistance (CVR), myocardial oxygen consumption (MVO<sub>2</sub>) and myocardial work (MW) in open-chest anaesthetized pigs. The recovery of the various parameters at 15 and 60 min after discontinuation of the infusions is also shown. Data are presented as mean and s.e. mean (n=5). \* P<0.05 vs. baseline.

*Plasma renin activity and angiotensin I and II levels.* Baseline PRA, Ang I and Ang II levels were in the normal range.<sup>31</sup> At doses of 2 mg.min<sup>-1</sup> and higher, remikiren lowered PRA and plasma Ang II to levels below the detection limit (Fig. 3). Plasma Ang I also fell to low levels, but remained detectable in 4 of the 5 pigs (Fig. 3).

After discontinuation of the infusion, PRA remained close to or below the detection limit during the 60 min post-infusion period in 4 of the 5 pigs. In one pig, PRA returned to approximately 30 percent of its baseline level in the post-infusion period. Plasma Ang I and Ang II increased in the post-infusion period, but remained below baseline in most pigs after 60 min (Fig. 3).

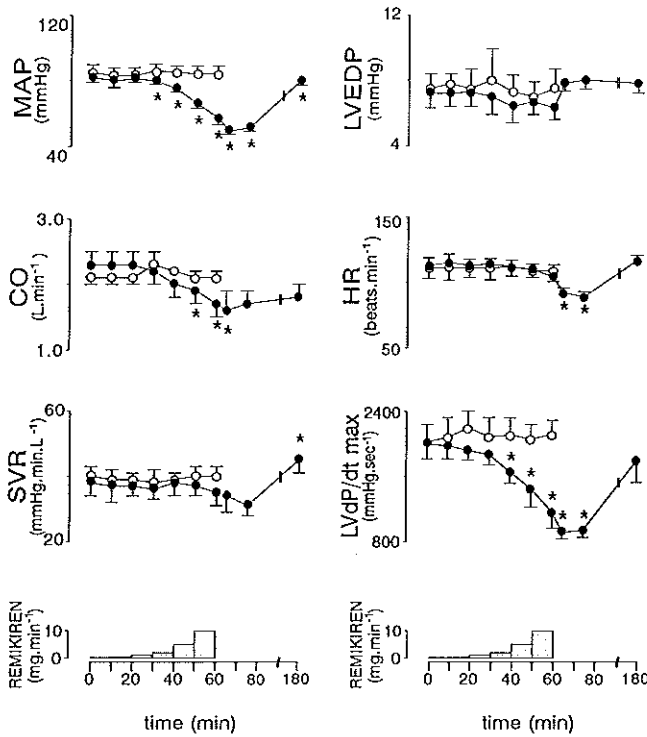


**Figure 3.** Effects of consecutive 10 min intravenous infusions of remikiren (2, 5, 10 and 20 mg.min<sup>-1</sup>) on plasma angiotensin I (ANG I), plasma angiotensin II (ANG II) and plasma renin activity (PRA) in open-chest anaesthetized pigs. The plasma levels of these components at 15 and 60 min after discontinuation of the infusions are also shown. The dotted line represents the limit of detection. Data are presented as individual data points.

*Intracoronary infusions without AT<sub>1</sub> receptor blockade*

*Systemic haemodynamics, myocardial function and oxygen consumption.* Intracoronary infusion of vehicle (methane sulphonic acid) did not cause significant changes in any of the systemic haemodynamic variables or in myocardial function parameters and myocardial oxygen consumption (Figs. 4 and 5).

MAP, CO, LV  $dp/dt_{max}$  and  $MVO_2$  were not affected by remikiren at 0.2, 0.5 and 1  $mg \cdot min^{-1}$  (Figs. 4 and 5), and were lowered at higher doses. At the highest dose, MAP decreased by  $31 \pm 4\%$  ( $P < 0.05$ ) and CO by  $26 \pm 6\%$  ( $P < 0.05$ ). The similar decrease in MAP and CO implies that SVR had not changed. Arterial pulse pressure, HR and LVEDP were also unaffected. LV  $dp/dt_{max}$  started to decrease during infusion of 2  $mg \cdot min^{-1}$  of remikiren, and had fallen to  $57 \pm 6\%$  of the predrug value ( $P < 0.05$ ) after the highest dose had been administered.

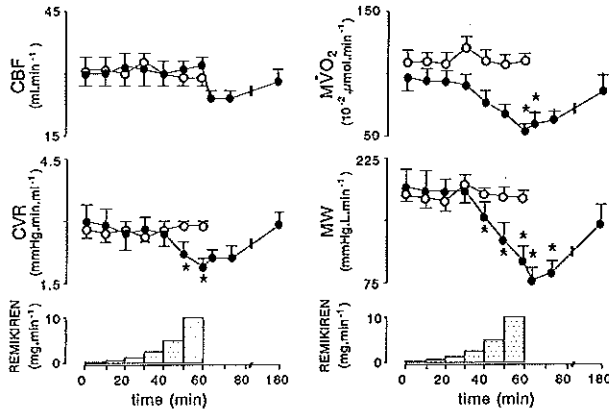


**Figure 4.** Effects of consecutive 10 min intracoronary infusions of remikiren (0.2, 0.5, 1, 2, 5, and 10  $mg \cdot min^{-1}$ ; closed symbols) or its vehicle (methane sulphonic acid in equivalent amounts; open symbols) on mean arterial pressure (MAP), cardiac output (CO), systemic vascular resistance (SVR), left ventricular end-diastolic pressure (LVEDP), heart rate (HR) and LV  $dp/dt_{max}$  in open-chest anesthetized pigs. The recovery of the various parameters at 15 and 120 min after discontinuation of the infusions is also shown. Data are presented as mean and s.e. mean ( $n=6$ ). \*  $P < 0.05$  vs. baseline.

CBF did not change at any dose of remikiren (Fig. 5). This implies that, because of the decrease in MAP, CVR was reduced at the higher doses of remikiren (by maximally  $36 \pm 7\%$ ,  $P < 0.05$ ). Myocardial oxygen extraction decreased significantly; coronary venous oxygen saturation was  $27 \pm 3\%$  at baseline and  $63 \pm 2\%$  after the highest dose ( $P < 0.05$ ).  $MVO_2$  decreased by maximally  $46 \pm 3\%$  ( $P < 0.05$ ) and MW by  $48 \pm 5\%$  ( $P < 0.05$ ). EDL and ESL in the distribution area of the LADCA

increased by maximally  $5 \pm 1\%$  ( $P < 0.05$ ) and  $10 \pm 2\%$  ( $P < 0.05$ ), respectively. SS was lowered at the highest dose of remikiren ( $P < 0.05$ ; Table 2). EDL, ESL and SS in the distribution area of the LCXCA remained unaltered (Table 1).

During the first 15 min post-infusion, HR and LV  $dP/dt_{max}$  decreased further to  $81 \pm 6\%$  ( $P < 0.05$ ) and  $47 \pm 4\%$  ( $P < 0.05$ ) of the predrug value, respectively. After two hours almost all variables had returned to their predrug values (Figs. 4 and 5).



**Figure 5.** Effects of consecutive 10 min intracoronary infusions of remikiren (0.2, 0.5, 1, 2, 5 and 10  $\text{mg}\cdot\text{min}^{-1}$ ; closed symbols) or its vehicle (methane sulphonic acid in equivalent amounts; open symbols) on coronary blood flow (CBF), coronary vascular resistance (CVR), myocardial oxygen consumption ( $\text{MVO}_2$ ) and myocardial work (MW) in open-chest anaesthetized pigs. The recovery of the various parameters at 15 and 120 min after discontinuation of the infusions is also shown. Data are presented as mean and s.e. mean ( $n=6$ ). \*  $P < 0.05$  vs. baseline.

*Plasma renin activity and angiotensin I and II levels.* Baseline PRA, Ang I and Ang II were in the normal range.<sup>31</sup> Already at a dose of  $0.2 \text{ mg}\cdot\text{min}^{-1}$ , remikiren had lowered PRA and plasma Ang II to levels below the limit of detection in 5 of the 6 pigs (Fig. 6). Plasma Ang I also fell to low levels, but remained detectable in most pigs.

In the 2-hour post-infusion period PRA remained below the detection limit in 5 of the 6 pigs; it increased to approximately 50% of baseline in one pig. Plasma Ang II remained below the detection limit during the entire post-infusion period in 4 pigs, whereas in two pigs it had returned to baseline after 60 min. Plasma Ang I rose to baseline levels within 60 min (Fig. 6).

