The intracardiac renin-angiotensin-system : new insights in its role in cardiac hypertrophy and wound healing

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THE INTRACARDIAC RENIN-ANGIOTENSIN-SYSTEM

new insights in its role in cardiac hypertrophy and wound healing

Cover: Photomicrograph of a rat heart, 4 days after myocardial infarction, immunohistochemically stained with a goat-anti-rabbit angiotensin I-converting-enzyme (ACE)-antibody. ACE-immunoreactivity (black) is predominantly present in endothelial cells in the border zone of the infarct area.

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THE INTRACARDIAC RENIN-ANGIOTENSIN-SYSTEM
new insights in its role in cardiac hypertrophy and wound healing

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aan de Rijksuniversiteit Limburg te Maastricht
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Cause I gotta have faith...

G. Michael, 1984.

Aan mijn ouders
Voor Sylvia

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SUMMARY

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LIST OF ABBREVIATIONS

ACE	Angiotensin I-converting-enzyme
ACTH	Adrenocorticotrophic hormone
ANF	Atrial natriuretic factor
ANG I	Angiotensin I
ANG II	Angiotensin II
ANG III	Angiotensin III
ANG IV	Angiotensin IV
AO	Angiotensinogen
AT receptors	Angiotensin II-receptors
AT1	Angiotensin II-receptor type I
AT2	Angiotensin II-receptor type II
DNA	Deoxyribonucleic acid
MI	Myocardial infarction
mRNA	messenger ribonucleic acid
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
RAS	Renin-angiotensin-system
RT	Reverse transcriptase
SDS	Sodium dodecylphosphate
SHR	Spontaneously hypertensive rats
SSC	Standard saline citrate
TGF β	Transforming growth factor β
WKY	Wistar Kyoto

CHAPTER 1

INTRODUCTION

1.1 GENERAL INTRODUCTION

Cardiac hypertrophy, defined as an increase in cardiac mass, is a powerful independent risk factor for cardiovascular complications (46,171,196). Hypertension is an important determinant for the development of cardiac hypertrophy. Also myocardial infarction (MI) results in hypertrophy of the non-infarcted myocardium. This hypertrophic response of the myocardium and other changes in ventricular composition (*e.g.* cardiac fibrosis) and architecture (*e.g.* ventricular dilatation and ventricular wall thinning) are referred to as "cardiac remodelling". Although cardiac hypertrophy is, at first, an adaptive response compensating for the increased hemodynamic load and/or loss of viable contractile tissue, it eventually leads to a worsened cardiac function, resulting in a higher mortality and morbidity. Over the past half century it has been established that regression of hypertension-induced cardiac hypertrophy can be achieved by reduction of blood pressure by several classes of antihypertensive drugs (66,69,76,131,198). However, it also became clear that blood pressure is not the only factor responsible for the development of cardiac hypertrophy. Comparisons of different classes of antihypertensive drugs demonstrated different effects on the regression of cardiac hypertrophy, while similar effects on blood pressure were observed (69,131,198,296). Particularly angiotensin I-converting-enzyme-inhibitors were found to be very effective in regressing cardiac hypertrophy, even without reducing the blood pressure (207). This suggested a role for the renin angiotensin system (RAS) in the development of cardiac hypertrophy. Tissue angiotensin II (ANG II) synthesis was suggested by the presence of ANG II in rat plasma after nephrectomy (3). In sheep and humans tissue ANG II synthesis was suggested by kinetic analysis of *in vivo* arterial-venous angiotensin I (ANG I) and ANG II differences (2,41). Indeed, components of the RAS have been detected in different organs, representing different functions. This is also true for the heart. The long-term responses to ACE-inhibitors, renin inhibitors and ANG II-antagonists are not predicted by plasma RAS activity (40,331). This has led to the assumption that the level of ANG II within the tissue may be affected differently by these agents than ANG II in circulating plasma and suggested the existence of a local RAS. ANG II has been shown to be a trophic factor for cardiac cells. All these arguments have led investigators to hypothesize that the intracardiac RAS is involved in the development of cardiac hypertrophy.

1.2 THE RENIN-ANGIOTENSIN-SYSTEM

1.2.1 The circulating renin-angiotensin-system

Biochemistry

The renin-angiotensin-system (RAS) has long been viewed as an endocrine system.

Figure 1. Pathways leading to ANG II formation. t-PA = tissue-plasminogen activator; LHRH = luteinizing hormone releasing hormone; CAGE = chymostatin-sensitive angiotensin II-generating enzyme. AO = angiotensinogen; ACE = angiotensin I-converting-enzyme; ANG I = angiotensin I; ANG II = angiotensin II.

The cascade of the circulating RAS starts with the production and secretion of the alpha-2 globulin, angiotensinogen (AO), in the liver. In the juxtaglomerular apparatus in the kidney, specialized cells of the distal tubule, macula densa cells, produce the enzyme renin. Renin is first synthesized as a precursor molecule, prorenin, which is then converted into active renin in the Golgi apparatus or in granules (166). Renin is an aspartyl protease and catalyzes the rate-limiting step of the RAS. AO is cleaved by renin to form the decapeptide ANG I. ANG I is then transported by the blood to the lungs where it is converted by angiotensin I-converting-enzyme (ACE) at the membrane of endothelial cells to form the octapeptide ANG II, the biologically most active component of the RAS (figure 1). That the lung is the main organ for the conversion of ANG I to ANG II is explained by the high flow and the presence of large numbers of endothelial cells in this organ. In addition, ACE also occurs as a circulating enzyme in blood plasma, where it can convert ANG I to ANG II. ANG II is degraded by aminopeptidase A and B resulting in a heptapeptide, des-Asp¹-ANG II (ANG III) and a hexapeptide des-Asp¹-Arg²-ANG II (ANG 3-8 or ANG IV), respectively. A prolyl endopeptidase cleaves the heptapeptide des-Phe⁸-ANG II

(ANG 1-7) from ANG I. All these angiotensin peptides still possess biological activity, like stimulation of aldosterone, vasopressin and prostaglandin synthesis by ANG II and ANG 1-7 (124). ANG IV exhibits biological activities that are opposite to those of ANG II and III (223,341).

In addition to the classical pathway of ANG II formation, also other pathways of ANG II formation are possible. Tissue-plasminogen activator (t-PA), kallikrein, tonin, plasmin, cathepsin G, elastase and endothelium derived prorenin activating enzyme (EDPAE) convert prorenin in active renin (283,302,305,336). Of interest, at least in humans, is chymase. This enzyme has been suggested to play a major role in ANG II formation (319,320). Urata *et al.* observed that more than 75% of the ANG II forming enzyme activity in human left ventricular tissue homogenates was not blocked by ACE-inhibitors, but was blocked by the serine proteinase inhibitor chymostatin and soybean trypsin inhibitor (319). Subsequent studies showed that the major ANG II-forming serine proteinase from the human cardiac ventricles was chymase (321,322). Furthermore, chymostatin-sensitive ANG II generating enzyme (CAGE), trypsin, cathepsin G, tonin, chymase and t-PA (90,148,242,320) are all able to generate ANG II directly from AO or from ANG I (figure 1).

In addition to the cleavage from ANG I to ANG II, ACE has also been shown to cleave other peptides like bradykinin, enkephalins, neurotensin, cholecystokinin, substance P, and the gonadotrophin luteinizing hormone releasing hormone (99,102,103,290).

Physiology

ANG II causes powerful arterial vasoconstriction and thereby it plays an important role in blood pressure regulation. Furthermore, ANG II stimulates synthesis and release of aldosterone from the adrenal glands. This results in sodium and water retention in the kidney. Water retention results in an increase of blood pressure. ANG II has also direct effects on renal function. ANG II stimulates proximal tubular sodium reabsorption (136). Glomerular filtration rate, which is dependent of renal blood flow, glomerular pressure and the glomerular filtration coefficient, is affected by ANG II. The glomerular filtration coefficient is decreased by ANG II through constriction of mesangial cells (24). Vasoconstriction of efferent glomerular arterioles by ANG II increases filtration pressure. The balance of these factors determines the glomerular filtration rate.

Activation of the sympathetic nervous system by ANG II also plays a role in blood pressure regulation (72,136,220). ANG II can facilitate the release of norepinephrine by a direct effect within the central nervous system. Alternatively, enhancement of sympathetic activity may also depend on stimulation of AT receptors on sympathetic nerve terminals, releasing norepinephrine. ANG II increases sympathetic outflow from the brain to both the vasculature and the myocardium (11). The rapid response of the circulating RAS on changes in fluid balance, blood flow and blood pressure provides immediate hemodynamic stability

Figure 2. ANG II-mediated functions. ANG II = angiotensin II; ↑ = stimulatory effect.

and prevents hypotension (figure 2). The principal function of the circulating RAS is to provide shortterm cardiorenal homeostasis. Except for states of severe cardiovascular decompensation, plasma renin-activity almost always returns to normal when homeostasis is achieved (89).

1.2.2 Angiotensin receptors

Differences in concentration-response relationship (248), binding properties for ANG II-analogs (82,130) and signal transduction systems (119) suggested the existence of different receptor subtypes for ANG II. One of the earlier reports on AT receptors was on the mas-oncogen product, expressed in xenopus oocytes (160). This mas-oncogen product was able to open a chloride channel in response to ANG II when transfected into xenopus oocytes.

The availability of selective compounds made it possible to identify the existence of at least two receptor subtypes for ANG II, defined as the AT1 and the AT2 receptor (55,332). The pharmacological classification of AT1 and AT2 receptors was based upon different binding profiles for the non-peptidergic ligands losartan (Dup 753) and PD123177 and the peptide CGP42112A. The angiotensin receptor that selectively binds losartan, was classified as the AT1 receptor (85), whereas

selective binding of PD123177 and CGP42112A was classified as the AT₂ receptor (55,332). Further distinction between the AT₁ and AT₂ receptors is possible by the inhibition of the AT₁ receptor and the potentiation of the AT₂ receptor by dithiothreitol (DTT) (56,261,294). Sasaki *et al.* (272) and Murphy *et al.* (228) cloned the AT₁ receptor from bovine adrenal glomerulosa cells and rat aortic vascular smooth muscle cells, respectively. The AT₁ receptor was found to belong to the seven-transmembrane-domain receptors superfamily and contains 359 amino acids (228,272). The subsequent cloning of the AT₁ receptor from the rat adrenal gland (154,271), showed an 8% difference in amino acid sequence when compared to the rat aortic AT₁ receptor (228), which indicated a distinction between AT₁ subtypes, the AT_{1a} and the AT_{1b}. A similar divergence between AT_{1a} and AT_{1b} has been found in humans (182,188,217) (table 1).

The AT₂ receptor was first cloned by Kambayashi *et al.* (170) and Mukoyama *et al.* (227) rat pheochromocytoma and rat foetus, respectively. These AT₂ receptors contain 363 amino acids and belong to the seven-transmembrane-domain receptor family. The AT₂ receptor displays a 32-34% homology with the AT₁ receptor. The highest homologies are found in the putative transmembrane areas.

Another group of AT receptors differs substantially from the AT₁ and the AT₂ receptor. These AT receptors do not bind either losartan or PD123177. However, CGP42112A, another AT₂ ligand, displayed a higher binding affinity than losartan and PD123177 for the AT receptor in radioligand binding studies (194). Binding sites for ANG II with these characteristics have been found in several tissues in different species, for example in the adrenal gland of the turkey, which has a 78% homology to the AT₁ receptor (45) and in the chorioallantoic membrane (CAM) of the chick embryo (194).

In human isolated cardiac fibroblasts from explanted end-stage failing human hearts, ANG II induced cellular proliferation, which could not be inhibited by losartan, PD123319, and CGP41112A (Dr. Neuss, personal communication) suggesting the existence of an as yet unknown AT receptor subtype (237).

1.2.3 Signal transduction systems coupled to AT receptors

The AT₁ receptor is coupled to at least five effector systems: phospholipase C, D, and A₂, adenylate cyclase and calcium channels (table 2). The activation of phospholipase C resulting in an increase of intracellular Ca²⁺ has been demonstrated in vascular smooth muscle cells (38,127). ANG II also activates phospholipase D (189,190). Activation of phospholipase D may be involved in cell growth (241,247). Activation of phospholipase A₂ has been demonstrated in different cell types (4,83,274) and results in free fatty acids, mostly arachidonic acid, and lysophospholipids. Arachidonic acid is the precursor for the synthesis of prostaglandins and leukotrienes. Inhibition of adenylate cyclase activity by ANG II was found in rat liver membranes (62,163,256), Leydig cells (174), cultured bovine adrenal membranes and cardiac myocytes or membranes in rat and rabbit (8,10).

Table 1. Differences of angiotensin receptors in protein sequence homology and pharmacological binding properties

ATrec	Origin	AT1 homology ¹	Pharmacological binding affinity ²	Reference
AT1a	human leukocytes	95%	ANG II ≥ losartan ≥ PD	(217)
AT1a	mouse genomic library	99%	ANG II ≥ losartan ≥ PD	(273)
AT1b	human placenta	90%	ANG II ≥ ANG III ≥ losartan ≥ ANG I ≥ PD	(182)
AT1b	rat adrenal cortex	95%	sarile ANG II ≤ ANG II = losartan ≤ ANG III ≤ PD = CGP	(271)
AT1b	mouse genomic library	94%	ANG II ≥ ANG III ≥ losartan ≥ ANG I ≥ PD	(273)
AT2	fetal mouse cDNA library	34%	sarile ANG II = CGP ≥ ANG II ≥ PD ≥ ANG I	(233)
AT2	fetal rat cDNA library	34%	sarile ANG II = CGP ≤ ANG II ≤ PD ≤ ANG I	(227)
AT2	rat pheochromocytoma cell line	32%	sarile ANG II ≥ CGP ≥ ANG II ≥ PD ≥ losartan ≥ ANG I	(329)
ATn	turkey adrenal gland	78%	ANG II ≥ sarile ANG II ≥ ANG III = CGP ≥ ANG I ≥ losartan ≥ PD	(45)
ATn ¹	chicken embryo CAM	N.D.	ANG II ≥ sarile ANG II ≥ ANG I ≥ ANG III ≥ CGP ≥ losartan ≥ PD	(194)
ATn ¹	human fibroblasts	N.D.	ANG II ≥ ANG II (1-7) = ANG IV ≥ losartan = PD ¹	(237)

1. Amino acid sequence homology compared to the AT1 of the rat vascular smooth muscle cell

2. Binding properties on basis of the IC50

Sarile ANG II = ligand [Sar¹Ile⁹]-angiotensin II; PD = AT2 antagonist PD123177; PD¹ = AT2 antagonist PD123319; CGP = AT2 ligand CGP42112A; losartan = AT1 antagonist; AT = angiotensin receptor; ATn = non-AT1/AT2 receptor; ANG I = angiotensin I; ANG II = angiotensin II; All = angiotensin III; ANG IV = angiotensin IV; CAM = chorioallantoic membrane; N.D. = not determined.

Table 2. *AT1 receptor mediated signal transduction systems*

Signal transduction	Tissue/cells	Species	Reference
↑ Phospholipase C	vascular SMC	rat	(38,127)
↑ Phospholipase D	vascular SMC	rat	(189,190)
↑ Phospholipase A2	umbilical vein SMC	human	(4)
	proximal tubular epithelium	rat	(83)
	mesangial cells	rat	(274)
↓ Adenylate cyclase	liver/adrenal/myocytes membranes	rat,rabbit	(8,10,163)
	Leydig cells	rat	(174)
↑ Calcium channels	aortic microsomes	pig	(31)
↓ Potassium channels	zona glomerulosa cells	rat,bovine,human	(258)

SMC = smooth muscle cells; ↑ = stimulation; ↓ = inhibition

Little is known about the signal transduction of the AT2 receptor subtype. In bovine cerebellum (36), human myometrium (257,332), and in different cultured cell lines (84,195,329) the AT2 receptor was expressed and was not coupled to G-proteins. In the rat brain two binding sites for the AT2 receptor were found. One was sensitive for γ GTP(S), DTT and pertussis toxin, while the other was insensitive to these agents (313,315). Furthermore ANG II was found to decrease or increase cGMP in neonatal rat brain cell cultures (298) and the N1E-115 cell line (343), respectively. AT2 receptors have also been shown to be involved in prostaglandin synthesis in human astrocytes (161). In rat pheochromocytoma PC12W cells, the AT2 receptor inhibited ANP-stimulated guanylyl cyclase through the activation of a phosphotyrosine phosphatase (35).

Functions mediated via the angiotensin receptors

Most known physiological actions of ANG II seem to be mediated through the AT1 receptor (309,339). The most important AT1 receptor mediated functions are summarized in table 3. In this thesis we focussed on the hypertrophic response of ANG II. ANG II-induced hypertrophy of the adult rat heart was mediated through the AT1 receptor (79). In rats following experimental MI, losartan regressed cardiac hypertrophy (292).