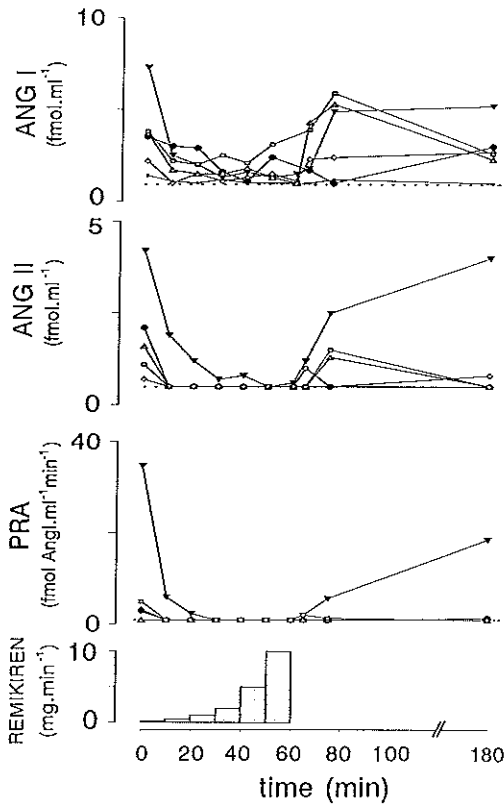
Vehicle (methane sulphonic acid) did not affect PRA, Ang I or Ang II (data not shown).



**Table 2.** Relative segment shortening (%) in distribution areas of LADCA and LCXCA after intravenous or intracoronary administration of remikiren

	baseline	ATI, receptor blockade	0.2	0.5	1.0	2.0	5.0	10.0	20.0	after recovery
<i>i.v. experiments</i>										
LADCA	16.7 ± 2.0	-	-	-	-	15.4 ± 2.6	13.9 ± 1.2	15.0 ± 2.1	12.4 ± 2.1	17.0 ± 2.5
LCXCA	9.9 ± 1.2	-	--	-	-	9.2 ± 1.4	9.3 ± 1.2	8.9 ± 1.1	8.6 ± 1.3	9.4 ± 1.4
<i>i.c. experiments without L-158,809</i>										
LADCA	15.5 ± 1.0	-	14.7 ± 1.1	14.9 ± 0.6	14.2 ± 0.9	13.5 ± 1.0*	12.9 ± 1.1*	11.6 ± 1.0*	-	11.6 ± 1.0*
LCXCA	13.3 ± 1.2	-	13.0 ± 1.2	13.8 ± 1.3	13.2 ± 1.2	11.9 ± 1.2	11.3 ± 1.6	11.0 ± 1.3	-	11.9 ± 0.4
<i>i.c. experiments with L-158,809</i>										
LADCA	15.2 ± 2.3	12.8 ± 2.0	-	-	-	14.4 ± 2.5	13.9 ± 2.4	12.2 ± 2.1	-	10.9 ± 2.4
LCXCA	13.9 ± 0.8	12.8 ± 0.5	-	-	-	11.7 ± 0.8	11.8 ± 0.6	11.5 ± 0.7	-	10.9 ± 0.7

Data are expressed as mean ± s.e. mean, \* P<0.05 vs. baseline. LADCA, left anterior descending coronary artery, LCXCA, left circumflex coronary artery.



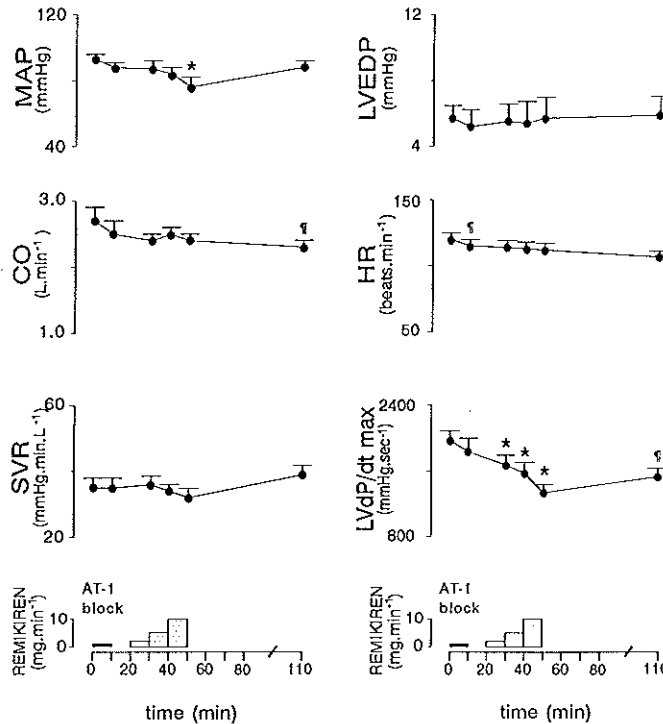
**Figure 6.** Effects of consecutive 10 min intracoronary infusions of remikiren (0.2, 0.5, 1, 2, 5 and 10  $\text{mg}\cdot\text{min}^{-1}$ ; closed symbols) on plasma angiotensin I (ANG I), plasma angiotensin II (ANG II) and plasma renin activity (PRA) in open-chest anaesthetized pigs. The plasma levels of these components at 15 and 120 min after discontinuation of the infusions are also shown. The dotted line represents the limit of detection. Data are presented as individual data points.

#### *Intracoronary infusions with $\text{AT}_1$ receptor blockade*

*Systemic haemodynamics, myocardial function and oxygen consumption.* Before administration of the  $\text{AT}_1$  receptor antagonist L-158,809, a series of i.v. bolus injections of Ang II (0.1, 0.3 and 1.0  $\mu\text{g}\cdot\text{kg}^{-1}$ ) increased MAP, CO, HR and LV  $dP/dt_{\text{max}}$  by maximally  $64\pm 10$ ,  $42\pm 13$ ,  $23\pm 8$  and  $99\pm 12\%$  ( $P<0.05$ ), respectively. Values returned to baseline within 5 minutes after each bolus injection. After i.v. infusion of 10 mg L-158,809 over a 10 min period, the haemodynamic responses to the same Ang II challenge were completely abolished (CO, HR and LV  $dP/dt_{\text{max}}$ ) or suppressed by at least 95% (MAP). At the end of the experiment, after a 60 min washout period, the haemodynamic responses to Ang II were still absent (CO, HR and LV  $dP/dt_{\text{max}}$ ) or suppressed by at least 70% (MAP). These data demonstrate that effective  $\text{AT}_1$  receptor blockade was maintained throughout the experimental protocol.

After administration of L-158,809 HR decreased by  $3\pm 1\%$  ( $P<0.05$ ). None of the other haemodynamic parameters were affected by the  $\text{AT}_1$  receptor antagonist

(Figs. 7 and 8). Subsequent i.c. infusion of remikiren at increasing doses (2, 5 and 10 mg.min<sup>-1</sup>) did not affect CO, SVR, LVEDP or HR (Fig. 7). Relative to its value after L-158,809 administration, MAP decreased by 14±4% (*P*<0.05) at the highest dose of remikiren (10 mg.min<sup>-1</sup>). Arterial pulse pressure did not change. LV *dp/dt*<sub>max</sub> already decreased at the lowest dose of remikiren. At the highest dose it had fallen to 73±5% (*P*<0.05) of its value before administration of the renin inhibitor (Fig. 7).

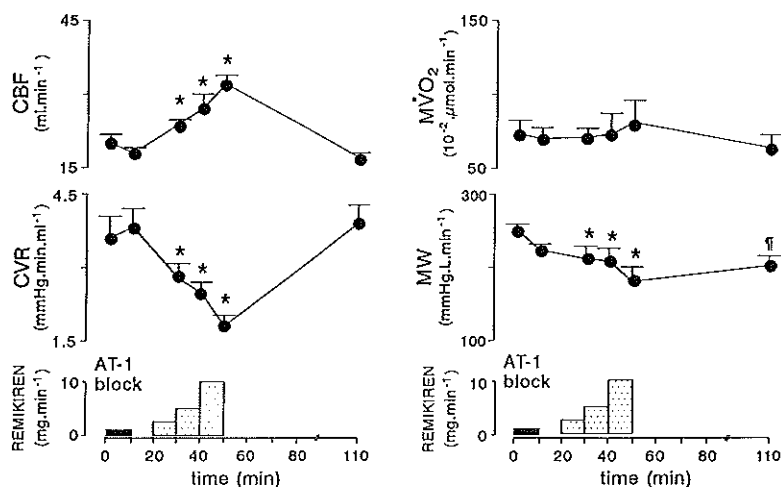


**Figure 7.** Effects of consecutive 10 min intracoronary infusions of remikiren (2, 5 and 10 mg.min<sup>-1</sup>) after AT<sub>1</sub>-receptor blockade with L-158,809 (10 mg i.v.) on mean arterial pressure (MAP), cardiac output (CO), systemic vascular resistance (SVR), left ventricular end-diastolic pressure (LVEDP), heart rate (HR) and LV *dp/dt*<sub>max</sub> in open-chest anaesthetized pigs. The recovery of the various parameters at 60 min after discontinuation of the infusions is also shown. Data are presented as mean and s.e. mean (n=5). \* *P*<0.05 vs. baseline after L-158,809; † *P*<0.05 vs. baseline before L-158,809.