Not much is known about AT2 receptor mediated functions of ANG II. Cogan et al. (60) demonstrated a reduction in bicarbonate, chloride and water absorption by proximal tubules in the rat in the presence of PD123177. Since the AT2 receptor is expressed transiently and at higher levels than the AT1 receptor in fetal developmental stages in different species (125,222,314), it has been suggested that the AT2 receptor

Table 3. *AT1 receptor-mediated functions*

Function	Species	Reference
↑ vasoconstriction	rat, dog, human	(58,59,337)
↑ aldosterone secretion	rat, dog, bovine, human	(49,59,134) (58,110)
↑ catecholamine release	rat, dog	(338,339)
↓ renin release	rat, monkey dog, human	(5,121) (58,59)
↑ vasopressin release	rat	(144)
↑ GFR	rat	(98)
↑ cell growth protein synthesis thymidine incorporation	rat, human	(1,57)
↑ drinking	rat	(340)

↑ = stimulation; ↓ = inhibition; GFR = glomerular filtration rate

plays a role in the proliferation and differentiation of cells. In agreement, in infarcted rats treatment with the AT₂-antagonist PD123319 inhibited DNA-synthesis in the non-infarcted septum when compared to untreated infarct animals, showing the involvement of the AT₂ receptor in the regulation of interstitial DNA synthesis after MI. Recently, an antiproliferative effect for the AT₂ receptor has been described (295). In cultured coronary endothelial cells from spontaneously hypertensive rats (SHR), ANG II inhibited basic fibroblast growth factor (FGF)-stimulated proliferation of the endothelial cells. This effect was prevented by PD123177 and CGP42112A (295).

AT₁ as well as AT₂ receptors have been described to be involved in the process of wound healing. A downregulation of the AT₁ receptor (176) and an upregulation of the AT₂ receptor (325) was observed in the rat skin after injury, suggesting that both the AT₁ and the AT₂ receptors are involved in the wound healing process. However, the precise role of ANG II in wound healing is not clear.

A different AT receptor, demonstrated in the CAM (*vide supra*), was involved in ANG II-induced angiogenesis, also one aspect of the wound healing process. This effect could not be blocked by losartan and PD123319. However, CGP42112A could block the ANG II-induced angiogenesis in the CAM completely (194). Also in a non-AT₁/AT₂ receptor in human isolated cardiac fibroblasts from explanted end-stage failing human hearts induced cellular proliferation after stimulation with ANG II. This effect could not be inhibited by losartan, PD123319, and CGP41112A (Dr. Neuss, personal communication), suggesting the existence of an as yet unknown AT receptor subtype (237).

1.2.4 The local RAS

In the last decade a new concept gained much interest. This concept involves, in addition to the circulating RAS, the existence of local renin-angiotensin-systems in a broad variety of tissues. The presence and synthesis of all components of the RAS, including AT receptors, in one organ is a prerequisite for reference as a local RAS. The evidence for the existence of these local renin-angiotensin-systems came from biochemical studies demonstrating the presence and activity of AO, renin-like enzymes, ACE, angiotensins and AT receptors in different tissues (42,89,118,149,255). Localization of the different components of the RAS in these tissues was performed by immunohistochemistry and radioligand binding assays (39,117). Although the presence and activity of the different components of the local RAS has been described, the uptake of these components from the circulation can not be excluded. Therefore, the prerequisite for the existence of a local RAS is the expression of the mRNA of the different components in these tissues. In a variety of tissues the production of the components at the mRNA level has been detected (94,108,213). However, little is known about possible functions of these local renin-angiotensin-systems. For the cardiovascular system it is thought that tissue RAS play a role in the long-term maintenance of the vascular and cardiac function and structure (114,142,166). The existence of the components of the RAS in organs, which play an important role in cardiovascular control, such as the heart, brain, kidney, adrenal, and blood vessels supports that hypothesis.

Blood vessels: In large and small arteries, like aorta and mesenteric arteries, enzyme activity and mRNA expression of the components of the RAS could be detected. AO mRNA and protein were detected in the peri-adventitial layer, renin activity and mRNA were particularly localized in the smooth muscle cells and endothelial cells (86,87), ACE mRNA and activity in endothelial cells (95), and AT receptors in smooth muscle cells. ANG II formation was demonstrated in the isolated rat hindlimb artery, in the absence of circulating renin (243). In addition, kinetic analysis of in vivo arterial-venous ANG I and ANG II differences in sheep and humans also supports the concept of vascular tissue ANG II production (2). Locally produced ANG II may influence vascular function directly via local hormonal effects or indirectly via modulation of norepinephrine release from sympathetic nerve endings. The vascular RAS may contribute to the regulation of vascular tone and blood flow and may be involved in the development of vascular hypertrophy (92).

Adrenal glands: AO, renin and ACE mRNA and protein have been detected in the adrenal glands (75,118,235). Also, AT receptors have been detected in the adrenal glands; the AT1 receptor primarily in the adrenal cortex, and the AT2 receptor is localized in the adrenal medulla (49,50). Adrenal angiotensin in the rat is involved in regulation of aldosterone biosynthesis, since saralasin inhibits basal aldosterone secretion rate in isolated adrenal glomerulosa cells (335). In rats, aldosterone

secretion is strongly correlated with adrenal renin activity (77). Increased adrenal renin activity was observed in SHR, suggesting a role for the adrenal RAS in the pathogenesis of hypertension.

Brain: AO, renin, ACE mRNA and AT receptors have been detected in the brain (61,168,212,250,316). It has been suggested that ANG II is primarily produced within neurones from neuronally synthesized AO (199). Total brain renin activity is regulated by factors independent of the circulating RAS (97). The brain RAS is possibly involved in blood pressure and thirst regulation as well as vasopressin and catecholamine release (117). Local ACE-inhibitor (ACE-I) treatment in the central nervous system resulted in reduction of the blood pressure and thirst, suggesting the involvement of a brain RAS in the thirst and blood pressure regulation (117).

Kidney: The demonstration of a 1000-fold higher ANG II concentration in the proximal tubular fluid than in plasma supports the existence of an intrarenal RAS (284). The intrarenal RAS may play a role in sodium reabsorption, pH regulation and glomerular hemodynamics (reviewed in (142)). AO mRNA is localized in renal cortical proximal tubules (151). Renin mRNA is primarily localized in the juxtaglomerular cells (133,307). Intrarenal ACE production has been demonstrated by the presence of ACE mRNA (267). ACE protein has been localized by immunohistochemistry in the brush border of the proximal tubule (47). In the proximal tubule, the site where ANG II is produced locally, also AT1 and AT2 receptors are found (82).

1.2.5 The Intracardiac RAS

Evidence for the existence of an intracardiac RAS

The presence of AO mRNA has been demonstrated in hearts from several species (26,94). AO mRNA concentration in the mouse heart was estimated to be 1% of mouse liver AO mRNA concentration, the main source for AO synthesis (94). AO mRNA and its translation product were also detected in isolated neonatal rat heart myocytes and fibroblasts (80). In addition, the release of the AO protein has been demonstrated in isolated perfused rat hearts (200). Renin mRNA was present in low quantities in the mouse and rat heart (94,96,268), as well as in isolated neonatal rat heart myocytes and fibroblasts (80). In accordance, the presence of renin protein was demonstrated in isolated ventricular myocytes from rats using a specific renin antibody, suggesting that renin synthesis occurs in cardiomyocytes. Not only its presence, but also renin activity has been described in isolated cardiomyocytes of the rat and in homogenates of mouse and rat hearts (96). Furthermore, ANG I-generating activity was found in left ventricular tissue of the canine heart. It is, however, not known whether this activity is due to the effects of renin or other ANG I-forming enzymes, such as cathepsin D (132) (see paragraph 1.2.1). Danser *et al.*

(71) showed evidence for angiotensin production in porcine cardiac tissue, although they concluded that cardiac renin is kidney-derived.

In addition to the cardiac AO and renin, also ACE mRNA has been demonstrated in the heart of different species (143,280). Furthermore, ACE protein was detected by immunofluorescent staining in rat neonatal cardiac myocytes, fibroblasts and endothelial cells (81,342). In accordance, conversion from ANG I into ANG II by local ACE has been described in the isolated rat heart (204). Treatment with an ACE-I blocked this intracardiac ANG II-formation (208). In nephrectomized rabbits, with undetectable levels of circulating ANG II, treatment with an ACE-I resulted in lowered concentrations of atrial ANG II (317), suggesting intracardiac formation of ANG II by ACE.

AT receptors have been identified in the heart in different species. In the human heart the most predominant subtype is the AT₂ receptor, whereas in animals the AT₁ receptor subtype is present in higher densities. Sympathetic nerve terminals (32), cardiomyocytes (1,25,260), endothelial cells (295) and fibroblasts (278,324) possess AT receptors. Whereas in coronary endothelial cells from rats AT₁ and AT₂ receptors are present (295), cardiac rat fibroblasts and myocytes exclusively express the AT₁ receptor subtype (239).

Although data strongly suggest the existence of a local intracardiac RAS, no direct biochemical evidence for its activity in vivo is available, since these data are difficult to obtain due to interference with the circulating RAS. The low levels of mRNA expression of the components of the cardiac RAS, especially renin mRNA expression, combined with the lack of in vivo data is the main reason why many researchers still question the function of an intracardiac RAS.

Topography of the intracardiac RAS

The topography of the components of the intracardiac RAS is summarized in table 4. In the adult rat, renin and AO mRNA have been detected in all compartments of the heart (26,52,96,187,201), with the highest mRNA levels for both components in the atria (96,187). However in neonatal rat hearts the highest renin and AO mRNA expression were found in the ventricles, with an overall lower level of renin mRNA expression (80). In the rat heart the highest concentrations of ACE were found in the valve leaflets (342). Furthermore high concentrations were detected in the coronary arteries and in the endothelial and adventitial layers of the aorta and pulmonary arteries. ACE concentrations were moderate in both atria and low in both ventricles (342). A high density of AT receptors was found in the atrioventricular (AV) node, in cells of the intracardiac ganglia and in parasympathetic nerve bundles. A moderate density was found in the AV bundle and low levels in the atria and ventricles (6).

Table 4. *Intracardiac localization of the renin angiotensin system*

	The heart in vivo	Celltypes	References
AO	high mRNA expression in atria; low in ventricles	fibroblasts, myocytes endothelial cells	(26,80,187)
renin	high mRNA expression in atria; low in ventricles	fibroblasts, myocytes endothelial cells	(80,96)
ACE	high density in valve leaflets and in endothelial cells of coronaries; moderate in atria; low in ventricles	endothelial cells; fibroblasts myocytes; macrophages	(81,342)
AT rec.	high density in AV node and para- sympathetic nerve bundles; moderate in AV bundle; low in myocardium	fibroblasts (AT1), myocytes (AT1) endothelial cell (AT1 and AT2), ganglia	(6,239,295)

AO = AO; ACE = angiotensin I-converting-enzyme; AT rec. = angiotensin receptors

1.2.6 Regulation of the systemic RAS

Steroids (48,101,141) and ANG II (28,140,175,282) upregulate the hepatic and the renal AO mRNA expression in the rat, respectively (282). Stimulating effects of ANG II on AO mRNA expression were shown both at pharmacological and physiological ANG II concentrations (156). Besides ANG II, also renin has been suggested to modulate AO production. Administration of renin was associated with decreases in plasma and liver AO levels (139,236,306).

Renal renin release is controlled by the sympathetic nervous system. Stimulation of β -adrenoreceptors on juxtaglomerular cells by sympathetic nerves or by circulating catecholamines increases renin release. Increases in renal perfusion pressure result in an inhibition of the renal release by intrarenal baroreceptors (11). ANG II-administration was shown to inhibit renal renin release and mRNA expression (229,282). Furthermore, low sodium intake, sensed by the macula densa cells, has been demonstrated to increase renal renin mRNA expression and renal release (11,268), whereas adrenocorticotrophic hormone potassium increase renin mRNA expression in cultured adrenal zona glomerulosa cells (328). Since renin is considered to be the rate-limiting enzyme, changes in activation of renin activity, has consequences for the complete systemic RAS.

Little is known about the regulation of the ACE gene. In addition to the effects on renin mRNA regulation, ANG II has also a negative feedback response on pulmonary ACE mRNA (281). This negative feedback of ANG II on ACE is also demonstrated by ACE-I treatment, resulting in induction of plasma and pulmonary ACE activity (115,159,281). Also glucocorticoids, thyroid hormones, androgens and β -adrenergic stimulation can increase ACE activity in different tissues (73,112,116, 232,323). Furthermore increased as well as decreased ACE activity has been observed during hypoxia (164,178).

ANG II is a potent factor in regulating mRNA expression and binding sites of AT receptors. In vascular smooth muscle cells ANG II decreased AT1 mRNA by 50 % (191) and in cultured rat ovarian granulosa cells AT2 binding sites were downregulated by ANG II. AT receptors in the anterior pituitary gland are downregulated by estrogen (51), which is primarily due to downregulation of the AT1b receptor mRNA expression. In contrast the AT1a receptor was not affected by estrogen treatment (169). Differences in AT receptor subtype regulation were also observed in the rat brain during dietary sodium changes (270). Sodium depletion resulted in an increased expression of the AT1b receptor, whereas the expression of the AT1a receptor was increased after a high sodium diet (270).

1.2.7 Regulation of the cardiac RAS

Effects of drugs on the intracardiac components of the RAS are summarized in table 5. Sodium depletion activates cardiac AO mRNA expression but not hepatic AO, indicating a tissue specific regulation (88). Glucocorticoids, which stimulate hepatic

AO mRNA expression (*vide supra*), also increase AO mRNA levels in the rat atria (43,94) and ventricles (94). The effects of ACE-I treatment on AO mRNA expression in the left ventricle of the rat are not clear. Both inhibitory (197) and stimulatory (26) effects have been described.

Increased cardiac renin mRNA levels and increased renin activity were observed in sodium depleted mice (93). Renal and adrenal renin expressions were also stimulated by sodium depletion, but submandibular or testicular renin expression were not, again indicating tissue specific regulation (93). In mice stimulation of β -adrenoceptors with isoproterenol increased cardiac and renal renin expression but not submandibular, testicular or adrenal renin in mice (96). Furthermore cardiac as well as submandibular renin levels were decreased after castration. Renin activity in cardiomyocytes of the rat was studied after treatment with different classes of antihypertensive drugs. After 7 days of treatment with a calcium blocker (nifedipine), ACE-inhibitors (CGS 14831, 14824A and 16617), a vasodilator (hydralazine) and a sympatholytic agent (α methyl dopa), myocytes were isolated from rat ventricles. While the ACE-inhibitors and the sympatholytic agent had no effect on renin activity, the calcium blocker decreased renin activity, and the nonspecific vasodilator increased renin activity (88,96). However, in whole-heart homogenates renin activity was increased after treatment with nifedipine (262).

Little is known about the regulation of cardiac ACE and the AT receptors. In rat ventricles ACE activity was increased after treatment with calcium-blockers or diuretics (262). An in vitro study demonstrated increased cardiac, but not pulmonary ACE activity after treatment with the oxidizing agent diamide (311). The glucocorticoid dexamethasone resulted in an increase of AT1a mRNA levels in isolated cardiac fibroblasts, while it did not affect the AT1b receptor mRNA levels (216). These data indicate that the AT receptor subtype expression in the rat heart can be regulated in a subtype-specific matter.

1.2.8 Functions of the intracardiac RAS

ANG II has direct and indirect physiological effects on the heart. These include coronary vasoconstriction and increased inotropy and chronotropy (29,251). Indirect effects are primarily due to modulation of the sympathetic nervous system. ANG II is known to facilitate adrenergic neurotransmission in the heart (29). The localization of the AT receptor in association with the sympathetic nervous system, supports the idea that ANG II exerts its effects, at least in part, indirectly. When sympathetic facilitation was avoided, dose-dependent positive inotropic effects of ANG II were demonstrated in isolated cardiac preparations in rats (234) and in isolated neonatal rat myocytes (7,78,238). In humans ANG I and ANG II displayed positive inotropic effects via AT receptors in atrial preparations from failing and non-failing hearts, which could be completely blocked by saralasin. The positive inotropic effect of ANG I could be blocked by enalaprilate. However right and left ventricular preparations of these patients did not reveal positive inotropic actions of ANG I and

Table 5. Effects of drugs on the intracardiac renin-angiotensin system

	AO			renin		ACE		AT rec.
	heart	liver	heart	kidney	heart	heart	lungs	
Sodium depletion	↑ (88)	↔ (88)	↑ (93,268)		↑ (93)			
Glucocorticoids	↑ (43,94)		↑ (48)		↑ (150)			AT1a ↑ (216) AT2,AT1b ↔ (216)
ACE-inhibitors	↑ ↓ (26,197)	↓ (26)	↔ (88,96)		↑ (155)		↑ (281)	
Isoproterenol			↑ (96)	↑ (96)				
Nifedipine			↑ ↓ (88,96)		↑ (262)			
Hydralazine			↑ (88,96)					
α-methyldopa			↔ (88,96)					
Diuretics					↑ (262)			
Diamide					↑ (311)		↔ (311)	

AO = angiotensinogen; ACE = angiotensin converting enzyme; AT rec. = angiotensin receptors; ↑ = increase; ↓ = decrease; ↔ = no change; (reference).

ANG II (145). The chronotropic effects of ANG II have been less consistent. In isolated atria from cats no chronotropic effects have been observed (181), whereas preparations from dogs (180), rabbits (308) and guinea pigs (345) demonstrated increased chronotropy by ANG II, although sympathetic facilitation could not be excluded in all experiments (240,345). It is believed that the inotropic and chronotropic effects of ANG II on the cardiomyocyte are mediated by its effect on calcium homeostasis and transmembrane calcium conductance (34,74).

In addition to the inotropic and chronotropic actions of ANG II, it may also play an important role in the regulation of cardiac growth, especially in the development of cardiac remodelling. This aspect of ANG II will be discussed below.

1.3 CARDIAC REMODELLING

1.3.1 Cardiac hypertrophy in pressure- and volume overload

Cardiac hypertrophy is defined as an increase of cardiac mass. Cardiac hypertrophy can be induced by pressure overload (prolonged), volume overload or a combination of both. It can also result from hyperthyroidism or from intensive exercise (physiological pressure overload). These latter two types of hypertrophy will not be further discussed here.