CBF increased by 82±16% (*P*<0.05) at the highest dose of remikiren (Fig. 8). Since MAP was only modestly lowered at the highest dose, it follows that CVR was reduced at all three doses of remikiren (by maximally 52±3%, *P*<0.05). Myocardial oxygen extraction decreased significantly; coronary venous oxygen saturation was 24±3% before remikiren and 49±7% after the highest dose (*P*<0.05). However, due to the concomitant increase in CBF, MVO<sub>2</sub> remained unaffected (Fig. 8). MW fell to 80±4% (*P*<0.05) of its value prior to remikiren administration.

During remikiren administration under AT<sub>1</sub> receptor blockade, EDL and ESL in the distribution area of the LADCA increased in parallel by maximally  $9 \pm 4\%$  and  $11 \pm 3\%$  ( $P < 0.05$ ). Consequently, SS in that area did not change (Table 2). EDL, ESL and SS in the distribution area of the LCXCA remained unaltered (Table 2).

After 60 min of recovery almost all parameters had returned to predrug values (Figs. 7 and 8).

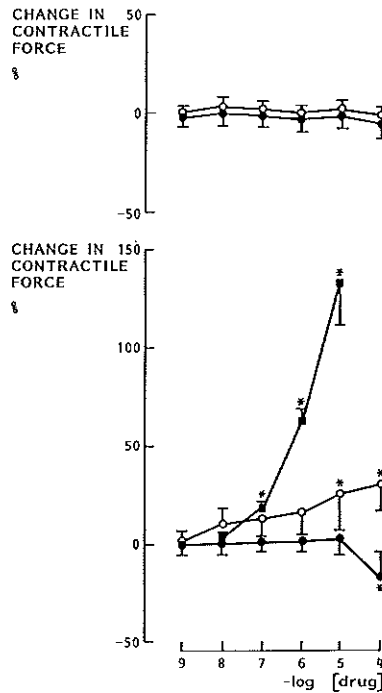


**Figure 8.** Effects of consecutive 10 min intracoronary infusions of remikiren (2, 5 and 10 mg.min<sup>-1</sup>) after AT<sub>1</sub>-receptor blockade with L-158,809 (10 mg i.v.) on coronary blood flow (CBF), coronary vascular resistance (CVR), myocardial oxygen consumption (MVO<sub>2</sub>) and myocardial work (MW) in open-chest anaesthetized pigs. The recovery of the various parameters at 60 min after discontinuation of the infusions is also shown. Data are presented as mean and s.e. mean (n=5). \*  $P < 0.05$  vs. baseline after L-158,809; †  $P < 0.05$  vs. baseline before L-158,809.

*Plasma renin activity and angiotensin I and II levels.* Baseline PRA, Ang I and Ang II were not different from the baseline values found in the previous i.v. and i.c. experiments. No significant changes were observed within the 30 min period after i.v. administration of L-158,809. Already at the lowest dose of remikiren used in these experiments, PRA, Ang I and Ang II reached levels at or below the limit of detection (data not shown).

#### *Experiments with isolated papillary muscle*

Neither vehicle (methane sulphonic acid) nor remikiren had any effect on the contractile force of left ventricular tissue precontracted with noradrenaline (Fig. 9). In tissues that were not precontracted, a slight decrease in baseline contractile force was observed with remikiren at its highest concentration ( $10^{-4}$  M), whereas with vehicle a small dose-dependent increase was seen (Fig. 9).



**Figure 9.** Top panel. Effect of increasing concentrations of remikiren (closed circles) or its vehicle (methane sulphonic acid; open circles) on the contractile force of isolated porcine left ventricular trabeculae precontracted with  $10^{-5}$  M noradrenaline. Data are presented as mean and s.e. mean ( $n=4$ ).

Bottom panel. Effect of increasing concentrations of remikiren (closed circles), its vehicle (methane sulphonic acid; open circles) or noradrenaline (closed squares) on non-precontracted isolated porcine left ventricular trabeculae. Data are presented as mean and s.e. mean ( $n=4$ ). \*  $P < 0.05$  vs. zero drug concentration.

## DISCUSSION

The role of the RAS in cardiac contractility is still uncertain. ACE inhibitors have been reported to reduce cardiac contractility when administered intracoronary, both *in vivo*<sup>65</sup> and *in vitro*.<sup>131</sup> Chronic treatment with the ACE inhibitor cilazapril depresses left ventricular contractile function in spontaneously hypertensive rats.<sup>26</sup> However, ACE inhibitors not only lower Ang II but may also increase bradykinin. Part of their cardiac effects may therefore be attributed to increased bradykinin levels. Especially in isolated hearts, where Ang I is no longer present,<sup>32,104</sup> increased bradykinin might contribute to the negative inotropic effects observed after ACE inhibition. In the present study we therefore examined the cardiac effects of the potent and specific renin inhibitor remikiren in the intact anaesthetized pig, and compared these regional responses with the systemic haemodynamic effects of remikiren. To exclude a non-specific, non-Ang II-dependent effect of the renin inhibitor, experiments were performed with and without Ang II receptor blockade, using the selective  $AT_1$  receptor antagonist L-158,809.

Intravenous infusion of remikiren lowered arterial blood pressure by peripheral vasodilation. The absence of a reflex-mediated increase in heart rate, as observed in the present study, is in agreement with previous reports.<sup>61,95,178</sup> Heart rate even

appeared to decrease at the highest dose of remikiren.

An important finding in the present study is the large decrease in LV  $dP/dt_{max}$  induced by remikiren. This parameter is a widely accepted index of global myocardial contractility. However, LV  $dP/dt_{max}$  is also sensitive to changes in heart rate and pre- and afterload. Because heart rate as well as left ventricular end-diastolic pressure did not change, the decrease in LV  $dP/dt_{max}$  must have been caused by a decrease in myocardial contractility and/or a decrease in diastolic arterial blood pressure. The reduced myocardial efficiency observed after intravenous administration of remikiren supports the former possibility. Further support comes from earlier studies, using the same model, of the second generation dihydropyridines nisoldipine and elgodipine,<sup>50,139</sup> and the potassium channel activator EMD 52692.<sup>140</sup> These agents caused similar decreases in mean arterial blood pressure as observed in the present study, without affecting heart rate and left ventricular end-diastolic blood pressure, and had no significant effect on LV  $dP/dt_{max}$ . It is therefore most likely that remikiren, in addition to its vasodilator action, depresses myocardial contractility at doses higher than 10  $\text{mg}\cdot\text{min}^{-1}$  (or  $370 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ ).

Our observations during intracoronary infusion of remikiren provide further evidence for a decrease in myocardial contractility. Intracoronary infused remikiren, at doses of  $2 \text{ mg}\cdot\text{min}^{-1}$  and higher, caused a decrease in LV  $dP/dt_{max}$  comparable to what was observed after intravenous remikiren at doses higher than  $10 \text{ mg}\cdot\text{min}^{-1}$ . In contrast with remikiren i.v. however, remikiren i.c. lowered cardiac output in parallel with arterial pressure, so that systemic vascular resistance did not change. Thus, the decrease in blood pressure after intracoronary infusion was mainly due to the cardiac effects of remikiren. In addition, intracoronary infused remikiren decreased regional systolic segment shortening in the distribution area of the vessel used for infusion, whereas no such effect was observed during intravenous infusion. It seems therefore that remikiren lowers blood pressure both through peripheral mechanisms (vasodilatation) and cardiac mechanisms (decrease in LV  $dP/dt_{max}$  and cardiac output). The remikiren blood levels required for cardiodepression appear to be higher than the levels causing peripheral vasodilatation. As coronary blood flow is about 4-5% of cardiac output, the cardiodepressant levels are probably at least one order of magnitude higher.

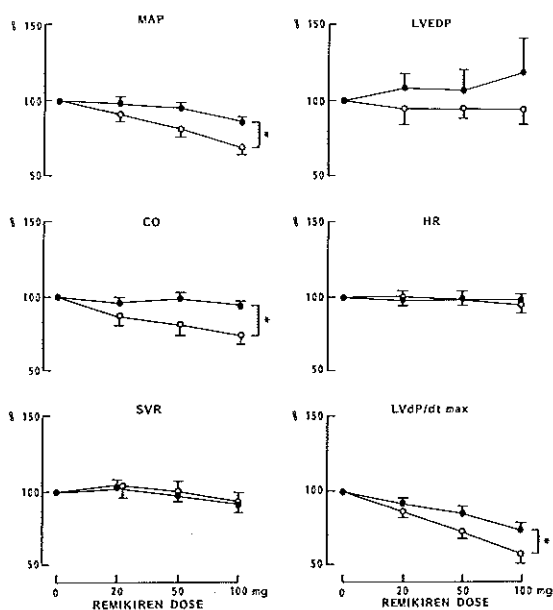
Ang II receptors are known to be present in cardiac tissue,<sup>135</sup> and Ang II has been reported to exert positive inotropic and chronotropic effects, both through actions on the heart itself<sup>99,105</sup> and via facilitation of noradrenergic neurotransmission.<sup>66</sup> Furthermore, there is growing evidence for renin-dependent Ang II formation in the heart,<sup>32</sup> and for the contention that locally generated Ang II, in addition to systemic Ang II, exerts an inotropic effect on cardiac muscle.<sup>105</sup> The cardiodepressive action of renin inhibition we observed is therefore not too surprising.

In patients with heart failure, remikiren has been given intravenously as a 0.3

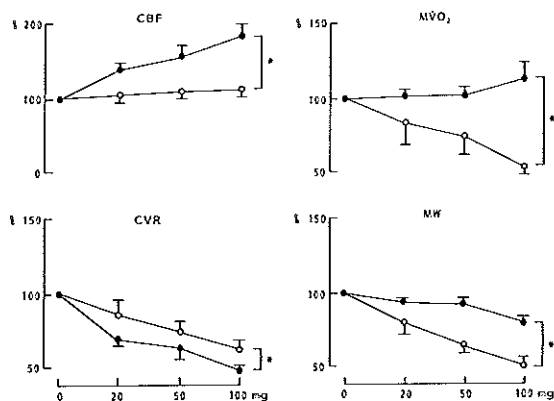
mg/kg bolus followed by infusion at a rate of 0.1 mg/kg per hour.<sup>95</sup> No effect on cardiac output was observed at these doses. The doses of remikiren, however, at which the cardiac effects were most prominent in the present study, were 4-8 times higher than the highest doses used in previous studies to lower blood pressure.<sup>95,178</sup> Because remikiren was given as its methane sulphonate salt, one could argue that, at the high doses we used, methane sulphonate was given in quantities sufficient to affect cardiac contractility. However, intracoronary infusions of methane sulphonic acid alone did not alter blood pressure or cardiac contractility (Fig. 4), nor did methane sulphonic acid reduce contractile force of isolated cardiac tissue (Fig. 9).

The maximal concentrations of remikiren reached in the coronary vascular bed during the infusions (approximately  $10^{-4}$ - $10^{-5}$  M) might be too high to exert only renin-specific effects. We used these high doses because remikiren is approximately 80 times less potent towards porcine renin than towards human renin; to obtain nearly 100% inhibition of porcine renin, concentrations of  $10^{-5}$  M and higher are required.<sup>30</sup> After administration of the  $AT_1$  receptor blocker L-158,809 at a dose (10 mg i.v.) that fully blocked the haemodynamic responses to systemically infused Ang II but had no significant haemodynamic effects by itself, the systemic haemodynamic and myocardial effects of subsequent i.c. infusion of remikiren were either abolished or significantly reduced (Figs. 10 and 11). The small effects of remikiren on blood pressure and LV  $dp/dt_{max}$  (Fig. 10) that were still seen in the presence of L-158,809 are most likely due to a small number of receptors not being blocked by L-158,809, either because the dose was not high enough or because these receptors are located at tissue sites which could not be reached by L-158,809. Remikiren is highly lipophilic and may penetrate more easily into the tissues. Coronary blood flow after i.c. remikiren did not change without  $AT_1$  receptor blockade, and increased during  $AT_1$  receptor blockade. This increase in coronary blood flow might be related to the fact that cardiac contractility was maintained under  $AT_1$  receptor blockade. Because of the increased coronary flow, myocardial oxygen delivery could also be maintained.

Our experiments with isolated cardiac tissue also argue against a non-specific cardiodepressive action of the renin inhibitor (Fig. 9). There is probably little or no Ang II formation in such tissue after it has been separated from the circulation.<sup>32</sup> In support of our *in-vitro* findings, other renin inhibitors, with a structure resembling that of remikiren, did not elicit negative inotropic responses in isolated rat Langendorff hearts.<sup>97</sup>



**Figure 10.** Comparison of the effects of consecutive 10 min intracoronary infusions of remikiren (2, 5 and 10 mg.min<sup>-1</sup>) in the absence (open symbols, n=5) or presence (closed symbols, n=5) of the AT<sub>1</sub>-receptor antagonist L-158,809 on mean arterial pressure (MAP), cardiac output (CO), systemic vascular resistance (SVR), left ventricular end-diastolic pressure (LVEDP), heart rate (HR) and LV dP/dt<sub>max</sub>. Data are presented as mean and s.e. mean, and are expressed as a percentage of the baseline level measured immediately before remikiren administration. \* P<0.05 without AT<sub>1</sub>-receptor blockade vs. with AT<sub>1</sub>-receptor blockade.



**Figure 11.** Comparison of the effects of consecutive 10 min intracoronary infusions of remikiren (2, 5 and 10 mg.min<sup>-1</sup>) in the absence (open symbols, n=5) or presence (closed symbols, n=5) of the AT<sub>1</sub>-receptor antagonist L-158,809 on coronary blood flow (CBF), coronary vascular resistance (CVR), myocardial oxygen consumption (MVO<sub>2</sub>) and myocardial work (MW). Data are presented as mean and s.e. mean, and are expressed as a percentage of the baseline level measured immediately before remikiren administration. \* P<0.05 without AT<sub>1</sub>-receptor blockade vs. with AT<sub>1</sub>-receptor blockade.



Are the observed systemic haemodynamic and cardiac effects of remikiren due to the reduction of tissue or plasma Ang II? PRA and Ang I and II were reduced to levels at or below the detection limit at doses of remikiren that were not high enough to affect systemic haemodynamics or myocardial function, both after i.v. and i.c. infusion. This suggests that these responses are related to the inhibition of non-circulating renin, *i.e.* renin at tissue sites that are more difficult to reach.<sup>61,62</sup> In addition, after the remikiren infusion had been stopped, the haemodynamic and cardiac parameters returned to baseline more rapidly than PRA and the plasma levels of Ang I and II. This again suggests that high concentrations of remikiren are required to inhibit tissue renin. Most likely remikiren is washed away from the tissues via the circulation.

The effect of remikiren on PRA appeared to be more pronounced and to last longer than the effect on the angiotensin levels. This phenomenon has also been observed in previous studies with renin inhibitors.<sup>36,39,46</sup> The angiotensinase inhibitors that are routinely used in PRA assays cause displacement of protein-bound remikiren, so that the free concentration of remikiren is increased.<sup>46</sup> To avoid this *in-vitro* artefact we measured PRA with an antibody trapping assay without angiotensinase inhibitors. In this assay the *in-vitro* generated Ang I is trapped by an excess of Ang I antibody and thereby protected from destruction by angiotensinases. Still, PRA was more suppressed than plasma Ang I and II. It is possible therefore that some *in-vitro* displacement of the renin inhibitor may occur also in the antibody trapping assay. Decreased Ang I and II levels are probably a better measure of renin inhibition.

In conclusion, our results support the contention that renin-dependent intracardiac angiotensin formation has a positive effect on contractility. While our observations on the renin inhibitor remikiren point towards an important local function of the renin-angiotensin system, they do not necessarily argue against the use of this inhibitor in hypertension and heart failure, because the inhibitor concentrations causing a reduction in cardiac contractility are higher than the concentrations required for peripheral vasodilatation.