The hemodynamic overload as in hypertension leads to increases of ventricular wall tension. According to LaPlace's law ($T = P \cdot r / 2h$: T = tension; P = pressure in the ventricle; r = radius of the ventricle; h = thickness of the ventricular wall) the cardiac response is directed to normalization of wall tension. Increased ventricular wall thickness as a response to increased ventricular pressure results in normalization of this wall tension. The pressure overload results in the development of concentric hypertrophy, i.e. an increase in ventricular wall thickness, without increases of the inner ventricular radius (224). Parallel sarcomere replication produces the increased wall thickness.

In volume-overload hypertrophy, as in an aortacaval shunt, the increased ventricular wall tension, resulting from increased volume, is normalized by cardiac hypertrophy by addition of new myofibrils in series (end by end). This is defined as eccentric hypertrophy.

Although volume- and pressure overload both lead to cardiac hypertrophy, differences exist with respect to prognosis. Prognosis is better for volume overload hypertrophy than for pressure overload hypertrophy. These differences in prognosis may be caused by structural or phenotypical alterations.

1.3.2 Changes in the infarcted ventricle following myocardial infarction

After MI, necrosis of myocytes is followed by a wound healing response consisting of hypermeability (vasodilatation), an inflammatory reaction, fibroblast proliferation, increased deposition of extracellular matrix components, angiogenesis and the

appearance of myofibroblasts (334). Further cellular changes associated with the cardiac remodelling involve infarct expansion. As a result of the infarction the heart does not contract synergistically towards the center (dyskinesis). This stimulates thinning and expansion of the infarcted area (147,253). Cellular changes responsible for the infarct expansion are the formation of wavy myocardial fibers as a result of inflammation and edema (109), a side to side slippage of myocytes, reduction and redistribution of cells across the infarcted zone (16,245,333).

1.3.3 Changes in the non-infarcted ventricle

After MI, dilatation of the heart is a compensatory response to the loss of functional myocardium to maintain cardiac function (104,218,253). This dilatation of the non-infarcted ventricle results in an increase of ventricular wall stress, which may further stimulate ventricular enlargement (253).

Cardiac hypertrophy after myocardial infarction

The loss of viable myocytes after MI results in an increased stroke work for the remaining myocytes. As soon as three days after MI cellular hypertrophy has been observed in left and right ventricles, with a greater response of the left ventricle. Besides a 29% increase of volume of the viable myocardium, the left ventricular mean myocyte cell volume per nucleus and myocyte length were increased by 28% and 14%, respectively (14). These changes are in accordance with characteristics of concentric as well as eccentric hypertrophy (12,128). The functional changes in the non-infarcted left ventricle are referred to as eccentric hypertrophy, as occurs in volume overload hypertrophy. However the loss of myocytes increases the stress on the remaining myocytes, suggesting that this hypertrophy may be seen as a pressure overload hypertrophy. Right ventricular hypertrophy was demonstrated by a 21% increase in mean cell volume per nucleus, without changes in mean cell length (14). These differences in cell volume but not in cell length are typical for concentric hypertrophy produced by pressure overload (15,17,128).

1.3.4 Cellular changes in cardiac remodelling

Cardiac myocytes

Approximately one third of all cardiac cells are myocytes, which make up approximately 67% of the volume of the heart. After birth the myocytes are thought to be terminally differentiated; they have often been compared with neurons for their loss of capability of cell division (303), although it has been suggested in animals (17,19,22,23,126,209) and humans (245,246) that myocyte hyperplasia is possible in a variety of pathological conditions, including cardiac hypertrophy. As a result of pressure overload or the loss of viable myocytes, volume, orientation and possibly

the number of myocytes change (13,44,246). However, the increase in myocyte mass, does not fully compensate for the loss of viable tissue.

Cardiac hypertrophy is also associated with cellular changes in the cardiomyocyte. Upregulation of gene and protein of the contractile protein, myosin light chain-2 has been observed in cardiomyocytes (192). Re-expression of the fetal phenotype is an important feature in the development of cardiac hypertrophy. For example, in rats the expression of α myosin heavy chain (V1), with a high myosin ATPase activity and rapid shortening velocity is switched to the expression of β myosin heavy chain (V3), with a low myosin ATPase activity and slow shortening velocity (157). Furthermore also skeletal alpha actin (30,210) and atrial natriuretic factor (ANF) mRNA are re-expressed. This re-expression of the fetal phenotype is associated with decreased contractility and lower energy demand of the cardiac muscle. This might explain the lack of functional improvement, in spite of an increase of myocyte mass.

The other cells in the heart like fibroblasts, endothelial cells, macrophages and smooth muscle cells (18) make up the largest number of cells, but only 33% of the total volume.

Endothelium

Proliferation of endothelial cells in the myocardium suggests the formation of new, or the growth of pre-existing capillaries. Cardiac hypertrophy induced by physical exercise or by chronic hypoxia results in an unchanged or even increased capillary density in the myocardium (215). Cardiac hypertrophy induced by pressure overload also induces capillary angiogenesis. It, however, does not completely compensate for the magnitude of the cardiac hypertrophy (310). This is also true for cardiac hypertrophy induced by MI: although growth of capillaries is observed in the non-infarcted myocardium, the ratio of capillaries to myocytes decreases (244). This increase in distance between capillaries impairs the diffusion of substrates, essential for the energetic processes in the hypertrophied myocardium. The re-expression of less energy consuming fetal contractile proteins in the (hypertrophied) myocardium may be seen as an adaptation for the impaired energy balance, although contractility is depressed (172,291).

Extracellular matrix

The extracellular matrix in the heart is mainly composed of a collagen network. The collagen in the myocardium predominantly consists of the fibrillar collagens type I and III. The collagen network serves to tether the cardiac myocytes to one another, thereby influencing their contraction (330). An increase of the concentration of fibrillar collagen, also referred to as myocardial fibrosis, is a major determinant of myocardial stiffness and results in diastolic and systolic ventricular dysfunction (330). Furthermore, arrhythmias can occur as a result of the increased collagen

deposition. This increase of collagen is a result of altered metabolic behavior and/or growth of cardiac fibroblasts (330).

Myocardial fibrosis occurs after MI in the non-infarcted and infarcted myocardium (183,221,326). Fibronectin, another component of the extracellular matrix, is increased after the induction of cardiac hypertrophy by MI (269,288). Furthermore, an increase of DNA synthesis was observed in the interstitium after induction of MI in the rat within the first two weeks in the non-infarcted left and right ventricles. This increase of DNA synthesis was predominantly observed in fibroblasts and endothelial cells (183,186).

In pressure-overload cardiac hypertrophy increased collagen concentrations have been demonstrated. This increase in collagen concentrations was correlated to the increase in pressure. In volume-overload hypertrophy no or a slight increase of collagen amounts were observed (137).

1.3.5 Factors involved in the development of cardiac hypertrophy

In recent years it has become evident that several factors are involved in the development of cardiac hypertrophy. In vitro models demonstrated that a variety of factors, like basic FGF and transforming growth factor beta (TGF β), can induce hypertrophy of cardiomyocytes (249,275). In addition, adrenergic agonists (179,344), endothelin-1 (289) and ANG II (25) elicit hypertrophic responses in cultured neonatal rat cardiomyocytes.

It has been proposed that mechanical factors, like stress, are involved in the development of cardiac hypertrophy. Stretch-induced hypertrophy of neonatal rat cardiac myocytes in vitro activated protein synthesis and gene transcription (167,265,276). Furthermore, conditioned medium of stretched myocytes in culture resulted in activation of immediate-early genes and activation of second messengers in non-stretched myocytes (264). Sadoshima *et al.* (266) found an increase in c-fos, EGR-1, skeletal alpha actin and ANF mRNA expression and protein synthesis in stretched myocytes. Interestingly, these responses could be completely inhibited by an AT1 receptor blocker (losartan) or [Sar-1 Ile-8]ANG II, but not by an AT2 receptor blocker (PD123319). In addition, the release of ANG II increased within 10 minutes after stretching the myocytes, which was associated with an upregulation of the AO gene 6 hours after stretching the myocytes. This shows that ANG II is an early mediator of the stretch-response in cardiac myocytes and may induce the hypertrophic response, either directly or indirectly by inducing other factors. Endothelin-1 may play a role in indirectly ANG II-induced hypertrophy in cardiomyocytes (153). ANG II upregulated endothelin-1 mRNA levels in neonatal rat cardiomyocytes. Furthermore, endothelin-1 stimulated leucine incorporation into the cardiomyocytes, which was dose dependently inhibited by an endothelin A receptor antagonist (BQ123) (153).

1.4 THE ROLE OF THE RAS IN CARDIAC REMODELLING

1.4.1 ACE-inhibition in cardiac hypertrophy

ACE-inhibition in experimental studies

There are several animal studies using different classes of antihypertensive drugs which show differences in reduction of cardiac hypertrophy, in spite of similar antihypertensive potency (66,67,113). The ACE-I captopril, given from 6-20 weeks of age, prevented the rise in blood pressure and cardiac hypertrophy in SHR. Treatment with the vasodilator dihydralazine also prevented the rise in blood pressure to the same extent as captopril, but cardiac hypertrophy was not prevented (207). ACE-inhibition regressed left ventricular hypertrophy in the two-kidney one-clip rat and in rats with aortic stenosis or banding (184,285,286). Also in volume overload induced cardiac hypertrophy in rats regression of cardiac hypertrophy was observed after treatment with an ACE-I (20). In this study volume overload hypertrophy was induced by an aortacaval fistula. After treatment with the ACE-I perindopril for 1 month a regression of cardiac hypertrophy was observed (20). Following MI it was also shown that treatment with an ACE-I resulted in regression of cardiac hypertrophy (252).

As mentioned before, the difficulty for obtaining evidence for the role of the intracardiac RAS in development of cardiac hypertrophy is the dissociation of effects of ACE-inhibitors on cardiac hypertrophy and blood pressure. However, Linz *et al.* (207) presented data supporting the role of the cardiac RAS in the development of cardiac hypertrophy *in vivo*. They compared the effects of equipotent antihypertensive doses of the ACE-I ramipril, the calcium antagonist nifedipine and the arterial vasodilator dihydralazine on cardiac hypertrophy in rats subjected to banding of the abdominal aorta after 6 weeks of treatment. In addition to the hypertrophy-regressing effect of these antihypertensive agents, the effect of a non-antihypertensive dose of ramipril (10 µg/kg/day) was studied on regression or prevention of cardiac hypertrophy. Only the ACE-I showed a complete regression of cardiac hypertrophy. Of interest was the similar effect on regression of cardiac hypertrophy observed after treatment with the low-dose ACE-I compared to the antihypertensive dose of the ACE-I, suggesting that other factors than blood pressure, like the RAS itself, are involved in the development of cardiac hypertrophy (207). However in SHR the ACE inhibitor enalapril was ineffective in regressing cardiac hypertrophy if the systolic blood pressure was not decreased (123,318).

ACE-inhibitors have, in addition to the inhibiting effect of ANG II formation, also an effect on other systems, like the kallikrein system. Inhibition of ACE results in inhibition of the degradation of bradykinin. The role of bradykinin in cardiac hypertrophy and function was studied by Linz and Scholkens (206). They found that a bradykinin B2 receptor antagonist, HOE 140, could abolish the beneficial effects of an ACE-I in rats with aortic banding, with respect to regression of cardiac

hypertrophy and cardiac function. However, in another study, the antihypertrophic effects of the ACE-I ramipril in SHR were not attenuated by chronic treatment with HOE 140 (122). These data suggest that the beneficial effects of ACE-I may be caused by its effect on the kallikrein system or by a combined effect on the kallikrein system and the RAS.

ACE-inhibition in clinical studies

ACE-inhibition appears to be very effective in regression of cardiac hypertrophy, not only in hypertensive animals but also in hypertensive patients with cardiac hypertrophy (21,91,100,111,231,279,287). The regression of cardiac hypertrophy, as was documented by echocardiography in these studies, is related to a decrease of left ventricular afterload (91,287). However, not all antihypertensive drugs have the same efficacy with respect to regression of the cardiac hypertrophy. ACE-inhibitors, β -blockers and calcium antagonists reduce left ventricular mass mainly through a reduction of wall thickness; ACE-inhibitors were found to be most effective (69). In a comparative long-term study, the ACE-I enalapril, but not hydrochlorothiazide, reduced cardiac hypertrophy, although the blood pressure reductions in both treatment groups were comparable (68). Short-term treatment with ACE-inhibitors, calcium antagonists, β -blockers and adrenergic agents all reduced cardiac hypertrophy, while α 1-adrenergic receptor blockers, diuretics and vasodilators did not (91,113,296). Again, these data indicate that the regression of cardiac hypertrophy is not only due to a reduction in hemodynamic overload.

1.4.2 ACE-inhibition: effects on cardiac function following MI

The beneficial effects of ACE-I therapy have been shown in experimental and clinical studies. Pfeffer *et al.* demonstrated that captopril treatment in rats after MI resulted in improved cardiac function and survival (252,254). In accordance increased survival was also observed with another ACE-I, enalapril, in rats after MI.

Also in large clinical trials the beneficial effects of ACE-I treatment after MI have been demonstrated. In the SOLVD (Studies of Left Ventricular Dysfunction) trial patients, of whom 65% had an MI, received enalapril or a placebo. Patients with infarcts less than 4 weeks old were excluded from the trial (293). In the treatment group the risk of mortality was reduced by 16%. In the AIRE (Acute Infarction Ramipril Efficacy) study treatment with the ACE-I ramipril was started in patients, 3-10 days after MI. The mortality risk in this study was reduced by 27% (135). In the Survival and Ventricular Enlargement (SAVE) study patients received the ACE-I captopril within 3-16 days of an acute MI (300). Patients, without clinical manifestations of heart failure, were followed for an average of 42 months. The study showed a 19% reduction in total cumulative mortality in the captopril treated group compared with the placebo group.

Although delayed treatment with ACE-inhibitors after MI clearly demonstrated

beneficial effects on morbidity and mortality, clinical trials focussing on the treatment of patients with an ACE-I in the early phase after MI were found to be less evident, which is in accordance with experimental studies. In experimental heart failure Schoemaker *et al.* (277) demonstrated depressed cardiac function when captopril treatment was given from 0-3 weeks after MI, however cardiac function was improved in the same study when captopril treatment was given from 3-5 weeks after MI. The Cooperative New Scandinavian Enalapril Survival Study II (CONSENSUS II) examined the effect of ACE-I treatment in 6090 patients with acute MI (301). ACE-I treatment with enalapril was started within 24 hours of the onset of symptoms. The trial was terminated early (six months) because of the higher mortality rate in the treated group compared with the placebo group.

In the SMILE (Survival of Myocardial Infarction Longterm Evaluation) study 1556 patients were randomized to receive the ACE-I zofenopril or placebo within 24 hours of acute anterior MI not treated by thrombolysis. After 6 weeks the risk of mortality was reduced by 22%, a difference which was not statistically significant. However, a significant reduction was observed in the frequency of refractory congestive heart failure (9). In the CATS (Captopril and Thrombolysis Study) only patients within 6 hours of a first acute anterior MI were selected for treatment with the ACE-I captopril. After 3 months no significant beneficial effects were found (106). In two megatrials GISSI-3 (Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico) (129) (18895 patients) and ISIS-4 (Fourth International Study of Infarct Survival) (152) (58050 patients) treatment with lisinopril or captopril, respectively, was started in patients within 24 hours of MI. Small differences (5-8 fewer deaths per 1000 patients treated for 6 weeks or 12 months with lisinopril or captopril, respectively) between control and treated groups were found. In the Chinese cardiac Study (CCS-1) (54) 13634 patients up to 36 hours after the onset of suspected acute MI were randomized between captopril or placebo treatment for one month. Approximately 5 fewer deaths per 1000 patients were observed after treatment with the ACE-I (a 5.6% proportional reduction in mortality).

The decrease of morbidity rate after early ACE-I treatment in patients with MI in large clinical studies suggested, although data concerning early ACE-I treatment are not consistent after MI, that ACE-I treatment may start in the early phase in patients with MI (54,152).

1.4.3 AT receptor blockade in cardiac hypertrophy

The broad variety of actions of ACE (see paragraph 1.2.1), makes it difficult to ascribe the effects of ACE-inhibitors to the inhibition of ANG II. However, with the use of AT receptor antagonists, specific actions of ANG II can be examined. A significant regression of left ventricular hypertrophy was found in SHR after treatment with the AT1 antagonist losartan (225). This effect was greater than after ACE-inhibition (53). In another study losartan and captopril similarly prevented cardiac hypertrophy in SHR (226). Long-term treatment with the vasodilator minoxi-

dil results in the development of volume-overload-induced cardiac hypertrophy in normotensive rats or humans (193,312). In this model 5 weeks treatment with the AT1 antagonist losartan was more effective in regressing cardiac hypertrophy than enalapril (263). Also in cardiac hypertrophy following MI complete regression of hypertrophy was observed (292). Mizuno *et al.* (225) demonstrated that left ventricular ANG II content decreased after losartan treatment, while plasma ANG II concentration and plasma renin activity were increased, suggesting the importance of the intracardiac RAS in the development of cardiac hypertrophy. However in rats, subjected to aortic stenosis, resulting in pressure-overload hypertrophy, ACE-inhibitors were more effective than AT1 antagonists in regression and prevention of cardiac hypertrophy (185,205). In patients with symptomatic heart failure and impaired left ventricular function, short-term and long-term losartan treatment resulted in beneficial hemodynamic effects (64)

The distinct effects of regression or prevention of cardiac hypertrophy after blocking the RAS in several models of hypertrophy, suggest the involvement of different factors in the development of cardiac hypertrophy, like bradykinin (*vide supra*), chymase (not in rats), the involvement of AT receptors, or the involvement of local RAS versus the circulating RAS.

1.4.4 Activation of the cardiac RAS in cardiac hypertrophy

The intracardiac mRNA expression of components of the RAS in different models of cardiac hypertrophy is summarized in table 6. The regression of cardiac hypertrophy by inhibitors of the RAS suggests that the activation of the RAS is changed in cardiac hypertrophy. In a pressure-overload model, induced by aorta constriction, AO mRNA was increased in atria and left ventricle, at 7 and 15 days after surgery, respectively (26). SHR, which develop marked cardiac hypertrophy, exhibit an increased cardiac expression of AO mRNA (197) when compared with Wistar Kyoto (WKY) rats. Similarly, MI resulted in an increase of AO mRNA in the non-infarcted left ventricle, 5 days after surgery. AO mRNA levels returned to base line at 25 days after infarction (202).

Little is known about the regulation of the renin gene in models of cardiac hypertrophy. In SHR an increased cardiac expression of renin mRNA was found in addition to the increased AO, when compared with WKY rats (203). In a volume-overload hypertrophy model in rats, Leenen *et al.* (33) demonstrated an induction of left ventricular renin mRNA expression at 7 days after surgery, while there was no increase of cardiac AO mRNA expression.

Aorta constriction increases ACE mRNA (280) and ACE activity. In a semi-quantitative study Hirsch *et al.* demonstrated a 2-fold increase in ACE mRNA expression in the right ventricle and septum 85 days after MI in the rat. Studer *et al.* (297) used a competitive PCR for ACE and found a 3-fold increase in ACE mRNA amount in human failing hearts. No difference in chymase mRNA expression between the control hearts and the human failing hearts was found. In accordance

Table 6: Activation of the intracardiac RAS in several animal models developing cardiac hypertrophy

	AO	renin	ACE	AT1	AT2
AC	↑ (26)			↑ (280)	
SHR	↑ (197)	↑ (203)			
MI	↑ (202)		↑ (297)	↑ (AT1a)(239) ↔ (AT1b)(239)	↑ (239)
VO	↔ (33)	↑ (33)			

AO = angiotensinogen; ACE = angiotensin I-converting-enzyme; AT1 = angiotensin receptor type 1; AC = aorta coarctation; SHR = spontaneously hypertensive rats; MI = myocardial infarction; VO = volume overload; ↑ = increase; ↓ = decrease; ↔ = no change; (reference)

Fabris *et al.* (105) found an increase of ACE activity in different compartments of the rat heart by radioligand binding studies, 4 weeks after MI. The highest increases of ACE activity were found in the infarcted area when compared to the sham-operated controls. In humans, Danilov *et al.* (70) found an increase of ACE immunoreactivity in the inflammatory zone of the infarcted left ventricle 7 days after MI.

Reiss *et al.* (259) isolated cardiomyocytes at 2-3 days after induction of experimental MI in rats. They found a 6- and 2-fold increase of AT receptor mRNA, without making a distinction in receptor subtypes, in left and right ventricular myocytes, respectively. Similarly, in rats a 2-fold increase of AT receptor mRNA expression, predominantly the AT1 receptor, was found 7 days after MI in left and right ventricular myocytes. This was associated with an increased ANG II-stimulated phosphoinositol turnover in these cells (219). In the rat heart Nio *et al.* (239) demonstrated an increase of AT1a and AT2 receptor mRNA expression and number of receptors 7 days after MI. Highest increases were observed in the infarcted left ventricle. AT1b receptor expression was not affected by MI. Treatment with an AT1 antagonist (TCV-116) reduced the increased mRNA levels of the AT1a and AT2 receptors after MI, whereas treatment with an AT2 antagonist (PD123319) had no effect on AT receptor mRNA levels, suggesting an AT1 receptor dependent feedback mechanism for the upregulation of the AT1a and AT2 receptors after MI (239).

1.4.5 The role of the RAS in cellular changes

Cardiac myocytes

Khairallah *et al.* (173) demonstrated that ANG II, without raising blood pressure, induced left ventricular hypertrophy in the rat. Baker and Aceto (25) demonstrated

an increase of protein synthesis and cell growth in cardiac myocytes after addition of ANG II. This effect was inhibited by saralasin. In the adult rat heart, *in vivo*, it was shown that the hypertrophic action of ANG II was independent of its effects on blood pressure and mediated entirely through the AT₁ receptor (79). Furthermore, ANG II induces the expression of the proto-oncogenes *c-myc*, *c-fos* and *c-jun* in the heart and other growth-related factors such as early growth response gene (EGR1), and TGF β 1 (158). In addition, ANG II increases cardiomyocyte DNA-, RNA- and protein-synthesis and content within 2-3 hours after incubation (173). As discussed earlier, stretch may be an important stimulus for the development of hypertrophy of cardiomyocytes by releasing ANG II.

Extracellular matrix

Another possible pathophysiological role of an activated intracardiac RAS is its involvement in the formation of fibrous tissue. This, in turn, increases cardiac stiffness and depresses cardiac function. Neonatal rat cardiac fibroblasts displayed a mitogenic response in the presence of ANG II (278). This response was mediated by the AT₁ receptor. In accordance with this study, AT receptors were identified on neonatal and adult rat cardiac fibroblasts (324). Johnson and Aguilera (165) demonstrated in freshly isolated fetal rat skin fibroblasts that both AT₁ and AT₂ receptors were present, with the AT₂ receptor as the most predominant AT receptor subtype. However, the proportions of the AT receptor subtypes tended to reverse during culture, with the AT₁ receptor as the most predominant AT receptor at day 6. ANG II induced, again via the AT₁ receptor, the mRNA expression of the extracellular matrix components, collagen (type I and III) and fibronectin (63). Chronic ANG II infusion in rats at sub-pressor doses induced fibrosis, even after only two days of treatment (304). The collagen synthesis after MI in the rat could be inhibited by ACE-I treatment (183,221) or AT₁ receptor blockade (292). In a chronic aorta coarctation model in rats (6 weeks) treatment with the ACE inhibitor enalapril resulted in regression of cardiac hypertrophy and a downregulation of type III collagen (214). The presence of ACE in tissue that is largely composed of collagen, like the heart valves, the adventitia of large vessels and intramyocardial arteries, corroborates the role of the RAS in formation of fibrous tissue. Sun *et al.* (299) found a high-affinity ACE binding in microscopic scars and perivascular fibrosis in the myocardium and atria after chronic treatment (4 and 6 weeks) with ANG II or aldosterone. The fact that inflammatory cells have not been observed in the vicinity of intramural vessels during the process of perivascular fibrosis, made them suggest that stimulation of fibroblasts could lead to production of ACE.

Wound healing

A role for the RAS in wound healing has been suggested before. ANG II in wound healing may depend on its ability to stimulate growth factors, like platelet derived

growth factor (PDGF) and TGF β . It has been demonstrated that ANG II is able to induce the expression of growth factors PDGF and TGF β in vascular smooth muscle cells (120,230). A paracrine/autocrine stimulation of TGF β or PDGF by ANG II may be involved in the wound healing process. A role of ANG II in wound healing has been suggested in the rat skin. During experimental wound healing in the adult rat, ANG II levels increased in the skin as early as 6 hours with a peak (10-fold increase) at 24 hours after injury (177), which was associated with a downregulation of the AT1 receptor during the initial 12-24 hours after wound healing. However, another study demonstrated an increase of AT receptors, mainly the AT2 receptor, in the same model of wound healing (325). Thus, both AT1 and AT2 receptor may be involved in the process of wound healing. Also in the heart activation of the RAS has been observed during wound healing. In the infarcted area increased amounts of ACE protein were found in the inflammation zone of scars of human infarcted hearts, 3 and 7 days after MI (70). In accordance, 4 weeks after experimental MI in the rat, increased ACE binding was observed in the infarcted left ventricle (105). The RAS is also involved in vascular injury. After balloon catheter denudation ANG II stimulated DNA synthesis of smooth muscle cells (65). In accordance, Janiak *et al.* (162) demonstrated suppression of myointimal thickening in balloon denudated rabbits after treatment with the ACE-I perindopril or losartan.

Endothelial cells

MI results in an increase of DNA-synthesis in the myocardium (183). About 30% of the proliferating cells in the myocardium are endothelial cells (186), which suggests vascular growth. The remaining proliferating cells are most probably mainly fibroblasts, which can produce matrix proteins. The AT1 receptor antagonist, losartan, could only partially inhibit proliferation. However, collagen synthesis was completely blocked by losartan (292). This suggests that the endothelial proliferation is mediated via another pathway. In accordance with this is the observation on the regulation of ANG II-induced angiogenesis in the CAM (194). This response could not be blocked by the AT2 antagonist PD123319 and the AT1 antagonist losartan, whereas the AT2 receptor ligand CGP42112A completely reduced the ANG II-induced angiogenesis (194). These data suggest the involvement of an AT receptor, different from the AT1 as well as the AT2 receptor, in the angiogenic response. In addition ANG II induced the formation of collateral vessels in the rat kidney in aortic stenosis (106) and the formation of new blood vessels in the rabbit cornea (107), supporting the hypothesis that ANG II is involved in angiogenesis. In agreement with these findings it has been reported that the ACE-I captopril reduces outgrowth of microvessels in the cremaster muscle of renal hypertensive and normotensive rats (327). In contrast with these findings is the increased myocardial capillary density after long-term treatment of SHR with the ACE-inhibitors ramipril or spirapril (318). Differences in the existence of the AT receptor subtypes may be an explanation for the differences in the effect ANG II in the angiogenic assays.

HYPOTHESIS

Above we discussed the RAS and its role in the development of cardiac hypertrophy and other aspects of cardiac remodelling. Cardiac remodelling and especially cardiac hypertrophy are associated with higher morbidity and mortality rates, and it is therefore crucial to obtain more insights in the process of cardiac remodelling in pathological circumstances. In the last decade the concept of an intracardiac RAS and its role in heart failure have gained an acceptance. Little is known about the regulation of the intracardiac RAS in pathological conditions resulting in cardiac hypertrophy. Knowledge about the regulation of this intracardiac RAS in pathological conditions may give insights in the possible functional significance of an intracardiac RAS. Studies of activation of the intracardiac RAS at the mRNA and protein level may provide answers on a possible autocrine/paracrine role of the intracardiac RAS and/or its relation to the circulating RAS in these pathological conditions. This thesis describes studies on the activation and regulation of the intracardiac RAS in experimental models of cardiac hypertrophy, such as abdominal aorta coarctation and MI.

Our working hypothesis was that the intracardiac RAS is an important modulator of the development of cardiac hypertrophy.

THIS THESIS

In this thesis we studied the role of the intracardiac RAS in the development of cardiac hypertrophy by investigating the expression of the RAS in different models of experimental cardiac hypertrophy in the rat. Gene transcription of the different components of the RAS was measured by quantifying the mRNA expression using a reverse transcriptase competitive PCR. Localization of mRNA and its protein was performed by in situ hybridization and immunohistochemistry, respectively. Furthermore, RAS activity was measured in plasma and tissue. Studies were performed in a rat aortic coarctation model, a model of hypertension-induced cardiac hypertrophy and in a rat MI model. The experimental MI model offers an opportunity of studying the wound healing process and the hypertrophic response in one model.

In the first experimental chapter (chapter 3) we studied the regulation of the expression of angiotensinogen and renin in the heart and other tissues after different interventions, like captopril treatment, nephrectomy and abdominal aorta coarctation, resulting in different plasma levels of the circulating RAS. In the chapter 4, 5 and 6 we describe the activation and localization of ACE, renin and AO mRNA in the different compartments of the myocardium at different times after MI, respectively. In chapter 4 tissue ACE protein and activity was studied in the compartments of the rat heart at different times after MI. In chapter 7 we studied the feedback regulation of the expression of the AO, renin and ACE in the heart and systemic organs (liver, kidneys, lungs) after MI during early captopril treatment. In chapter 8 the findings are discussed.

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CHAPTER 2

MATERIALS AND METHODS

2.1 ANIMALS

Adult male Wistar rats (chapter 4) or Wistar Kyoto (WKY) rats (chapters 3, 5, 6 and 7) were used (body weight 250-300 g at the start of the experiments). The experiments were performed according to the institutional guidelines for care and use of laboratory animals. All rats were given standard rat chow and water ad libitum.

2.2 SURGERY

2.2.1 Myocardial infarction

Animals were anesthetized with pentobarbital (60 mg/kg i.p.) and ventilated by positive pressure through an endotracheal tube attached to a Harvard small animal respirator. Via a left-sided thoracotomy and opening of the pericardium, the left anterior descending coronary artery (LAD) was ligated, which results in infarction of the free left ventricular wall (6,8). After closing the chest, Lidocaine (Xylocaine, ASTRA, Rijswijk, The Netherlands) was given i.m. (2 mg/kg) to reduce the incidence of ventricular arrhythmias. In the sham procedure a superficial suture was placed in the epicardium of the left ventricle, near the LAD.

2.2.2 Bilateral nephrectomy

Animals were anesthetized with ether. Two dorsal lateral incisions were made and both kidneys were removed and the wounds were closed. In the sham group two dorsal incisions were made, but kidneys were not removed. Twenty-four hours later the animals were sacrificed in ether anesthesia.

2.2.3 Abdominal aorta coarctation

The animals were anesthetized with ether. The abdominal aorta was exposed through a midline abdominal incision. In the experimental group a needle with a diameter of 0.6 mm was placed adjacent to the abdominal aorta between the renal arteries. A ligature was tightened around the aorta and the needle. The needle was removed, resulting in a constricted aorta with the diameter of the needle. In sham-operated rats the abdomen was closed after exposure of the aorta. Seven days after surgery the animals were sacrificed in ether anesthesia.

2.2.4 Captopril treatment

Animals were anesthetized with ether. Rats received the ACE-inhibitor captopril (0.5 mg/kg/hr) for 24 hours (chapter 3) or for 7 days (chapter 7) by subcutaneously implanted osmotic minipumps (Alzet model 2001, Alza Corp., Palo Alto, Ca, USA). Before implantation the minipumps were placed in 0.9% NaCl at 37°C for 4 hours.

2.3 TISSUE PROCESSING

At the end of the experiments animals were sacrificed by deep ether anesthesia. The tissues of interest were removed. After myocardial infarction or sham-surgery a slice of tissue, which was taken at the mid-sagittal level and parallel to the base of the heart, was formalin fixed overnight in 10% phosphate buffered formalin (pH 7.4), routinely processed and paraffin embedded for determination of the infarct size. The rest of the heart was divided in the (infarcted) left ventricle, septum and right ventricle and quickly frozen in liquid nitrogen and stored at -70°C for RNA isolation or angiotensin I-converting-enzyme (ACE)-activity determination.

2.3.1 Morphometric analysis

The infarct size was determined on AZAN stained sections (4 μ) from the slice of the heart using a computerized morphometric system (Quantimet 570, Leica, Cambridge, U.K.). Infarct size was expressed in percentage of the left ventricular circumference (2).

2.3.2 RNA Isolation

Total RNA was extracted using the LiCl/urea method (9). Tissues were homogenized in 3 M LiCl/6 M urea and centrifuged afterwards. The pellet was dissolved in 10 mM Tris pH 7.8/0.5% SDS (sodium dodecyl sulfate). After homogenizing the tissues and phenol/chloroform/isoamylalcohol (IAA) extraction, RNA was precipitated with 2.5 volumes of ethanol and 0.1 volume of 3 M NaAc (pH 5.3). The RNA concentration was measured by spectrophotometry at 260 nm. RNA integrity was verified by gel electrophoresis.

2.4 COMPETITIVE REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION

Competitive reverse transcriptase PCR (RT-PCR) uses an internal standard. This standard was created by making a shorter fragment (deletion) with restriction enzymes in the target sequence. This results in identical primer sequences for the target and the internal standard fragments. Tissue total RNA and internal standard RNA are converted in the same tube into cDNA, which is subsequently amplified in the presence of specific primers by a DNA polymerase. By performing a competitive RT-PCR of the target fragment and its competitor in the same tube, differences in amplification efficiencies can be minimized (figure 1).

Figure 1. The competitive reverse transcriptase polymerase chain reaction (RT PCR). Internal standard (Δ) and total RNA are converted into cDNA in the same tube and subsequently amplified by PCR. Total RNA is also brought on a agarose gel for measuring the ribosomal RNA bands (18 and 28S).