*References are presented in the general reference list.*



*Chapter 7*

**SUMMARY AND  
CONCLUDING REMARKS.**

*Thesis. 1997.*

## SUMMARY

### *Introduction*

A remarkable aspect of angiotensin II is the wide diversity of physiological responses this small peptide induces in many different organs. One common characteristic of these angiotensin II-induced effects is that they are all related to the maintenance of cardiovascular homeostasis. For many years angiotensin II has been regarded as an endocrine factor, generated in the blood from its precursor angiotensin I by angiotensin-converting enzyme (ACE). Angiotensin I was also thought to be formed in the blood only, through cleavage of liver-derived angiotensinogen by renin from the kidney.

Following the introduction of inhibitors of the renin-angiotensin system, the ACE inhibitors in particular, it became clear that the beneficial effects of these drugs in cardiovascular diseases could not be explained entirely by their inhibitory effects on the circulating renin-angiotensin system. A new concept of the renin-angiotensin system therefore emerged: formation of angiotensins does not only occur in the circulation, but also at tissue sites, and the long-term beneficial effects of blockers of the renin-angiotensin system are due to inhibition of angiotensin II formation in tissues.

Biochemical and molecular-biological studies confirmed the presence of renin, angiotensinogen, ACE and angiotensin I and II in tissues. However, the reported extrarenal tissue levels of renin mRNA were in most cases close to or below the limit of detection. Moreover, the release of angiotensins from tissue sites into the circulation, both *in vivo* and *in vitro*, appeared to depend on the presence of kidney-derived renin. Thus, controversy arose whether formation of angiotensins at tissue sites did occur and to what extent it depends on *in-situ* synthesized renin.

In view of the widely acknowledged beneficial effects of ACE inhibitors in heart failure and post-infarction myocardial remodelling, effects which are at least partly independent of the blood-pressure lowering effects of these drugs, it was the aim of the present thesis to investigate the production and function of angiotensins in the heart.

### *Chapter 2*

To assess the origin of renin-angiotensin system components in the heart, we compared the cardiac tissue levels of renin, angiotensinogen and angiotensin I and II with the levels in blood plasma in healthy and in nephrectomized pigs. The cardiac renin and angiotensin I levels were similar to the plasma levels of these components, and angiotensin II in tissue was higher than in plasma. Thus, the presence of renin and angiotensin I and II in cardiac tissue cannot be accounted for by trapped plasma or simple diffusion from plasma into the interstitial fluid.

Tissue levels of renin and angiotensin I and II were directly correlated with plasma levels, and both in tissue and in plasma the levels became undetectably low after bilateral nephrectomy, indicating that most, if not all, renin in cardiac tissue originates from the kidney. Angiotensinogen levels in cardiac tissue were 10-25% of the levels in plasma, which is compatible with the assumption that this component enters the tissue by diffusion into the interstitial fluid. Renin, but not angiotensinogen, was enriched in a purified cardiac membrane fraction prepared from left ventricular tissue.

Taken together, these results suggest that in the healthy heart, angiotensin production depends on plasma-derived renin and that plasma-derived angiotensinogen is a potential source of cardiac angiotensins. Binding of renin to cardiac membranes may be part of a mechanism by which renin is taken up from the circulation.

### *Chapter 3*

In order to study the cardiac uptake and localisation of renin, angiotensinogen and angiotensin I, as well as the site of angiotensin I generation in the heart, a modification of the isolated perfused rat heart was used. This modification allowed the separate collection of coronary effluent and interstitial transudate. The levels that were measured in interstitial transudate were considered to be representative for the levels in the cardiac interstitial fluid. Both renin and angiotensinogen, during perfusion of the heart with these components, slowly reached the interstitial fluid, at a rate similar to that reported previously for other large molecules. The steady-state levels of renin and angiotensinogen in interstitial fluid were not different from those in coronary effluent. Measurement of tissue levels of renin and angiotensinogen, under steady-state conditions, suggested that these components were located mainly in the extracellular fluid compartment of the tissue. During angiotensin I perfusion, angiotensin I rapidly appeared in interstitial transudate, reaching steady-state levels within 5 minutes that were 3-4 fold lower than in coronary effluent. The tissue level of angiotensin I under steady-state conditions was much lower than expected on the basis of its presence in the extracellular fluid, suggesting that angiotensin I is rapidly degraded in cardiac tissue.

Angiotensin I was not present in interstitial transudate or coronary effluent during perfusion with buffer or angiotensinogen. It was very low in interstitial transudate during perfusion with renin, and rose to much higher levels in interstitial transudate and coronary effluent during combined renin/angiotensinogen perfusion. The total production rate of angiotensin I present in interstitial fluid could be largely explained by the renin-angiotensinogen reaction in the fluid phase of the interstitial compartment. In contrast, the net ejection rate of angiotensin I via coronary effluent was higher than could be

## Chapter 7

explained by angiotensin I formation in the fluid phase of the intravascular compartment.

These data reveal two tissue sites of angiotensin I production, *i.e.* the interstitial fluid and a site closer to the blood compartment, possibly vascular surface-bound renin. The release of locally produced angiotensin I into coronary effluent and interstitial transudate appeared to depend on blood-derived renin and angiotensinogen.

## Chapter 4

To study uptake of circulating angiotensin I and II by tissues we measured the levels of  $^{125}\text{I}$ -labelled angiotensin I and II that were reached in various tissues and in blood plasma during the infusion of these peptides into the left cardiac ventricle of pigs. During infusion of  $^{125}\text{I}$ -angiotensin I, both  $^{125}\text{I}$ -angiotensin I and II were present in blood, but only  $^{125}\text{I}$ -angiotensin II could be detected in tissue. Within 5 minutes of infusion, steady-state levels of  $^{125}\text{I}$ -labelled peptides were reached in the circulation, whereas a steady state at the tissue level was reached after 60 minutes of infusion. The steady-state concentration of  $^{125}\text{I}$ -angiotensin II in heart, kidney and adrenal was approximately 100, 450 and 2000% of its steady-state concentration in arterial plasma. Little difference was observed in the tissue levels of  $^{125}\text{I}$ -angiotensin II, when expressed as a percentage of the arterial plasma levels, between the animals subjected to  $^{125}\text{I}$ -angiotensin I infusions with and without ACE inhibitor treatment. There was also little difference between the results obtained after  $^{125}\text{I}$ -angiotensin I infusion and those after  $^{125}\text{I}$ -angiotensin II infusion. This indicates that most of  $^{125}\text{I}$ -angiotensin II in the tissues was derived from arterially delivered  $^{125}\text{I}$ -angiotensin II and not, via conversion, from arterially delivered  $^{125}\text{I}$ -angiotensin I.

The amount of accumulated  $^{125}\text{I}$ -angiotensin II in the various tissues (adrenal » kidney » heart) corresponds with the reported  $\text{AT}_1$  receptor densities of these organs. Blockade of the receptor with a specific  $\text{AT}_1$  receptor antagonist prevented the accumulation of  $^{125}\text{I}$ -angiotensin II in tissue almost completely. After discontinuation of infusion,  $^{125}\text{I}$ -angiotensin II disappeared much slower from tissue ( $t_{1/2} \approx 12\text{-}15$  min) than from the circulating blood ( $t_{1/2} \approx 0.5$  min).

Thus, angiotensin II from the circulation, but not angiotensin I, is accumulated by heart, kidney and adrenal, and this is mediated by  $\text{AT}_1$  receptors. The time course of this process and the long half life of the accumulated angiotensin II suggest that this angiotensin II is internalised after its binding to the  $\text{AT}_1$  receptor, so that it is protected against rapid degradation by endothelial peptidases.

## Chapter 5

As shown in chapter 4, the uptake of angiotensins from the circulation by the heart and other organs can be quantified by measuring the steady-state levels of  $^{125}\text{I}$ -

angiotensin I and II during systemic infusion of  $^{125}\text{I}$ -angiotensin I or II. By comparing the levels of the radiolabelled angiotensin I and II with the levels of endogenous angiotensin I and II in cardiac tissue, it is possible to calculate what part of the endogenous levels is synthesized in cardiac tissue and what part is taken up from the circulation. This approach was followed in untreated and captopril-treated pigs.

Captopril caused a parallel increase in the plasma and cardiac levels of renin and angiotensin I. Plasma angiotensin II showed the expected decrease, whereas cardiac angiotensin II did not change following captopril. In all pigs, cardiac angiotensin II was 5-10 fold higher than plasma angiotensin II, and cardiac angiotensin I resembled plasma angiotensin I. Angiotensinogen remained unaltered in the circulation, but decreased in cardiac tissue after captopril.