2.4.1. Selecting specific primers

angiotensinogen

Angiotensinogen (AO) primers were chosen at the second and third exons of the rat AO cDNA sequence (5). The sequence of the upper (sense) primer was 5' CAAG-AGGTGTAGCCAGTGGG 3', base pairs (bp) 344 to 363, and of the lower (antisense) 5' AGCTGGGAGAAGCCTCTCAT 3' (bp 835 to 854). A second set of specific AO primers was used (nested primers) for increasing the amount of the specific AO amplification product. In the second set of primers the sequence of the upper primer was 5' GGGCCGTCCTCTCTCCA 3' (bp 362 to 378) and of the lower 5' GCCTCTCATCTTCCCTTGG 3' (bp 825 to 843), spanning the second and the third exon. Amplification by sequentially using these two sets of primers resulted in a 482 bp PCR fragment. Southern blotting of this PCR fragment and hybridizing with a ^{32}P labeled AO rat probe, revealed the specificity of this fragment (data not shown).

renin

Renin primers were selected at the sixth and ninth exon of the rat renin cDNA sequence (1). The sequence of the upper primer was 5' CTGGGAGGCAGT-GACCCTCAACATTACCAG 3' (bp 747 to 776) and of the lower 5' GAGAGCCAG-TATGCACAGGTCATCGTTCCT 3' (bp 1089 to 1118). Amplification by using these primers resulted in a 372 bp PCR fragment. Renin specificity of the PCR-fragment was demonstrated by Southern blotting of the PCR fragment and hybridizing with a ³²P labeled rat renin probe (data not shown).

angiotensin converting enzyme

The human ACE cDNA sequence (10) was used for selecting ACE-primers. The sequence of the upper (sense) primer was 5' GCCTCCCCAACAAGACTGCCA 3' (bp 492 to 512) and of the lower (antisense) 5' CCACATGTCTCCCAGCAGATG 3' (bp 860 to 880). The cDNA sequence of the lower ACE primer was identical to the recently cloned rat ACE cDNA sequence (3). The cDNA sequence of the upper primer revealed 1 mismatch, when compared to the rat ACE cDNA sequence. Amplification of lung cDNA resulted in a 389 bp fragment. The specificity of this product was checked by sequencing (Sanger method), by hybridizing the PCR-fragment (after Southern blotting) with a ³²P labeled human ACE cDNA probe (10), and by Northern blot hybridization, hybridizing total rat lung RNA with the ³²P labeled PCR fragment. Sequencing of the 389 bp PCR fragment resulted in a homology of 100% with the rat ACE cDNA sequence (3). Southern and Northern blot hybridizations resulted in positive signals at the appropriate heights (data not shown).

2.4.2 Internal standards

After gel electroelution, the PCR-fragments were digested with specific restriction enzymes. The ACE PCR fragment was digested by Hha I (Promega), the renin PCR fragment was digested by Apal and NaeI (Promega), and the AO PCR fragment was digested by HpaII (Promega). Digestion of the PCR fragments resulted in three fragments of different lengths. Again by gel electroelution the 5' and 3' fragments of each gene were separately isolated and consequently ligated with 1 U of T4 DNA ligase (Promega) at 15°C overnight. Subsequently, the ligation product was amplified (35 cycles), using the following cycle profile for all genes: 1 min at 94°C, 1 min at 65°C, and 1 min at 72°C. This was concluded with 10 min at 72°C, which results in a 3' adenosine nucleotide overhang at the amplified products. This deletion fragment was checked by agarose gel electrophoresis for length and purity. Afterwards the deletion fragment was ligated for 15 hours at 15 °C with 2 U T4 DNA ligase in the presence of the TA-vector (Stratagene, molar ratio 3:1, 1:1 and 1:3), containing a 3' thymidine overhang and a T7 and Sp6 RNA polymerase site. After linearizing the plasmid with HindIII (Promega), RNA was transcribed with 1 U T7

Table 1: *Length in base pairs of the target DNA and the internal standard DNA fragments of the different components of the RAS for the competitive RT-PCR*

	Angiotensinogen	Renin	ACE
Target length (bp)	482	372	389
Internal standard length (bp)	372	263	291

bp = base pairs; ACE = angiotensin I-converting-enzyme

RNA polymerase (Promega), 1 hour at 37°C. Plasmid DNA was removed by electroelution, followed by DNase treatment with 5 U of RQ1 DNase (Promega), 30 min at 37°C. The concentration of the internal standard RNA was measured by spectrophotometry at 260 nm and integrity was checked on a 1% agarose gel. Possible plasmid DNA contamination was ruled out by amplification of the RNA. For stabilizing the standard RNA, 30 µg/ml tRNA was added. The lengths of the internal standards of the different components of the RAS are shown in table 1.

2.4.3 Reverse transcription

In the same tube, total RNA was mixed together with increasing amounts of the standard RNA (1 fg to 50 fg AO standard RNA per 500 ng total RNA, 10 fg to 400 fg renin standard RNA per 500 ng total RNA, 200 fg to 5 pg ACE standard RNA per 500 ng total RNA, 6 concentrations/RNA sample) in 1 x RT-buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl and 3 mM MgCl₂), 1 µM of random hexanucleotide primers (Promega) and 2 mM dNTP (Pharmacia). Sterile water was added to a volume of 10.5 µl. This was placed at 72°C for 3 min and cooled at 42 °C. Hereafter 10 mM dithiothreitol, RNase inhibitor (10 U; 1.6 U/100 ng total RNA; Promega) and M-MLV-RT (50 U; 8 U /100 ng total RNA; Gibco) were added to a final volume of 12.5 µl and incubation was performed for 1 hour at 42°C.

2.4.4 Polymerase chain reaction

The cDNA fragments from each reverse transcription reaction was amplified in duplicate. Five µl of the reverse transcription mixture was added to 20 µl of a PCR-mix, containing 20 mM Tris-HCL, pH 8.3, 50 mM KCl, 20 pmol of both the lower and upper primers (*vide supra*) and 1.25 U AmpliTaq DNA polymerase (Perkin & Elmer). The final MgCl₂ concentration varied for the different components (2 mM MgCl₂ for the first PCR of AO, 1 mM MgCl₂ for the second PCR; 3 mM MgCl₂ for renin; 1 mM MgCl₂ for ACE). The amplification profile for the different components of the RAS was as follows: AO; 0.5 min 94°C, 0.5 min 62°C, 1 min 72°C, 20 cycles. After amplification, 3 µl was used for a second PCR with nested primers in 22 µl of the

PCR-mix. The profile of the second PCR was also 0.5 min 94°C, 0.5 min 62°C, 1 min 72°C, 20 cycles. For renin the amplification profile was 0.5 min 94°C, 0.5 min 65°C, 1 min 72°C, 40 cycles. For ACE the amplification profile was 0.5 min 94°C, 0.5 min 65°C, 1 min 72°C, 30 cycles.

2.4.5 Quantification of the PCR fragments

After amplification, 10 µl of the PCR fragments were separated by electrophoresis on a 1.5% agarose gel, containing ethidium bromide. By UV irradiation the two fragments were detected and photographed (Polaroid positive/negative, type 665). The negative film was used for measuring the optical density of the two fragments by a densitometer (Pharmacia). The value of the optical density of the internal standard was multiplied by the ratio of target DNA bp/standard DNA bp, thereby correcting for the difference in ethidium bromide density, caused by the difference in length between the target fragment and the internal standard. The mean values of the duplicate samples were plotted as the logarithm of the ratio of the internal standard to the target fragment against the logarithm of the known amount of internal standard RNA in each tube. The line of best fit and the correlation coefficient (r) were calculated. If r^2 was below 0.95 data were discarded. The logarithm value of zero on the y-axis represents the equivalence point, indicating the equal amount of internal standard RNA and target RNA. Equal amounts of RNA, as used in the competitive RT-PCR, were brought in duplicate on a 1% agarose gel. The total amount of 500 ng of standard total RNA and sample RNA were brought onto gel in triplicate and duplicate, respectively. The mean values of the RNA samples were related to the mean of the standard total RNA. The mRNA amount is expressed in femto (f)g or atto (a)g/100 ng of total RNA. The mean mRNA amounts ($\pm$ SEM) of 3 animals are given. The mRNA amounts in the compartments of the heart at the different time points in the infarcted and the sham group are evaluated. The inter- and intra-assayvariation of the competitive RT-PCR were 13% and 11% for AO, 15% and 10% for renin and 15% and 12% for ACE, respectively. The variation between the duplicate values after PCR was less than 5%.

2.5 NORTHERN HYBRIDIZATION ANALYSIS

15 µg total RNA was dissolved in 30 µl sample buffer containing 10 mM sodium phosphate (pH 7), dimethylsulfoxide (DMSO) and glyoxal. After denaturation (1 hour at 50°C) the samples were electrophoresed in a 1% agarose gel with a 10 mM sodiumphosphatebuffer (pH 7) for 3 hours at 100 V and subsequently transferred to a Hybond N⁺ membrane.

2.5.1 Labeling of cDNA and hybridization

The cDNA probes used in the experimental chapters are shown in table 2. The

Table 2: Length in base pairs of the probes used for Northern hybridization analysis.

	AO	Renin	ACE	ANF	18S	28S
Probe length (bp)	1634	1425	389	697	750	2100

bp = base pairs; AO = angiotensinogen; ACE = angiotensin-converting-enzyme; ANF = atrial natriuretic factor.

probes were labeled by the random priming method using α - ^{32}P dCTP. After 1 hour prehybridization the radioactive probe was added (a total of 300,000 counts per minute of the radioactive probe per ml hybridization mix) and hybridized for 18 hours at 42°C, followed by washes in 2 X standard saline citrate (SSC). After exposure of the autoradiogram the blot was stripped in bidest at 100°C for 30 minutes and rehybridized with another cDNA probe. By using the 18S (4) or 28S probe for rehybridization, autoradiographic signals of the probes of interest could be related to the 18S or 28S probe autoradiographic signal (*vide infra*). Washing and hybridization of this probe were performed at 65°C instead of 42°C.

2.5.2 Autoradiography

Hybridization signals were visualized by exposing the membrane to a X-ray film (Kodak) at -70°C in the presence of an intensifying screen. This autoradiogram was scanned with the use of a densitometer. The 18S signal was used as the internal standard, and the ratios of the signals of the probes of interest and the 18S or 28S probe was determined. On every blot one probe/18S or probe/28S ratio of a sham-operated animal was given the value of 1. All other probe/18S or probe/28S ratios on that blot were normalized to that value.

2.6 IN SITU HYBRIDIZATION

The PCR fragments of the different components of the RAS, described above, were ligated in the TA-vector. A ^{35}S - or ^{33}P -UTP-labeled antisense riboprobe was created by transcription using T7 RNA polymerase. Plasmid DNA was removed by RQ1 DNase treatment and the probe was precipitated after phenol/chloroform extraction. Sections (4 μm) of formalin fixed, paraffin embedded hearts were cut from infarct and sham hearts 4 days after surgery and dried for 5 min at 55 °C. Pretreatment consisted of 10 min fixation in 4% paraformaldehyde, followed by proteinase K (2 $\mu\text{g}/\text{ml}$) treatment for 10 min at room temperature. Hereafter the sections were incubated for 1 hour in a prehybridization buffer (50% formamide, 0.3 M NaCl, 20 mM Tris pH 8, 5 mM EDTA, 0.02% polyvinyl-pyrrolidone, 0.02% Ficoll, 0.02% BSA, 10% dextran sulphate, and 10 mM DTT) at 37°C. Hybridization was performed

overnight at 55°C after addition of 600.000 cpm of the ^{35}S or ^{33}P labeled probe per section. This was followed by a wash in 2 X SSC, 1 mM EDTA and 10 mM β -mercaptoethanol for 10 min, RNase treatment and 2 washes in SSC. After dehydration and drying, the sections were dipped in NTB-2 nuclear emulsion (Kodak, Technorama, Zürich, Switzerland) in the dark. The sections were developed after 3-6 weeks with Kodak D19 and fixed in 0.8 M sodium thiosulfate and 0.09 M potassium disulfate. Finally, the sections were counterstained with hematoxylin and examined by bright-field and dark-field microscopy.

2.7 BIOCHEMICAL ASSAYS

2.7.1 Plasma angiotensin II concentration

A total of 2 ml blood was drawn, just before sacrifice, via a catheter in the abdominal aorta and collected in heparinized tubes containing EDTA. The plasma concentrations of ANG I or ANG II were determined by radioimmunoassay. At the end of the incubation periods antibody-bound ANG I or ANG II were separated from unbound ANG I or ANG II using anti-sheep (donkey) coated cellulose in suspension as the solid phase. Following a brief incubation and centrifugation, the unbound ANG I or ANG II was decanted and the antibody-bound ^{125}I radiolabeled ANG I or ANG II was measured in a gamma counter. Using a standard curve, sample concentrations were expressed in picomoles per ml. The lower limit of detection was 1.1 fmol/tube for ANG I and 0.7 fmol/tube for ANG II. Intra-assay coefficient of variation was 7.3% and inter-assay variation was 14.1%

2.7.2 Plasma renin activity

A total of 2 ml blood was drawn, just before sacrifice, via a catheter in the abdominal aorta and collected in heparinized tubes containing EDTA. Plasma renin activity was measured by incubation of plasma at 37°C and 0°C for 2 hours in the presence of AO and expressed as the formation of ANG I (pg/l.min), measured by radioimmunoassay.

2.7.3 Tissue ACE activity

This method was described by Pinto *et al.* (7). Briefly, after rinsing the tissue, it was homogenized in 50 mM K_2HPO_4 homogenate (pH 7.5). The sample was diluted and 100 μl was transferred into a 0.5 M K_2HPO_4 buffer (pH 7.5). As a substrate, 100 μl of 12.5 mM hippuryl-His-Leu (Sigma), which is converted by ACE into His-Leu, was then added and incubated at 37°C for exactly 10 min. In this amount the substrate is present in excess and is thus not rate-limiting. The conversion of the substrate was stopped by adding 1.45 ml of 0.28 M NaOH, after which 100 μl of 1% phtaldialdehyde was added. This adheres to the His-Leu product. The amount of tagged His-Leu

was then determined fluorimetrically at an excitation wavelength of 364 nm and an emission wavelength 486 nm. This is a measure of the amount of His-Leu generated by ACE in the sample. In blank samples, conversion of the substrate was prevented by prior addition of NaOH. The sensitivity of this assay is less than or equal to 0.02 nmol.mg⁻¹ tissue.min⁻¹; the generation of the product His-Leu is linear from 0.02 to 15 nmol.mg⁻¹.min⁻¹ in each tissue (7).

2.8 IMMUNOHISTOCHEMISTRY

Four μ m sections were cut from paraffin embedded hearts from the sham group and infarct group, at 4 days after the operation. The sections were deparaffinized in xylene and 100% ethanol, followed by blocking the endogenous peroxidase in 0.3% H₂O₂ in methanol. After washing in Trisbuffered saline (0.14 M NaCl, 4 mM Tris-HCl pH7.4; TBS) the sections were incubated with a 1:1000 dilution of the ACE antibody (goat- α -rabbit lung; polyclonal, gift of Dr. Soffer) for 1 hour at room temperature. Sections were washed in TBS, followed by incubation with a rabbit-anti-goat peroxidase labeled conjugate (1:1000; DAKO) for 45 min at room temperature. 3,3'Diaminobenzidine was used as the chromogen. The sections were counterstained with hematoxylin, dehydrated and mounted.

2.9 STATISTICAL ANALYSIS

All data are presented as mean $\pm$ SEM. Statistical differences were calculated using the nonparametric Mann-Whitney U test (chapter 3), two way analysis of variance (ANOVA) (chapter 4, 5 and 6) or Kruskal-Wallis test (chapter 7). ANOVA was followed by Bonferroni's t-test if differences existed between the groups (11). Statistical Significance was accepted at the level of $p < 0.05$.

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CHAPTER 3

**DIFFERENTIAL REGULATION OF ANGIOTENSINOGEN AND RENIN
MESSENGER RNA FOLLOWING INTERVENTIONS IN THE RENIN
ANGIOTENSIN SYSTEM IN RATS**

ABSTRACT

The present study was designed to characterize the regulation of the angiotensinogen (AO) and renin mRNA in different tissues. Experiments were performed in 3 month-old Wistar Kyoto (WKY) rats. In the first experiment a coarctation of the abdominal aorta was made and the animals were sacrificed 7 days later. These animals exhibited significant cardiac hypertrophy. In the second experiment normal animals received the angiotensin converting enzyme inhibitor (ACE-I) captopril (0.5 mg/kg/hr). In the third experiment the rats were subjected to bilateral nephrectomy or sham surgery. In the last two experiments the rats were sacrificed after 24 hours. In all experiments plasma angiotensin II (pANG II) concentrations and plasma renin activity (PRA) were measured and the tissues of interest were removed for determination of AO and renin mRNA expression by Northern blot hybridization. Nephrectomy significantly increased AO mRNA expression in the liver and in the brain (190% and 160% of sham, resp.) but had no effect on AO mRNA expression in the other tissues. In contrast, both aorta coarctation and captopril treatment significantly decreased AO mRNA expression in the atria (25% and 83% of sham, resp.) but had no effect on AO mRNA expression in liver and brain. Furthermore, we found a 67% increase of renal renin mRNA expression after captopril treatment, while none of the other tissues examined showed a difference in renin mRNA expression. Abdominal aorta coarctation and nephrectomy revealed no difference in renin mRNA expression in all tissues examined. These observations are consistent with a negative feedback of renin mRNA expression by ANG II in the kidney, but not in other tissues. From these data we conclude that there is a differential regulation of AO and renin mRNA in the different tissues examined: while AO mRNA in the liver and the brain appears to be tonically inhibited by renin, AO mRNA in the atria is not. Furthermore we conclude that myocardial hypertrophy is not necessarily associated with an increased AO mRNA expression.

3.1 INTRODUCTION

The renin-angiotensin-system (RAS) plays an important role in blood pressure and hydromineral homeostasis, with ANG II as the biologically active component of the RAS. In the classical concept (systemic RAS), the RAS is a circulating hormonal system. In addition, expression of components of the RAS has been shown in several tissues, like heart, aorta, adrenal gland, brain and kidney (4,5,10,15,26,32), suggesting the existence of a local RAS. Several authors (17,28) suggest the involvement of a cardiac RAS in cardiovascular disorders. For instance, increased cardiac AO mRNA and angiotensin-converting enzyme (ACE) activity and mRNA expression were found after aortic banding (1,33) or myocardial infarction (12,18,25).