$^{125}\text{I}$ -angiotensin I was virtually undetectable in cardiac tissue in untreated and captopril-treated pigs. Tissue  $^{125}\text{I}$ -angiotensin II during  $^{125}\text{I}$ -angiotensin I and II infusion was 100% and 75%, respectively, of  $^{125}\text{I}$ -angiotensin II in arterial plasma. Captopril reduced the amount of  $^{125}\text{I}$ -angiotensin II derived, by conversion, from arterially delivered  $^{125}\text{I}$ -angiotensin I from 23% to less than 2% of  $^{125}\text{I}$ -angiotensin I in arterial plasma. Taken together, it appears that most or all of cardiac angiotensin I and 75-90% of cardiac angiotensin II is produced at tissue sites.

The heart can maintain its angiotensin II production when the production of plasma angiotensin II is effectively suppressed by captopril, either because the ACE inhibitor dose was too low to block cardiac ACE completely, because enzymes other than ACE might be involved in cardiac angiotensin I-to-angiotensin II conversion, or because captopril does not reach the tissue site of angiotensin II production.

### *Chapter 6*

To investigate the role of the renin-angiotensin system in the regulation of myocardial contractility, the haemodynamic and biochemical effects of systemic and intracoronary infusions of the renin inhibitor remikiren were compared in open-chest anaesthetised pigs. Intravenous administration of remikiren decreased blood pressure by inducing peripheral vasodilation; cardiac output was not affected. Intracoronary administration of remikiren did not affect systemic vascular resistance, but decreased blood pressure by reducing cardiac output. It is unlikely that these observations were caused by a non-specific action of remikiren, since 1) the renin inhibitor had no effect on contractile force of porcine isolated cardiac trabeculae and 2) systemic  $\text{AT}_1$  receptor blockade either abolished or reduced the haemodynamic and myocardial effects of subsequent intracoronary infusions of remikiren.

Plasma renin activity and angiotensin I and II were reduced to levels below the limit of detection at doses of remikiren that were not high enough to affect

systemic haemodynamics or myocardial function, both after intravenous and intracoronary infusion. This suggests that a decrease in circulating angiotensin II is not the sole explanation for the remikiren-induced haemodynamic findings. Taken together, the results support the contention that intracardiac renin-dependent angiotensin formation contributes to the contractile state of the heart.

## CONCLUDING REMARKS

The experiments described in this thesis focus on the origin and distribution of components of the renin-angiotensin system in the heart. The cardiac levels of renin correlated closely with the levels of renin in the circulation, and after a bilateral nephrectomy, renin became virtually undetectable both in the circulation and the heart. Thus, it appears that the kidneys are the main source of cardiac renin under normal circumstances. In our studies with the isolated rat Langendorff heart it was observed that the level of renin in cardiac tissue could largely be explained by its presence in the extracellular fluid. However, the washout of renin from the heart followed a biphasic pattern, suggesting that renin may also be present in a second compartment. Indeed, since renin was found to be enriched in a purified membrane fraction prepared from freshly obtained porcine hearts, it is possible that renin, in addition to its presence in extracellular fluid, is bound to cardiac membranes. This could explain why the level of renin in the heart *in vivo* (expressed per g tissue) is as high as its level in blood plasma (expressed per mL plasma). Recent data on the existence of renin-binding proteins or receptors in isolated membrane fractions support the possibility of renin binding.<sup>21,116,152,170</sup> Specific binding of renin to cells has been proposed as a mechanism by which formation of angiotensins at tissue sites can be regulated.<sup>21</sup>

In the isolated heart, angiotensinogen, like renin, was mainly present in extracellular fluid. Its levels in cardiac tissue *in vivo* are also in agreement with the contention that angiotensinogen from the circulation enters the tissue by diffusion into the interstitial fluid. No evidence for cellular binding of angiotensinogen was found.

The above conclusions on renin and angiotensinogen apply to normal healthy animals. We cannot exclude that local synthesis of renin and/or angiotensinogen does occur under pathological circumstances, for instance following myocardial infarction.<sup>106,124</sup>

Studies in the isolated perfused rat heart showed that angiotensin I from the perfusion fluid enters the interstitial fluid more rapidly than renin and angiotensinogen, most likely due to its smaller size. The steady-state levels of angiotensin I in the interstitial fluid were 5-6 fold lower than in the arterial fluid. This contrasts with our findings on renin and angiotensinogen. It appears therefore



that angiotensin I is rapidly degraded when entering the tissue. In support of this conclusion, virtually no *intact*  $^{125}\text{I}$ -angiotensin I could be demonstrated in cardiac tissue during  $^{125}\text{I}$ -angiotensin I infusions in pigs. Thus, arterially delivered angiotensin I does not accumulate in cardiac tissue. In contrast,  $^{125}\text{I}$ -angiotensin II was present in cardiac tissue in detectable amounts, both during  $^{125}\text{I}$ -angiotensin I and II infusions. Its accumulation in the heart occurred slowly and could be blocked with an  $\text{AT}_1$  receptor antagonist. Moreover, once present in the heart,  $^{125}\text{I}$ -angiotensin II had a half life of 12-15 min, as compared with a half life of  $<1$  min in the circulation. Taken together, these data suggest internalisation of plasma-derived angiotensin II after its binding to the  $\text{AT}_1$  receptor.

From measurements of the levels of radiolabelled angiotensins during  $^{125}\text{I}$ -angiotensin I and II infusion, combined with measurements of endogenous angiotensin I and II, it could be calculated that virtually all angiotensin I and 75-90% of angiotensin II in the heart was synthesized *in situ* at tissue sites. This local synthesis appears to depend on renal renin, since cardiac angiotensin I and II became undetectably low following a bilateral nephrectomy.

Summarizing, the following picture emerges from our observations. Circulating renin is taken up by the heart, by binding to membrane binding sites and by diffusion into the interstitial fluid. This renin can react with angiotensinogen, present in blood and/or interstitial fluid, to form angiotensin I, which is then converted to angiotensin II by ACE (Figure 1). Our studies with the renin inhibitor remikiren show that cardiac angiotensin synthesis has functional importance, since the cardiodepressant actions of remikiren were unrelated to its effects on the circulating renin-angiotensin system.

An important question that emerges needs to be addressed. Where in the heart are angiotensins being formed and what is their localisation in cardiac tissue? Three possible sites will be discussed here (Figure 1).

### *Interstitial fluid*

According to our studies in the isolated rat heart, the components required for angiotensin synthesis are present in interstitial fluid. During combined angiotensinogen/renin perfusion, the level of angiotensin I in interstitial fluid was 2-3 fold higher than its level in the intravascular compartment, suggesting that angiotensin generation had occurred in the interstitium. Such high interstitial levels would be compatible with our *in-vivo* observations that the cardiac tissue levels of angiotensin I (expressed per g wet weight) are as high as the plasma levels of angiotensin I.

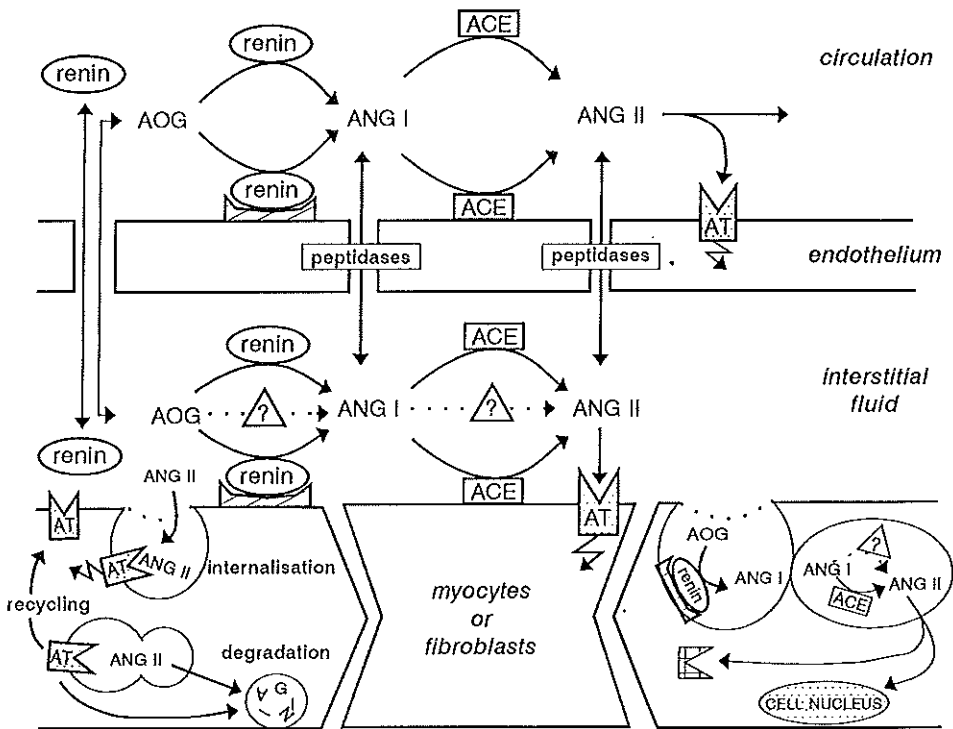
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### *Cell membrane*