Although local synthesis of AO is a prerequisite for a local RAS, little is known about the regulation of the AO gene. The most obvious findings were obtained with

steroids (8,11) and ANG II (2,17,23,24,30,34), which upregulate the hepatic or, in case of ANG II, the renal AO mRNA expression of the rat (34). Effects of ANG II on AO mRNA expression were shown both at pharmacological and physiological ANG II concentrations (21). Besides ANG II, also renin has been suggested to modulate AO production. Administration of renin was associated with decreases in plasma AO (31,36) and liver AO levels (16). Several studies suggest that ANG II is also a potent regulator of renin synthesis (9,13). ANG II-administration was shown to inhibit renal renin release and mRNA expression (34). Furthermore, low sodium intake has been demonstrated to increase renal renin mRNA expression, whereas ACTH and potassium increase renin mRNA expression in cultured adrenal zona glomerulosa cells (38).

The present experiments were performed to determine the regulation of AO mRNA and renin mRNA in different tissues by manipulating the plasma concentrations of the different components of the RAS, by nephrectomy, ACE-inhibition and aorta coarctation.

3.2 MATERIALS AND METHODS

Surgery.

In all experimental groups and sham-operated groups 15 WKY rats were used (chapter 2.1). The animals were randomly assigned to the different groups. Nine rats in each group were used for determining the AO and renin mRNA expression. Six rats in each group were used for determination of pANG II (chapter 2.7.1) concentrations and PRA (chapter 2.7.2). Body weight was measured before and after the experiments.

Nephrectomy.

Rats were subjected to bilateral nephrectomy as described in chapter 2.2.2. Twenty-four hours later the animals were sacrificed in deep ether anesthesia. Left and right ventricle, atria, brain, liver, kidneys and lungs were removed, frozen in liquid nitrogen and stored at -70°C.

Abdominal aorta coarctation.

Seven days after surgery (chapter 2.2.3) sham and coarctation animals were sacrificed in deep ether anesthesia. The atria were separated from the ventricles. The ventricles were weighed and ventricle/body weight ratio was used as an indicator of cardiac hypertrophy. The left ventricle, right ventricle, atria, and liver were frozen in liquid nitrogen and stored at -70°C.

Captopril treatment.

Twenty-four hours after captopril treatment (chapter 2.2.4) rats were sacrificed in deep ether anesthesia. Left and right ventricle, atria, brain, liver, kidneys and lungs were removed, frozen in liquid nitrogen and stored at -70°C.

Plasma ANG II.

The assay used for measuring the plasma ANG II concentrations are described in chapter 2.7.1

Plasma renin activity.

The assay used for measuring PRA is described in chapter 2.7.2

RNA isolation.

Because of the low amount of RNA in the atria, the atria of two or three animals in each experiment were pooled. Total RNA was extracted using the LiCl/urea method as described in chapter 2.3.2.

Northern hybridization analysis.

In chapter 2.5 this procedure is described. The probes used in this chapter were the cDNA inserts to AO (1634 kb) , 18 S rRNA (750 bp) and renin (1425 bp).

Statistics.

Data are presented as means $\pm$ SEM and comparisons were made by Mann-Whitney U test. Statistical significance was accepted at the level of $p < 0.05$ (chapter 2.9).

3.3 RESULTS

AO and renin mRNA expression were detected at 1.8 kb and 1.6 kb, respectively. AO mRNA expression was highest in the liver and renin mRNA expression was highest in the kidney. The mRNA expression of AO in the atria was very low, near the level of detection. AO mRNA levels in the ventricles were even lower and could not be detected by the Northern hybridization analysis. Using Northern hybridization analysis renin mRNA, although present in the other tissues, could also not be detected in the compartments of the heart.

Figure 1. Northern blot of rat liver and brain total RNA (15 μ g) hybridized with radiolabeled angiotensinogen (AO) and 18S cDNA probes, 24 hours after nephrectomy (NX), or captopril (CAP) treatment and in sham (SH) rats.

Angiotensinogen mRNA expression

Figure 2. Densitometric analysis of messenger RNA levels for angiotensinogen in atria, brain, liver, kidneys, lungs, testis in sham (SH) rats, 24 hours after nephrectomy (NX), or captopril treatment (CAP). All values are normalized to the densitometric angiotensinogen (AO)/18S ratio of the sham rat.* $P < 0.05$, significantly different from sham.

Nephrectomy.

Twenty-four hours after bilateral nephrectomy we observed a 1.9 fold increase of liver (n=9) and a 1.6 fold increase of brain AO mRNA (n=6; figures 1 and 2), the two major organs for AO production, when compared to the sham animals. In all the other tissues examined, no difference in AO mRNA expression was found between the sham and nephrectomized animals (figure 2). Renin mRNA expression was not affected in any of the tissues examined.

Abdominal aorta coarctation.

Seven days after abdominal aorta coarctation a significant increase ($9.1 \pm 0.9\%$) in the ventricle/body weight ratio was observed in the coarctation animals when compared to the sham animals. No change in body weight was seen after surgery in both groups, indicating that the increase in the ventricle/body weight ratio was due to hypertrophy of the ventricles. AO mRNA expression in the atria was significantly decreased after aorta coarctation (25% of sham, n=3; figure 3). AO mRNA expression in the liver was not different in abdominal aorta coarctation versus sham (figure 3). Aorta coarctation did not affect renin mRNA expression in all tissues examined.

Captopril treatment.

After 24 hours of captopril treatment AO mRNA expression in the atria was significantly decreased when compared to the sham animals (83% of sham, n=3). None of the other tissues examined, showed a difference in AO mRNA expression after captopril treatment (figure 2). Renal renin mRNA (figure 4) expression was significantly increased by captopril treatment (1.7 fold, n=6), while no effect on renin mRNA expression was observed in the other tissues.

PRA and pANG II

In table 1 PRA and pANG II levels are shown after the different interventions. After nephrectomy pANG II decreased to 10% of sham-operated rats, whereas PRA was not detectable in the experimental group. After 7 days of abdominal aorta coarctation there was no increase in pANG II as well as PRA, when compared with the sham-operated animals. After captopril treatment pANG II was 45% of sham-operated rats, while there was an 8-fold increase in PRA compared to sham group.

Angiotensinogen mRNA expression

Figure 3. Densitometric analysis of messenger RNA levels for angiotensinogen mRNA in atria and - liver, 7 days after abdominal aorta coarctation (CO). All values are normalized to the densitometric angiotensinogen (AO)/18S ratio of the sham (SH) rat. * $P<0.05$, significantly different from sham.

Renal renin mRNA levels

Figure 4. Densitometric analysis of messenger RNA levels for renin in the kidney in sham (SH) and captopril (CAP) treated animals. All values are normalized to the densitometric renin/18S ratio of the sham rat. * $P<0.05$, significantly different from sham.

Table 1: Plasma renin activity and plasma ANG II following aorta coarctation, nephrectomy or captopril treatment

	SH	CO	SH	NX	CAP
PRA	31 ± 6.1	26 ± 15	28 ± 7.0	N.D.	223 ± 62*
pANG II	38 ± 8.2	35 ± 13	38 ± 4.4	3.9 ± 0.31*	17 ± 3.6*

SH = sham; CO = aorta coarctation; NX = nephrectomy; CAP = captopril treatment; PRA = plasma renin activity; pANG II = plasma ANG II levels; N.D. = not detectable; *p<0.05

3.4 DISCUSSION

The present study was designed to analyze the regulation of AO and renin mRNA expression in several tissues of the rat, by experimentally manipulating the different components of the RAS. The data of our study are summarized in table 2. Little is known about the regulation of AO and renin mRNA expression. Increased AO mRNA synthesis was found following glucocorticoid administration to rats (22), a rat hepatoma cell line (8), or fat cells (5). In a study in intact rats, Campbell and Habener (4) did not observe any regional specificity, but rather an overall increase in all tissues examined. In contrast, androgens may differentially affect tissues, since testosterone administration to female or castrated male rats increases renal but not hepatic AO mRNA expression (11). Different factors have also been reported to regulate renin mRNA expression in the kidney. Sodium deprivation has been demonstrated to increase renal renin mRNA expression (7,20,39). In addition an upregulation of renin mRNA was also found in cultured adrenal zona glomerulosa cells by ACTH and potassium (38).

In addition also the components of the RAS have been suggested to modulate AO and renin mRNA expression. Both renin and ANG II themselves were demonstrated to influence hepatic production or plasma levels of AO. Administration of ANG II increases hepatic (30,34) and renal (34) AO mRNA expression in vivo and also increases hepatic AO protein synthesis in vivo (23,31) and in vitro (16,24), indicating the existence of a positive feedback of ANG II on the production of AO. As mentioned above, also renin has been shown to modulate AO, since administration of renin decreases plasma levels (31,36) and liver concentrations (16) of AO, indicating the negative feedback of renin on AO production. ANG II has also been found to regulate renin mRNA expression and production. ANG II administration exerted a negative feedback on renal renin mRNA expression and renin release (29,34). This was confirmed by studies performed with the ANG II-antagonist Dup753 (40) or captopril (19,27), which revealed an increase in plasma renin concentrations and renal renin mRNA expression.

Table 2. Effects of nephrectomy, aorta coarctation and captopril treatment on systemic RAS or intracardiac angiotensinogen and renin.

			Liver		Brain		Atria		Kidney	
	pANG II	PRA	AO	renin	AO	renin	AO	renin	AO	renin
NX	↓↓	↓↓	↑	↔	↑	↔	↔	↔		
CAP	↓	↑↑	↔	↔	↔	↔	↓	↔	↔	↑
CO	↔	↔	↔	↔		↔	↓	↔	↔	↔

NX = nephrectomy (24 hours); CO = aorta coarctation (7 days); CAP = captopril treatment (24 hours); AO = angiotensinogen; pANG II = plasma angiotensin II levels; PRA = plasma renin activity; ↑ = increase; ↓ = decrease; ↔ = no change.

In the present study we observed an increase of hepatic and brain AO mRNA following nephrectomy. None of the other tissues examined showed a difference in AO mRNA expression following nephrectomy. These observations are in accordance with a tonic inhibition of hepatic and brain AO production by renin, since nephrectomy is associated with both plasma renin and ANG II were extremely low (table 1). Similar observations were previously made by Campbell and Habener (4). Nephrectomy did not result in differences in renin mRNA expression in the different tissues examined.

Aorta coarctation as employed in the present study (i.e. similar to two-kidney one-clip hypertension) induced ventricular hypertrophy, but did not result in increased PRA and ANG II plasma levels. At least after 7 days of coarctation PRA and pANG II were not different from sham-operated rats, although abdominal aorta coarctation initially leads to an increase of these parameters (28), indicating the dynamics of the RAS. In our study AO mRNA expression was decreased in the atria, following coarctation. None of the other tissues showed a difference in AO mRNA expression. The effect differs from the increase of AO mRNA expression in the atria, reported by Baker *et al.* (1); in their model, however, they ligated the aorta above the renal bifurcation, which resulted in a volume dependent hypertension model (similar to one-kidney one-clip). These authors, in their model of coarctation (*vide supra*) observed increased AO mRNA in atria after fifteen days. They also noted normal plasma renin concentration, and presumed normal ANG II levels. However they could not detect a difference in atrial AO mRNA expression after 7 days after aorta coarctation. This emphasizes the dynamics and the variability of the RAS in time. In this model no difference in renin mRNA expression was detected in all tissues examined.

Treatment with converting enzyme inhibitors has yielded more variable results with respect to hepatic AO mRNA levels. Long-term administration in rats has been shown to result in increased (1) or unchanged (6,14,34) AO mRNA expression in

liver. In at least two other studies, reductions were noted as soon as 4 hours after administration (21,30). In the present study, 24 hours infusion of captopril resulted in a decrease of atrial AO mRNA but did not affect AO mRNA levels in the other tissues examined. This discrepancy in the different studies may depend upon the dynamics of the responses of renin and ANG II to ACE inhibitors. In our study 24 hours after captopril treatment, circulating ANG II levels are reduced, whereas renin levels are increased (table 1). However during chronic treatment ANG II levels return to normal (3), while renin levels remain increased. Thus, depending upon the duration of administration and the dosage-interval the balance of the effects of renin and ANG II may differ. Baker *et al* (1) administered enalapril for 1 to 2 weeks and noted increased left ventricular AO mRNA levels. Again, this may depend upon the dynamics of the response to the drugs (*vide supra*) and the duration of administration. The increased PRA in our study may be responsible for the decreased AO mRNA expression in the atria by a negative feedback. The hypothesis that a tonic inhibition (*vide supra*) in non-atrial tissues, like liver and brain, plays a role in the regulation of AO mRNA expression, is strengthened by the lack of difference in AO mRNA expression in those tissues.

Renal renin mRNA expression was increased after captopril treatment. This is in accordance with the increase in plasma renin concentrations and renal renin mRNA expression after treatment with an ANG II-antagonist or captopril (19,27). Our data confirm that ANG II is an important factor in controlling renal renin mRNA expression. Renin mRNA expression was not different in the extrarenal tissues examined. Studies on the regulation of extrarenal renin mRNA expression are scarce. In a study by Iwai & Inagami (19) renin mRNA expression levels in the brain increased during 7 day administration of captopril. In the present study there was no increase in brain renin mRNA. However the captopril treatment in our study lasted only 24 hours and the captopril dose was 8 times lower, although this dose causes a 17-fold shift of the pressor dose-response curve to angiotensin I (35), indicating effective inhibition of the enzyme.

The present study provides further evidence for the differential regulation of local AO and renin mRNA expression in different tissues by components of the RAS. The brain RAS has been implied in cardiovascular control and has been shown to be regulated independently from the circulating system (37). Our results and those of others (4) suggest that this is not the case for the feedback regulation by the components of the system, although we have not addressed the influence of physiological stimuli as low or high sodium intake. The cardiac RAS has been implied in cardiac hypertrophy. In the present study, aortic coarctation was found to be associated with ventricular hypertrophy. This was associated with decreased atrial expression of AO mRNA in the present study, but increased expression in another (1). This suggests that cardiac hypertrophy is not necessarily associated with increased AO mRNA expression.

In conclusion, we observed differential expression and regulation of expression of AO and renin mRNA in different tissues of the rat. In liver and brain, and possibly

also in the atria AO mRNA expression seems to be controlled by both renin and ANG II levels in plasma. The dynamics of the RAS and the differential responses of the different tissues make it difficult to predict the regulation of their genes at a certain time point. The balance between the two hormones determines the outcome for expression. Furthermore, we show that hypertrophy is not necessarily associated with increased cardiac expression of AO.

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CHAPTER 4

**ACTIVATION OF ANGIOTENSIN I-CONVERTING-ENZYME GENE AND
PROTEIN IN THE INFARCT ZONE FOLLOWING MYOCARDIAL
INFARCTION IN THE RAT**

ABSTRACT

In the present study we quantified angiotensin I-converting enzyme (ACE) mRNA and localized ACE mRNA and protein in the infarcted rat heart. Wistar rats underwent ligation of the left descending coronary artery, resulting in myocardial infarction (MI), or a sham-operation. At different times (1-90 days) after surgery (n=3 each) the heart was removed and divided into right ventricle (RV), septum (SE) and left ventricle (LV). ACE mRNA was quantified by competitive reverse transcriptase polymerase chain reaction (RT-PCR). At 4 and 7 days after MI we found a 2.8-fold respectively 2.7-fold increase of ACE mRNA (n=3; $p<0.05$) in the infarcted LV compared to the LV of the sham-group. No increases of ACE mRNA were found in the non-infarcted hypertrophied compartments. ACE activity increased 2.6-fold and 3.6-fold in the infarcted LV at 7 and 90 days after MI, respectively. In situ hybridization and immunohistochemistry showed increased ACE mRNA and protein density in the border zone of the infarct area, predominantly in the endothelial cells lining capillaries. In the non-infarcted myocardium ACE mRNA and protein were confined to endothelial cells of the larger vessels. From these data we conclude that the intracardiac ACE is not necessarily associated with hypertrophy in the rat heart after MI. Furthermore, the data suggest that the intracardiac ACE is involved in the healing of the scar after MI in the rat, possibly giving rise to neovascularization and/or fibrosis.

4.1 INTRODUCTION

Angiotensinogen, ACE, and angiotensin II receptors have all been detected at the mRNA and protein levels in the heart, indicating local synthesis of these components (11,23). The intracardiac RAS is thought to be involved in the development of cardiac hypertrophy since angiotensin II (ANG II) itself is a stimulus for growth. In vitro, ANG II induces not only the mRNA expression of the proto-oncogenes c-fos, c-myc and c-jun in vascular smooth muscle cells (28,42), cardiac myocytes and cardiac fibroblasts (33), but also hypertrophy and/or hyperplasia of these cells (2,4,8,33). In experimental MI in the rat, which induces cardiac hypertrophy (1), increased angiotensinogen mRNA levels were found in the non-infarcted LV (24). Increased ACE mRNA and activity (13,17) were found in all compartments of the heart after MI. Also in humans an increased ACE mRNA expression was found after MI (38). Increased cardiac ACE mRNA and enzyme-activity were also demonstrated in another model of cardiac hypertrophy, i.e. aortic stenosis (34). In accordance with a functional role for ACE in hypertrophy are the observations that even a non-hypotensive dose of an ACE-inhibitor (ACE-I) regresses cardiac hypertrophy (25).

Since the localization of ACE protein in the heart is closely related to myocardial fibrosis induced by chronic aldosterone and/or ANG II treatment (40), it has also been suggested that the intracardiac RAS plays a role in wound healing and fibrosis.

This is supported by the findings of increased ACE immunoreactivity in the infarct area in human and rat hearts (9,14).