Synthesis of angiotensins may also occur on the cell membrane. We found renin to be enriched in cardiac membrane preparations. Moreover, the release of angiotensin I from the isolated rat heart into the perfusion fluid was several times higher than expected on the basis of the renin-angiotensinogen reaction in the intravascular fluid. Interstitial angiotensin production was too low to account for this difference. There was also no evidence that angiotensin I from the perfusion fluid compartment made an important contribution to the level of angiotensin I in the interstitial fluid compartment. This suggests that cleavage of angiotensinogen might have occurred at a site close to the blood compartment, possibly the surface of vascular cells. This would result in formation of angiotensin I, which can then be released into the circulation, either directly or after its conversion to angiotensin II. Conversion of angiotensin I that is synthesized *in situ* at tissue sites, however, does not appear to contribute to the angiotensin II level in the coronary venous blood.<sup>2,31</sup>

### *Intracellular compartment*

Our studies in pigs showed that the levels of endogenous angiotensins in cardiac tissue did not rapidly fall when the tissue was stored at 37°C after the heart had been removed from the body. Therefore, these angiotensins may not be located in the interstitial fluid compartment, since angiotensins in that compartment are susceptible to rapid degradation. Plasma <sup>125</sup>I-angiotensin II, taken up by the heart by an AT<sub>1</sub> receptor-mediated process, has a long half life in cardiac tissue, most likely because angiotensin II bound to the AT<sub>1</sub> receptor is protected against rapid degradation. The absence of a change in the cardiac angiotensin II levels during *ex vivo* storage might thus be explained by assuming that endogenous angiotensin II is also located in the cells, either because it is synthesized within the cell, or because, following its synthesis outside the cell, it is internalised via AT<sub>1</sub> receptors (Figure 1). The latter theory implicates that the cardiac angiotensin II levels should decrease following chronic AT<sub>1</sub> receptor blockade. A study in rats showed that this was not the case.<sup>22</sup> Thus, intracellular production of angiotensins is a realistic option, although at present it is unknown how such intracellular production might occur. One possibility is that renin is transported to the intracellular compartment following its binding to renin receptors. When, concurrently with renin, angiotensinogen is taken up from the interstitial fluid via bulk fluid endocytosis, a scenario for intracellular angiotensin I generation is provided. This *de-novo* formed angiotensin I may then be converted to angiotensin II within the cell (Figure 1).



**Figure 1.** Cardiac angiotensins: local generation and uptake from plasma. Tissue generation of angiotensins may occur in the interstitial fluid, on the cell membrane, or within cells. Renin-angiotensin system components are assumed to exchange freely between intravascular fluid and interstitial fluid. Plasma-derived and/or locally generated angiotensin II enters cardiac cells by receptor-mediated endocytosis and may exert intracellular effects. Dotted arrows indicate alternative (renin- and ACE-independent) angiotensin-forming pathways. AOG: angiotensinogen; ANG: angiotensin; ACE: angiotensin-converting enzyme; AT: angiotensin II receptor.

Intracellular angiotensin II may serve some important functions. High-affinity binding sites for angiotensin II have been detected in cytosol<sup>137</sup> and nuclei of liver cells,<sup>172</sup> and accumulation of angiotensin II has been demonstrated in nuclei of vascular smooth muscle cells and cardiomyocytes.<sup>72,136</sup> Once internalised, the peptide may prolong activation of signal transduction pathways<sup>67</sup> or bind to nuclear receptors in order to regulate gene transcription.<sup>53</sup> Injection of angiotensin II into vascular smooth muscle cells induced a sustained elevation of intracellular  $Ca^{2+}$  levels, an effect that could be blocked by intracellular, but not extracellular, administration of an  $AT_1$  receptor antagonist.<sup>72</sup> When angiotensin II or renin were dialysed into cultured cardiomyocytes, the conductance of the adjacent myocytes decreased.<sup>41,42</sup> The reduction of conductance was amplified when renin was infused

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together with angiotensinogen and attenuated when a renin inhibitor was co-administered, suggesting that these effects are mediated by renin-dependent angiotensin II formation within the cell.

More detailed knowledge of the actual site and regulation of angiotensin production in the heart will further illuminate the role of the renin-angiotensin system in cardiac function, growth and remodelling. Our observations that angiotensin II, when bound to  $AT_1$  receptors, is protected against rapid metabolism, and that cardiac uptake of circulating angiotensin II is prevented by  $AT_1$  receptor blockade are reasons to believe that  $AT_1$  receptor antagonists might potentiate the therapeutic effects of ACE inhibitors. Whereas ACE inhibition and  $AT_1$  receptor blockade separately do not reduce cardiac angiotensin II, combined treatment might achieve this goal by reducing both the cardiac angiotensin II generation and the formation of complexes of the  $AT_1$  receptor with its agonist angiotensin II. This could conceivably have important implications for the clinical use of these drugs.

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## NEDERLANDSE SAMENVATTING

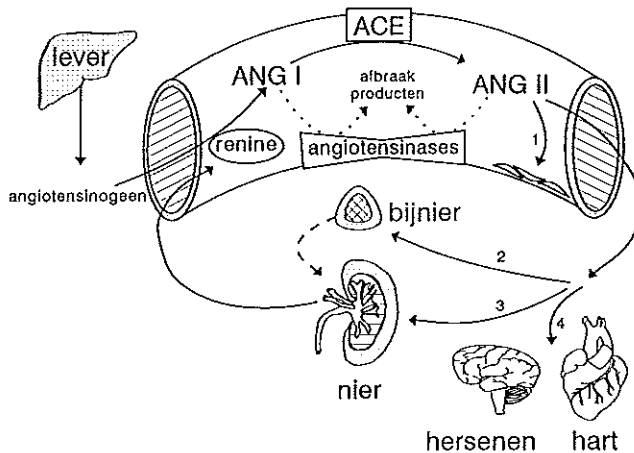
### *Algemeen*

Hormonen zijn moleculen die aangemaakt en uitgescheiden worden door gespecialiseerde cellen in een organisme. Na transport via de bloedbaan kunnen ze een effect bewerkstelligen op enige afstand van hun oorsprong. Sommige hormonen hebben effect op één specifiek doelorgaan; anderen hebben effect op meerdere doelorganen. Nadat een hormoon heeft gebonden aan een, voor dit hormoon specifieke receptor, zal er een respons optreden in de cel waarop de receptor zich bevindt. Deze respons wordt mede bepaald door het genetische programma van de cel. Een hormoon kan hierdoor verschillende effecten bewerkstelligen in verschillende organen.

### *Het renine-angiotensine systeem*

Traditioneel wordt het renine-angiotensine systeem beschouwd als een hormonaal systeem. Echter, angiotensine II, de effectieve component van het systeem, is een uniek hormoon. In tegenstelling tot veel andere hormonen wordt angiotensine II niet aangemaakt en uitgescheiden door één bepaald orgaan, maar vindt de vorming plaats in het bloed.

In de bloedbaan wordt eerst angiotensin I gevormd door de reactie van het enzym renine (afkomstig van de nieren) met het substraat angiotensinogeen (afkomstig van de lever). Dit angiotensine I wordt vervolgens omgezet in angiotensine II door angiotensine-converterend enzym (ACE). Angiotensine I en II worden beide snel afgebroken door zogenaamde 'angiotensinases', zodat hun concentraties in het bloed binnen bepaalde grenzen blijven (Figuur 1).



**Figuur 1.** Productie van angiotensine I en II (ANG I en II) in de bloedbaan. Angiotensine II dat in het bloed wordt gevormd stimuleert vernauwing van de vaatwand (1) en regelt de water- en zouthuishouding van een organisme via de bijnier (2) en nier (3). Angiotensine II heeft ook effecten op andere organen zoals de hersenen en het hart (4).

### *Nederlandse samenvatting*

Het renine-angiotensine systeem is betrokken bij de handhaving van de bloeddruk. Angiotensine II heeft een sterk vernauwend effect op bloedvaten en reguleert de water- en zouthuishouding van een organisme. Daarnaast stimuleert het de groei van het hart en de bloedvaten. De meeste effecten van angiotensine II worden gemedieerd via binding van dit peptide aan een specifieke receptor (de 'AT<sub>1</sub> receptor').