Although there is some evidence available for the role of ACE in cardiac hypertrophy and wound healing, quantitative data combined with ACE localization are lacking. In the present study we quantified ACE mRNA expression with the highly sensitive competitive RT-PCR and localized ACE mRNA and its protein by in situ hybridization and immunohistochemistry, respectively, at different times after MI, a model for studying the development of hypertrophy and wound healing. We found an increase in ACE mRNA expression in the infarcted LV in the early phase after MI, but no increase in the hypertrophied non-infarcted myocardium. ACE mRNA and protein were predominantly localized in the border zone of the infarct area and in the endothelial cells of the larger vessels in the non-infarcted myocardium.

4.2 MATERIALS AND METHODS

Surgery

Adult male Wistar rats were used, weighing 250-300 g at the beginning of the experiments. Rats underwent induction of MI (chapter 2.2.1) or sham-surgery. At 1, 2, 4, 7, 14, or 90 days after surgery infarct and sham rats (n=3 per time point per group) were killed by ether overdose. In a second series of experiments, animals (n=3 per group) underwent the same surgery as described above. Since the highest ACE mRNA amounts were found at 4 days after MI, this time point was chosen for in situ hybridization and immunohistochemistry. In a third series of experiments, MI and sham animals (n=6 per group) were sacrificed at 7 and 90 days after surgery by ether overdose for determination of cardiac ACE activity.

Tissue processing

In the first and third series of experiments the hearts were removed and a slice of tissue parallel to the base was cut, formalin fixed overnight and paraffin embedded for determination of the infarct size (chapter 2.3.1). The rest of each heart was divided in infarcted or non-infarcted (sham) LV, SE and RV. These latter tissues were subsequently frozen in liquid nitrogen and stored at -70°C for RNA isolation and tissue ACE activity determination.

In the second series of experiments, the hearts of the MI or sham-operated animals were removed and cut in 2 parts parallel to the base and a slice of tissue was taken parallel to the base. All parts were formalin fixed overnight and paraffin embedded for determination of infarct size (chapter 2.3.1), in situ hybridization (chapter 2.6) and immunohistochemistry (chapter 2.8).

RNA isolation

Total RNA was extracted using the LiCl/urea method (35) as described in chapter 2.3.2.

Competitive RT-PCR for ACE

The competitive RT-PCR was used for quantifying ACE mRNA. The selection of the ACE primers, creation of the internal standard, the reverse transcription and the PCR is described in chapter 2.4.. The PCR-cycle used for ACE was 0.5 min. at 94°C, 0.5 min. at 65°C, 1 min. at 72°C, 30 cycles.

In situ hybridization

The rat ACE PCR fragment of 389 bp, described in chapter 2.4.1, was ligated in a TA-vector. A ³³P-UTP-labeled antisense riboprobe was created by transcription using T7 RNA polymerase (Promega). Sections of 4 µm, formalin fixed, paraffin embedded hearts were used for the in situ hybridization as described in chapter 2.6.

Immunohistochemistry of ACE

The paraffin embedded hearts from the sham group and infarct group were stained with 1:1000 dilution of the ACE antibody (goat/anti-rabbit/lung, polyclonal, gift of Dr. Soffer) as described in chapter 2.8.

ACE activity

Tissue ACE activity was determined (chapter 2.7.3) in LV, SE and RV in sham and infarcted animals at 7 and 90 days after surgery (n=6 per group).

Statistical analysis

All data are presented as mean ± SEM. Statistical significance was calculated using the ANOVA and Bonferroni's t-test. Statistical significance was accepted at the level of p<0.05 (chapter 2.9).

Figure 1A. Gel electrophoresis of the PCR products after competitive RT-PCR of ACE performed in duplicate, showing the target DNA (389 bp) and the standard DNA (291 bp). A constant amount of 500 ng total RNA was used together with increasing amounts of standard RNA (200fg-5pg).

Figure 1B. Competitive RT-PCR of ACE. The logarithmic value of the standard (del=deletion) DNA/target DNA ratio after densitometry of duplicate experiments are given on the y-axis. The logarithmic value of the starting amount of internal standard RNA is given on the x-axis. The correlation coefficient and the line of best fit are shown. By intrapolation of 0-value, the equivalence point at the y-axis, original ACE mRNA is calculated.

4.3 RESULTS

Competitive RT-PCR of ACE after MI

Electrophoresis of the PCR products demonstrated the expected two fragments of 389 (target fragment) and 291 bp of length (internal standard) (figure 1A). The mean of the duplicate logarithmic ratios of the sample were plotted to the logarithm of the amount of standard RNA and the linear correlation coefficient (r) was calculated (figure 1B). If r^2 was below 0.95 data were discarded. ACE mRNA expression could easily be detected by the competitive RT-PCR in each RNA sample of the heart.

In sham-operated rats the mean value ($\pm$ SEM) of ACE mRNA expression at the different time points after surgery was 439 ± 54 fg/100 ng total RNA in the LV, 403 ± 98 fg/100 ng total RNA in SE and 547 ± 113 fg/100 ng total RNA in the RV. In the sham group, there was no significant difference in ACE mRNA expression at the different time points after surgery. At 4 days after MI ACE mRNA expression increased 2.8-fold in the infarcted LV compared to the non-infarcted LV in the sham group at that time point. ACE mRNA expression remained increased (2.7-fold) at 7 days after MI in the LV. Thereafter, ACE mRNA expression in the infarcted LV returned to base line values (figure 2A), and was no longer significantly different from the values obtained in the sham group. In the MI animals no significant change in ACE mRNA expression was observed in the SE and RV (figure 2B and C, respectively) at the different time points.

In situ hybridization

At 4 days post-MI, the ^{33}P labeled rat ACE riboprobe showed a dense labeling in the infarct area. ACE mRNA was localized predominantly in endothelial cells lining capillaries in the border zone of the infarct area (figure 3A and B). In the non-infarcted area, ACE mRNA was found in endothelial cells of the larger vessels as well as a low expression in adventitial cells (figure 3C). Expression was also present in the epicardial and endocardial layer. In sham animals ACE was restricted to the endothelial cells of the larger vessels. Control experiments, performed by sense hybridization, revealed no specific labeling (data not shown).

Immunohistochemistry

Immunohistochemistry of ACE revealed the same pattern as observed when compared to the localization of the ACE mRNA obtained by in situ hybridization, meaning an increased ACE-immunoreactivity in the border zone of the infarct (figure 4A and B). Again ACE-immunoreactivity was predominantly found in endothelial cells. In the non-infarcted myocardium ACE immunoreactivity was observed in the pericardial and endocardial layer and in endothelial cells of large but not small vessels (figure 4C). In sham animals the same pattern was observed as in the non-

A

B

C

Figure 2. Time course of ACE mRNA amount in infarcted (MI) or sham-(SH) operated rats in left ventricle (A), septum (B) and right ventricle (C). * P values<0.05, significantly different from sham.

Figure 3. Photomicrograph (dark-field) in situ hybridization on heart tissue sections, 4 days after MI, performed with a ^{32}P -labeled antisense ACE riboprobe. **A)** Overview of ACE labeling in the border zone of the infarct area (lower arrowheads) and in endothelial cells of large vessels (upper arrowhead). Bar represents 72 μm . **B)** ACE labeling of endothelial cells (arrowhead) of a large vessel in the myocardium. Bar represents 18 μm . **C)** ACE labeling of the non-infarcted myocardium (E = epicardium). Bar represents 72 μm .

Figure 4. Photomicrograph of immunohistochemistry of ACE on heart tissue sections, 4 days after MI, performed with a polyclonal goat anti rat ACE (1:1000), counterstained with hematoxylin. **A)** Overview of ACE immunoreactivity in the border zone of the infarct area (I=infarct area, Ep=epicardium). Bar represents 72 μ m **B)** ACE immunoreactivity in endothelial cells (arrowheads) of large and small vessels in the border zone of the infarct area myocardium (I=infarct area). Bar represents 18 μ m **C)** ACE immunoreactivity (arrowheads) in the endothelial cells of larger vessels and endocardium (En) in the non-infarcted myocardium. Bar represents 72 μ m.

Table 1: *Tissue ACE activity in MI or sham animals*

	7 days		90 days	
	SH	MI	SH	MI
LV	49.6 ± 5.8	131 ± 20.3*	37.7 ± 3.6	134 ± 25.9 [#]
SE	39.5 ± 4.2	51.7 ± 5.7	30 ± 2.4	85.1 ± 24.1
RV	52.3 ± 6.1	64.3 ± 7.1	42.1 ± 4.7	49.3 ± 4.5

ACE activity in U/g/min in the infarcted left ventricle (LV), non-infarcted septum (SE) and right ventricle (RV) at 7 and 90 days after MI or sham-operation. * $P < 0.05$, [#] $P < 0.01$, significantly different from sham.

infarcted myocardium, i.e. in the endocardial layer and in endothelial cells of the larger vessels.

ACE activity

The mean infarct size in the experimental MI groups was $40.4 \pm 1.2\%$ and $38.4 \pm 1.0\%$ at 7 and 90 days after MI, respectively. This infarct size was representative for all MI animals in this study. Table 1 shows the ACE activity at 7 and 90 days after MI or sham-operation in the different compartments of the rat. ACE activity was significantly increased in the infarcted LV at 7 days (2.6 fold) and 90 days (3.6 fold) after MI when compared to LV of the sham group. No significant differences in ACE activity were observed in the non-infarcted SE and RV at the two time points after MI, compared to the sham-group.

4.4 DISCUSSION

Although there is evidence that the intracardiac RAS may play a role in pathological processes like cardiac hypertrophy and wound healing, quantitative analysis of the components of the RAS combined with the localization of these components in the heart in pathological circumstances is lacking. In this study we quantified ACE mRNA in the different compartments of the rat heart after MI. Ligation of the LAD results in a mean infarct size of 40% and a 30% increase in mass of RV, SE and viable LV (1); thus the model seems appropriate for studying cardiac hypertrophy and wound healing. The quantification of ACE mRNA and ACE activity was combined with the localization of ACE mRNA and ACE protein.

ACE mRNA levels increased 3-fold in the infarcted LV at 4 and 7 days after MI. ACE activity also increased approximately 3-fold in the infarcted LV at 7 and 90 days after MI. This increase in left ventricular ACE activity after MI is in accordance with the increase of ACE mRNA amounts. Furthermore the increased ACE activity in the

infarcted LV at 90 days after MI, suggests a low turnover of the ACE enzyme. We can not exclude a possible contribution of plasma ACE activity, although this is not likely since plasma ACE levels are not elevated in these stages of MI (32). In situ hybridization and immunohistochemistry localized ACE mRNA and protein in the border zone of the infarcted LV. In a qualitative study in rats, using a similar infarct model as in our study, Hirsch *et al.* (17) found a 2-fold increase of right ventricular ACE mRNA expression and enzyme-activity and a 2-fold increase of interventricular septal ACE-activity, 85 days after infarction, with normal plasma ACE and renin levels. Pinto *et al.* (32) observed an increase of ACE activity in the apex at 3, 5 and 80 days after MI and in the RV at 3 days after MI. The infarcted LV was, however, not included in their analysis. In another study (13) in the same model an increase of cardiac ACE, estimated by radioligand binding was observed in all the compartments of the rat heart, with the highest increase (4-fold) in the infarct region. Sun *et al.* (39) found an increase of ACE binding in the infarcted LV from 1 week after MI. In the free left ventricular wall of failing human hearts, Studer *et al.* (38) demonstrated a 3-fold increase of ACE mRNA by competitive RT-PCR, while there was no increase of gene expression of chymase, an alternative ANG II forming enzyme. These latter studies are in agreement with our study, indicating an activated intracardiac RAS after MI. The timing and site of activation of ACE demonstrated by Hirsch *et al.* (17) was different from our study. In our study, however, ACE mRNA amounts were measured by the quantitative competitive RT-PCR from 1-90 days, while in the study from Hirsch *et al.* (17) ACE mRNA expression was estimated with a qualitative PCR at one time after MI.

Also other components of the intracardiac RAS are increased after MI, including a transient increase in angiotensinogen (AO) mRNA (24) in the non-infarcted LV of the rat, and an increase of angiotensin receptor density at sites of MI and pericardial fibrosis as early as three days after MI (39).

In chronic experimental aortic stenosis, cardiac hypertrophy was associated with a 4-fold increase of ACE mRNA expression and a 30% increase of ACE-activity in the hypertrophied LV. The intracardiac conversion rate from angiotensin I (ANG I) to ANG II was doubled in isolated hypertrophied hearts, while treatment with the ACE-inhibitor enalaprilat significantly decreased the conversion from ANG I to ANG II (34). Experimental and clinical studies have demonstrated that ACE-I treatment in heart failure results in a decrease of hypertrophy, LV dilatation and mortality (29,30), even with a non-hypotensive dose of an ACE-I (25). Furthermore ACE inhibition reduces interstitial DNA synthesis in the early phase after MI (19). In chapter 3 we described decreased levels of atrial AO mRNA, 7 days after aorta coarctation. In the same model of pressure overload in rats, increased AO mRNA expression (3), ANG II concentrations and increased AT1a mRNA levels and angiotensin receptor density (41) were detected in the myocardium. These observations confirm the importance of the intracardiac RAS in pathological circumstances, like pressure overload cardiac hypertrophy and MI.

Although the above mentioned studies suggest that the RAS is involved in the

development of hypertrophy, the lack of increase in ACE mRNA and activity in the non-infarcted hypertrophied SE and RV after MI in the present study indicates that cardiac hypertrophy is not necessarily associated with activation of intracardiac ACE in the rat heart after MI. The activation of ACE both at the mRNA, protein and activity level in the infarcted LV suggest, however, a role for the intracardiac RAS in the healing of the infarct.

A role for the RAS in wound healing has been suggested before. In the rat skin tissue ANG II levels increased as early as 6 hours with a peak (10-fold increase) at 24 hours after injury (31), which was associated with a downregulation of the AT1 receptor (18). Increased amounts of ACE protein were also found in the inflammation zone of scars of human (9) and rat (14,39) infarcted hearts, 3 and 7 days after MI, the same time period of increase as in the present study. Increased ACE-immunoreactivity following MI in the rat was predominantly localized in endothelial cells of capillaries in the border zone of the infarct, whereas in the non-infarcted myocardium ACE-staining was observed in arterioles and arteries (14). Although we localized the ACE mRNA and ACE protein predominantly in the endothelial cells, we can not exclude the presence of ACE mRNA and protein in other cells like macrophages or (myo)fibroblasts. It is possible that (myo)fibroblasts produce increased amounts of ACE, because they express the components of the RAS (10,43). Furthermore myofibroblasts appear within 4 days after infarction (44). ANG II has been shown to induce the expression of collagen, TGF β and PDGF (6,27,37), which have been implicated to play a role in wound healing and which are produced by these myofibroblasts (12,16).

Another important protein in wound healing is collagen and ACE may be involved in collagen deposition in the infarct zone. Increased collagen I and III mRNA expression was demonstrated after MI (7). This increase runs in parallel with the increase in ACE mRNA expression, 4 and 7 days after MI in this study. Furthermore collagen deposition could be suppressed by the ACE inhibitors captopril (19) and perindopril (26) and by the AT1-antagonist losartan (36). In cultured adult rat cardiac fibroblasts ANG II directly stimulated collagen synthesis and inhibited collagenase activity (5), resulting in accumulation of collagen. The presence of ACE in tissue, largely composed of collagen, such as in the heart valves, the adventitia of larger vessels and intramyocardial arteries supports this role (45). Sun *et al.* (40) found a high-affinity ACE binding in microscopic scars and perivascular fibrosis in the myocardium and atria after chronic treatment (4 and 6 weeks) with ANG II or aldosterone.

The localization of ACE in the capillary endothelium in the border zone of the infarct area, suggests a role in neovascularization. Kuizinga *et al.* demonstrated that 30% of the proliferating cells following MI are endothelial cells, suggesting vascular outgrowth after MI (20). ANG II has been shown to induce formation of new blood vessels in the rabbit cornea (15). Furthermore a dose-dependent induction of angiogenesis was found in the chick embryo chorioallantoic membrane after treatment with ANG II (21). This response was resistant to the AT1-antagonist

angiogenesis was found in the chick embryo chorioallantoic membrane after treatment with ANG II (21). This response was resistant to the AT1-antagonist losartan and the AT2-antagonist PD123319, but could be blocked by the AT2-antagonist CGP42112A (22).

In conclusion, our data suggest that the intracardiac ACE does not play a dominant role in the development of cardiac hypertrophy in the experimental MI model in the rat, since there was no activation of ACE in the hypertrophied SE and RV. On the other hand the activated ACE in the border zone of the infarct suggests the involvement of intracardiac ACE in the wound healing of the infarct area. The intracardiac RAS could thereby play an important role in the development of fibrosis and neovascularization after MI.