### *Lokale renine-angiotensine systemen*

Na de introductie van ACE remmers als bloeddruk-verlagende geneesmiddelen, werd duidelijk dat de werking van deze klasse van farmaca niet geheel gewijd kon worden aan een verminderde angiotensine II vorming in het bloed. Het idee rees dat de vorming van angiotensine I en II niet enkel beperkt blijft tot de circulatie, maar dat ook lokaal, in weefsels, angiotensines geproduceerd kunnen worden. ACE remmers zouden in dat geval niet alleen de vorming van angiotensine II in de circulatie tegengaan, maar ook lokaal, binnen een orgaan. De componenten die benodigd zijn voor de productie van angiotensines (renine, angiotensinogeen, ACE) bleken aanwezig te zijn in diverse organen, waaronder het hart. De oorsprong van die componenten was echter onduidelijk; zowel opname uit het bloed als lokale vorming zou de aanwezigheid van die componenten in weefsel kunnen verklaren.

ACE remmers worden tegenwoordig niet alleen met succes toegepast als middelen tegen hoge bloeddruk, deze farmaca blijken ook gunstig als therapie voor de behandeling van hartfalen en het proces van 'remodelling' van de hartspier dat optreedt na een hartinfarct. Deze effecten op het hart zijn slechts gedeeltelijk toe te schrijven aan de bloeddruk-verlagende werking van ACE remmers, hetgeen wederom suggereert dat lokale vorming van angiotensines in het hartweefsel een belangrijke rol speelt.

Er is niet veel bekend over de oorsprong, distributie en productie van componenten van het renine-angiotensine systeem in het hart. Het onderzoek, zoals dat staat beschreven in dit proefschrift, had ten doel om de concentraties van componenten van het renine-angiotensine systeem te bepalen in het hart onder gewone omstandigheden en tijdens blokkade van het renine-angiotensine systeem. Daarnaast was het de bedoeling om de opname van renine-angiotensine systeem componenten vanuit het bloed in het hart te kwantificeren. Tenslotte diende de distributie en lokalisatie van renine-angiotensine systeem componenten te worden vastgesteld en werd het belang van lokale vorming van angiotensine II voor de hartspier onderzocht. Voor deze studies is gebruikt van zowel ratten als varkens.



*Oorsprong, distributie en productie van renine-angiotensine systeem componenten in het hart*

De renine concentratie in het hart was hoger dan verwacht kon worden op grond van de hoeveelheid bloed die zich normaal in het hartweefsel bevindt. Dit suggereert dat renine in het hart ook buiten de bloedbaan aanwezig is. Echter, dertig uur na verwijdering van de nieren was renine niet meer aantoonbaar in zowel bloed als hartweefsel. Kennelijk is renine in hartweefsel dus afkomstig van de nieren. Mogelijk wordt dit 'nierrenine' uit de bloedbaan opgenomen via zogenaamde renine receptoren of renine-bindende eiwitten aan de buitenkant van hartcellen.

De concentratie van angiotensinogeen in het hart was lager dan in het bloed. Er zijn geen duidelijke aanwijzingen dat actieve opname van angiotensinogeen in het hart plaatsvindt, zoals dat bijvoorbeeld wel voor renine het geval lijkt te zijn. Waarschijnlijk bevindt angiotensinogeen in het hart zich alleen in bloed en in de vloeistof die zich tussen de cellen bevindt ('interstitiële vloeistof').

De opname van angiotensine I vanuit het bloed in hartweefsel bleek verwaarloosbaar klein, terwijl angiotensine II vanuit het bloed juist wel in meetbare hoeveelheden in het hart terecht kwam. Met behulp van infusen met radio-actief gemerkt angiotensine II ( $^{125}\text{I}$ -angiotensine II) kon worden vastgesteld dat  $^{125}\text{I}$ -angiotensine II na één uur infusie dezelfde concentratie in hartweefsel bereikte als in bloed. Deze opname in hartweefsel bleek af te hangen van binding aan AT<sub>1</sub> receptoren. Interessant was dat  $^{125}\text{I}$ -angiotensine II na binding aan deze receptoren maar langzaam werd afgebroken, terwijl het in bloed juist zeer snel (binnen enkele minuten) verdwijnt. Kennelijk biedt binding aan de receptor bescherming tegen afbraak.

Na correctie voor de opname van angiotensine I en II uit het bloed kon worden berekend dat vrijwel al het angiotensine I en meer dan 75% van het angiotensine II in het hart lokaal in hartweefsel wordt gemaakt, niet alleen onder normale omstandigheden, maar ook na voorbehandeling met een ACE remmer. Onze studies tonen daarmee voor het eerst in een levend dier aan dat angiotensine I en II in het hart gemaakt worden. Angiotensine II dat in het hart wordt gemaakt blijkt onder andere de contractiekracht van het hart te stimuleren.

Nog onbekend is waar in het hart angiotensine I en II worden gevormd. Mogelijke plaatsen zijn: 1) de interstitiële vloeistof, 2) op de hartcellen en 3) in hartcellen. Toekomstig onderzoek zal hier uitsluitsel over moeten geven, alsmede over de regulatie van angiotensine vorming in het hart. Daarmee zal een beter inzicht worden verkregen in de rol die angiotensine II speelt in het hart en de werking van blokkers van het renine-angiotensine systeem bij hoge bloeddruk en hartfalen.

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## CURRICULUM VITÆ

De auteur van dit proefschrift werd geboren op 23 maart 1967 te Jaquariuna, Brazil.

Voorbereidend wetenschappelijk onderwijs (Atheneum B) werd genoten op het Niels Stensen College in Utrecht. In 1985 werd aangevangen met de studie medische biologie aan de Rijksuniversiteit Utrecht. Deze studie werd kort onderbroken voor een uitstap naar de Landbouw Universiteit Wageningen (propadeuse humane voeding). Uiteindelijk werd in augustus 1992 het doctoraal examen medische biologie met succes afgelegd.

Zijn hoofdvak, moleculaire neurobiologie, werd uitgevoerd op het Rudolf Magnus Instituut voor Farmacologie, te Utrecht. Onder begeleiding van prof. J.P.H. Burbach werd de invloed van schildklierhormoon op de oxtocine en vasopressine gen-expressie onderzocht. Zijn eerste bijvak, onder supervisie van dr. P.R. Bär, werd gevolgd op het laboratorium van de vakgroep Neurologie van het Academisch Ziekenhuis Utrecht. Daar bestudeerde hij de groei-bevorderende effecten van neuropeptiden op gekweekte zenuwcellen. Een tweede bijvak werd gevolgd op het Department of Pharmacology, University of Toronto, Canada, onder supervisie van dr. H.H.M. van Tol. In deze periode was hij betrokken bij de moleculair-farmacologische karakterisatie van de dopamine D<sub>4</sub> receptor. Zijn doctoraal-scriptie behandelde de functie van receptoren voor neurotrofe groeifactoren.

Sinds februari 1993 is hij werkzaam als assistent in opleiding op de afdelingen Inwendige Geneeskunde I en Experimentele Cardiologie, welke deel uitmaken van de Cardiovasculaire Onderzoekschool Erasmus Universiteit Rotterdam (COEUR). Het in dit proefschrift beschreven onderzoek aan het cardiale renine-angiotensine systeem is daar uitgevoerd onder supervisie van zijn promoteres prof. M.A.D.H. Schalekamp en prof. P.D. Verdouw en co-promotor dr. A.H.J. Danser.

## BOOKS AND AFTERWORDS

(for 66 little pigies)

“He took his house and he built a wall right in the middle of the house.

In the apartment on one side:  
clothes, furniture, food, books,  
radio.

In the apartment on the other side:  
different clothes, different books,  
different furniture, ... TV set.

He'd live on this side for a while until  
he got tired of it. Put his book down,  
took his clothes of and walked over to

this side, and lived on this side for  
a while, until he got tired of it.  
And he flip-flopped

to this side, and lived over here for  
while until he got tired of it. And he  
did this back and forth until he died.

After he died they went in to clean  
the house so they could rent it out to  
college students. These people went  
back in closet, at the very back of this  
house. They opened the door and the  
whole closet was filled with these  
books.

Each book was exactly the same,  
and this man had written these  
books and had them all published,  
all stacked in his closet. He never  
sold one or given one away.

The name of the book was: life, ... and how to live it.”

*Michael Stipe*, MC Vredenburg Utrecht, 1987.