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CHAPTER 5

ACTIVATION OF THE RENIN GENE IN THE INFARCT ZONE
FOLLOWING MYOCARDIAL INFARCTION IN THE RAT

ABSTRACT

Data on the activation of renin, the rate-limiting enzyme of the renin-angiotensin-system (RAS) cascade, after myocardial infarction (MI), are lacking. In the present study we therefore quantified and localized renin mRNA in the infarcted rat heart. WKY rats underwent ligation of the left descending coronary artery, resulting in MI, or a sham-operation. At different time points after MI, ranging from 1 to 90 days ($n=6$ per group), hearts were removed and divided into infarcted left ventricle (LV), non-infarcted septum (SE) and right ventricle (RV). The tissues were used for total RNA isolation or formalin fixed for in situ hybridization. Renin mRNA content was quantified in the different compartments by the competitive reverse transcriptase polymerase chain reaction (RT-PCR). Furthermore, atrial natriuretic factor (ANF) mRNA expression, a marker for cardiac hypertrophy, was determined by Northern blot hybridization analysis in the different compartments of the heart, at 1, 7 and 90 days following surgery. We found a 3.8, 17 and 8.4-fold increase ($p<0.05$) in renin mRNA in the infarcted LV at 2, 4 and 7 days after MI, respectively. At 4 days after MI a slight increase of renin mRNA expression was observed in the SE. At other time points after MI or sham-surgery, renin mRNA levels were near the limit of detection. The results of the in situ hybridization studies were in accordance with these findings, showing increased renin mRNA expression around the infarct area, at 4 days after MI. Renin mRNA was not localized in cardiomyocytes and, in contrast to our findings with angiotensin I-converting enzyme (ACE) (chapter 4), renin mRNA was not predominantly localized in endothelial cells. Renin mRNA expression in the non-infarcted myocardium was very low and not different from shams. ANF mRNA increased approximately 2-fold in all compartments of the heart at 1, 7 and 90 days after MI. The low amounts of renin mRNA in the non-infarcted hypertrophied myocardium, indicate that the intracardiac synthesis of renin does not play a dominant role in the development of cardiac hypertrophy in the rat heart after MI. In addition, the increased renin mRNA expression in the border zone of the infarcted LV early after infarction suggests a role for intracardiac synthesized renin in infarct healing.

5.1 INTRODUCTION

Although the intracardiac synthesis of most components of the RAS is well established now, the existence of an increased intracardiac synthesis of renin is still not clear. In the isolated perfused rat Langendorff heart, both angiotensin I and angiotensin II production were dependent on extracardiac renin (11). Moreover, in a nephrectomy study Danser *et al.* (5) concluded that cardiac renin originates from the kidney. While several investigators could detect renin mRNA expression in normal hearts (6,14), others did not find cardiac renin mRNA expression (9).

In addition to the ongoing discussion on the possible intracardiac synthesis of renin, little is known about the activation of the renin gene in cardiac hypertrophy.

Boer *et al.* (4) recently described an up to 40-fold increase of cardiac renin mRNA expression, 7 days after induction of volume-overload cardiac hypertrophy. Also in spontaneously hypertensive rats (SHR) increased cardiac renin and angiotensinogen mRNA expression were demonstrated.

Similarly, activation of other components of the RAS in cardiac hypertrophy has been demonstrated. In the previous chapter (chapter 4) activation of the ACE gene and protein were described after MI in the border zone of the infarct area. Others also demonstrated increased ACE and angiotensinogen mRNA in the myocardium following MI (8,12). In addition, cardiac ACE and angiotensinogen mRNA and ACE activity were increased in pressure-overload induced cardiac hypertrophy (3,17).

No data are available on the regulation and localization of renin mRNA after MI, a model which results in cardiac hypertrophy. In the present study no increase of renin mRNA was found in the non-infarcted hypertrophied segments, which exhibited elevated levels of ANF mRNA, a marker of cardiac hypertrophy. Renin mRNA amounts were, however, elevated in the border zone of the infarct area, suggesting a role for intracardiac synthesized renin in wound healing after MI.

5.2 MATERIALS AND METHODS

Surgery

Adult male WKY rats were used, weighing 250-300 g at the beginning of the experiments. Rats underwent induction of MI (chapter 2.2.1) or sham-operation. At 1, 2, 4, 7, 14, 21 or 90 days after surgery infarct and sham rats (n=6 per time point per group) were killed by ether. In a second series of experiments, animals (n=3 per group) underwent the same surgery as described above. Since the highest renin mRNA amounts were found at 4 days after MI, this time point was chosen for in situ hybridization.

Tissue processing

The heart was removed and a slice of tissue parallel to the base was cut and formalin fixed overnight and paraffin embedded for determination of the infarct size (chapter 2.3.1). The rest of each heart was divided in infarcted or non-infarcted (sham) LV, SE and RV. The tissues were subsequently frozen in liquid nitrogen and stored at -70°C.

MI or sham-operated animals (n=3 per group) of the second series of experiments were sacrificed by ether overdose. The hearts were removed and cut in 2 parts parallel to the base. Both parts were formalin fixed overnight and paraffin embedded for determination of infarct size and in situ hybridization (chapter 2.6).

RNA isolation

Total RNA was extracted using the LiCl/urea method (18) as described in chapter 2.3.2.

Competitive RT-PCR for renin

The competitive RT-PCR was used for quantification of renin mRNA. The selection of the renin primers, creation of the internal standard, the reverse transcription and the PCR is described in chapter 2.4. The PCR-cycle used for renin was 0.5 min at 94°C, 0.5 min at 65°C, 1 min at 72°C, 40 cycles.

In situ hybridization

The rat renin PCR fragment of 372 bp, described in chapter 2.4.1, was ligated in the TA-vector. A ³⁵S-UTP-labeled antisense riboprobe was created by transcription using T7 RNA polymerase (Promega). Four µm sections, formalin fixed, paraffin embedded hearts were used for the in situ hybridization as described in chapter 2.6.

Northern hybridization analysis

ANF mRNA expression was used as an indicator for cardiac hypertrophy in the different compartments of the hearts in infarct and sham rats, 1, 7 and 90 days after surgery. A total of 15 µg total RNA was used for the Northern blotting hybridization (chapter 2.4.3). Specific primers for rat ANF were chosen. The upper primer was 5' ACCAGAGAGTGAGCCGAGAC 3', the lower primer 5' AGTGGGAGAGGTAAGG-CCTC 3'. Amplification resulted in a PCR fragment of 697 bp in length. Dideoxy sequencing showed a 100% homology with the rat ANF cDNA sequence (data not shown). This ANF PCR-fragment was used as a probe. The other probe used was the cDNA insert to 28S rRNA. Labeling and hybridization of the probes are described in chapter 2.5.

Statistical analysis

All data are presented as mean ± SEM. Statistical differences were calculated using ANOVA and Bonferroni's t-test. Statistical significance was accepted at the level of p<0.05 (chapter 2.9).

5.3 RESULTS

Infarct size and ANF mRNA expression

The mean infarct size in the experimental MI group at different time points was 44 ±

Figure 1. ANF mRNA expression at 1, 7 and 90 days after myocardial infarction (MI) or sham (SH)-surgery in left ventricle (LVSH and LVMI), septum (SESH and SEMI), and right ventricle (RVSH and RVMI). The ANF/28S ratios of the MI animals were related to the sham ratios. * $P < 0.05$, significantly different from sham.

3%. There were no differences in infarct sizes between the different groups. ANF mRNA expression was used as a marker for hypertrophy of the non-infarcted myocardium. ANF mRNA expression was significantly higher in the different compartments at all time points after MI. At 1 day after MI ANF mRNA was 2.4, 1.6 and 1.5 fold higher, at 7 days after MI 2.4, 1.8 and 1.9 fold higher and at 90 days after MI ANF mRNA was 2.9, 3 and 2.6 fold higher in LV, SE and RV, respectively, when compared to the ANF mRNA expression in the different compartments of the sham groups (figure 1). This suggests that cardiac hypertrophy occurred in the different compartments after MI.

Competitive RT-PCR

To first evaluate the levels of renin mRNA expression, a qualitative RT-PCR, i.e. RT-PCR without an internal standard, was performed in duplicate on the RNA samples in the different compartments of the hearts at all time points after surgery. Renin mRNA expression was very low in all compartments of the sham-hearts at the different time points after surgery (1-90 days). In the MI group they were high in the

Figure 2. A) Gel electrophoresis of the PCR products after competitive RT-PCR of renin in the infarcted (MI) or sham (SH) left ventricle (LV), showing the target DNA (372 bp) and internal standard DNA (263 bp). A constant amount of 500 ng total RNA was used together with increasing amounts amounts of internal standard RNA (10 to 400 fg). B) Renin mRNA amounts in MI or SH rats in LV, from 1 to 90 days after surgery. * $P < 0.05$, significantly different from sham.

infarcted left ventricles, at 2, 4 and 7 days after MI and in the SE at 4 days after MI. Therefore quantification of renin mRNA amounts by competitive RT-PCR was restricted to these time points.

In the sham group renin mRNA levels were above the level of accurate quantification, in only 2 out of 6 left ventricles at 4 and 7 days after sham-surgery, whereas at 2 days after sham-surgery 4 samples were above the level of detection. Reducing the amounts of internal standard did not solve this problem. At 2 days after sham-surgery the mean renin mRNA levels in LV were 7.0 ± 1.5 fg/100 ng total RNA. At 4 and 7 days after sham-operation mean renin mRNA levels in LV were 2.6 and 2.7 fg/100 ng total RNA, respectively. Renin mRNA levels after MI were increased in the infarcted LV. At 2 days after MI renin mRNA levels were 26.6 ± 7.6 fg/100 ng total RNA in the infarcted LV, which indicates a more than 4-fold increase in renin mRNA levels in the infarcted LV when compared to the sham group (figure 2A and B). The renin mRNA levels in the 4 day infarcted LV were 35.6 ± 7.6 fg/100 ng total RNA, indicating a more than 17-fold increase as compared to sham. At 7 days after MI renin mRNA levels in the infarcted LV were 21.8 ± 5.3 fg/100 ng total RNA, a more than 8-fold increase when compared to sham animals.

In the SE of the sham animals renin mRNA levels were below the level of accurate quantification, whereas in the SE of the infarcted group at day 4 in 4 out of 6 animals renin mRNA levels could be quantified. Mean renin mRNA levels were 13.6 ± 4.4 fg/100 ng total RNA. At other time points after MI in the SE and RV no increases of renin mRNA were found.

In situ hybridization

Since the highest renin mRNA levels were found at 4 days after MI, this time point was used for the localization of the renin mRNA in the infarcted heart and compared to sham hearts. The results of the *in situ* hybridization with the ^{35}S labeled renin probe were in accordance with the data obtained by the competitive RT-PCR. High renin mRNA expression was found around the infarct area in the LV at 4 days after MI (figure 3). Renin mRNA was most probably localized in (myo)fibroblasts and/or endothelial cells around the infarct area. Furthermore, we found that renin mRNA was not localized in cardiomyocytes of the infarcted and non-infarcted myocardium. In the non-infarcted myocardium renin mRNA expression was very low and not distinguishable from the background labeling. Also in the sham-operated hearts at day 4 renin mRNA expression was very low and not distinguishable from the background labeling.

5.4 DISCUSSION

In the present study we observed increased renin mRNA amounts in the infarcted LV, 2, 4 and 7 days after MI. Renin mRNA levels were especially increased around the necrotic area in the infarcted LV. Cardiomyocytes were negative for renin mRNA

Figure 3. Photomicrograph (dark-field) of in situ hybridization on heart tissue sections, 4 days after MI, performed with a ^{35}S -labeled antisense renin riboprobe. **A)** Overview of renin labeling in the border zone of the infarct area (arrowhead). I = infarct area; bar represents 72 μm . **B)** Renin labeling in the border zone of infarct area (arrowhead). E = epicardium; bar represents 18 μm .

expression. In contrast to a slight increase of renin mRNA in the SE at 4 days after MI, low to undetectable levels of renin mRNA amounts were found in the non-infarcted hypertrophied myocardium of infarct animals and in the ventricles of all sham hearts.

Experimental MI is a model that includes cardiac hypertrophy as well as infarct healing. The mean infarct size of the infarction group was 44%. Although we did not measure hypertrophy in our animals as heart weights, MI of this size have been reported to result in a 20-40% mass increase of the non-infarcted myocardium (1,2). The hypertrophic response of the myocardium after MI was corroborated in our studies by a significant increase of ANF mRNA expression in all compartments of the heart at 1, 7 and 90 days after MI, when compared to sham hearts. ANF is induced in the early phase of cardiac hypertrophy and is used as a marker for cardiac hypertrophy (16).

The presence of renin mRNA has been detected in all compartments of the heart (6,7), with highest mRNA levels in the atria. Elevated ventricular renin mRNA levels were found in neonatal rat hearts, but not in adults (6). However expression was very low, demonstrating the need for sensitive techniques, like RT-PCR. Cellular localization of renin mRNA was described by Dostal *et al.* (6). They found renin mRNA expression and protein synthesis in isolated neonatal rat heart myocytes and fibroblasts. However the localization of renin in pathophysiological circumstances is not known.

In other models of cardiac hypertrophy, activation of the components of the intracardiac RAS has been observed. Recently, Boer *et al.* (4) demonstrated an increase of renin mRNA in a volume-overload induced cardiac hypertrophy model in rats (4). At 1 day after surgery no increase of renin mRNA was observed. However, 7 days after aortacaval shunting a more than 40-fold increase of renin mRNA levels were observed by competitive RT-PCR. Angiotensinogen mRNA was not increased in that model. In spontaneously hypertensive rats (SHR), which develop marked cardiac hypertrophy, increased cardiac mRNA expression of both renin and angiotensinogen genes were demonstrated. In addition, increases of angiotensinogen and ACE mRNA levels, ACE activity, AT1a mRNA and angiotensin receptor density have been observed in pressure-overload induced hypertrophy (3,13,17,19).

To the best of our knowledge this is the first study demonstrating activation of the intracardiac renin gene following MI. Our data suggest that the intracardiac synthesis is not necessary for the development of cardiac hypertrophy after MI in the rat, since renin was not activated in the hypertrophied non-infarcted myocardium. Activation of renin around the necrotic area in the early phase after MI, suggests, however, a role for intracardiac renin in the infarct healing process, which includes proliferation of endothelial cells and (myo)fibroblasts (10,20).

In addition to the increased renin mRNA expression in the infarcted LV, we described in the previous chapter (chapter 4) the localized activation of the ACE gene and protein in the border zone of the infarct area in the early phase after MI,

whereas in the SE and RV no changes in ACE gene and protein activity were observed. In chapter 4 we suggested a role for the intracardiac ACE in wound healing after MI by possibly affecting processes like cardiac fibrosis and angiogenesis, but a less important role in the development of cardiac hypertrophy after MI. The activation of the renin gene in the border zone of the infarct area in the early phase after MI is in agreement with the ACE data. In contrast to ACE, renin mRNA could not be localized in the endothelial cells of smaller and larger coronary vessels. However, in a paracrine/autocrine way the intracardiac renin may play a role in angiogenesis and fibrosis after MI in the rat.

In summary, this study demonstrates activation of the renin gene around the necrotic area in the early phase after MI, while no increase in renin mRNA levels was observed in the hypertrophied non-infarcted myocardium. From these data we conclude that intracardiac synthesis of renin does not play a dominant role in the development of cardiac hypertrophy following MI in the rat. The presence of renin mRNA around the necrotic area suggests a role for intracardiac renin synthesis in wound healing. The increased ACE mRNA expression (chapter 4) in the infarcted LV (15) in the same period in which renin activation occurs after MI, indicates activation of the intracardiac RAS in the infarcted LV in the early phase after MI.

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CHAPTER 6

**ANGIOTENSINOGEN MESSENGER RNA EXPRESSION IN THE RAT
HEART AFTER EXPERIMENTAL MYOCARDIAL INFARCTION**

ABSTRACT

The activation of angiotensin I-converting enzyme (ACE) and renin after myocardial infarction (MI), has been described in chapter 4 and 5. In the present study we quantified and localized angiotensinogen (AO) mRNA in the infarcted rat heart. At different time points after MI, ranging from 1 to 90 days (n=6 per group), hearts were removed and divided in infarcted left ventricle (LV), non-infarcted septum (SE) and right ventricle (RV). The tissues were used for total RNA isolation or formalin fixed and paraffin embedded for in situ hybridization. AO mRNA contents were quantified in the different compartments by the competitive reverse transcriptase polymerase chain reaction (RT-PCR). Furthermore, ANF mRNA expression, was determined by Northern blot hybridization analysis in the different compartments of the heart, at 1, 7 and 90 days following MI (as described in chapter 5). Cardiac AO mRNA levels were extremely low. Mean cardiac AO mRNA levels were 435 ag/100 ng total RNA. No differences were observed between AO mRNA levels in sham and infarcted hearts in the different compartments at any time after surgery. ANF mRNA increased approximately 2-fold in all compartments of the heart at 1, 7 and 90 days after MI. In situ hybridization revealed an overall low density of AO mRNA in the myocardium, with somewhat higher mRNA expression in the epicardium. These data demonstrate that intracardiac AO is not activated after experimental MI, suggesting that intracardiac synthesis of AO does not play a dominant role in the development of cardiac hypertrophy or infarct healing in the rat heart after MI.

6.1 INTRODUCTION

Little is known about the activation of the AO gene in cardiac hypertrophy. Lindpaintner *et al.* (5) reported a transient activation of AO mRNA in the non-infarcted LV of the rat, with peak levels at 5 days after MI. AO mRNA levels had returned to sham levels 25 days after MI (5). In spontaneously hypertensive rats (SHR) increased cardiac AO mRNA expression was demonstrated (3). In contrast to the activation of the AO gene in MI- and hypertension-induced hypertrophy, Boer *et al.* (2) recently described that AO mRNA levels were not changed at 1 and 7 days after induction of volume-overload induced cardiac hypertrophy.

No quantitative data and localization of AO mRNA after MI are available. In the present study AO mRNA levels were measured at different time points after surgery by competitive RT-PCR. At all time points measured, no differences of cardiac AO mRNA amounts were observed between infarcted and sham animals. Using in situ hybridization, AO mRNA was found to be localized in the epicardium of sham and infarcted rats, whereas AO mRNA was not detected in the rest of the hypertrophied myocardium of infarcted and sham rats. These data suggest that activation of the intracardiac AO gene does not play a dominant role in the development of cardiac hypertrophy and wound healing after MI in rats.

6.2 MATERIALS AND METHODS

Surgery

Adult male WKY rats were used, weighing 250-300 g at the beginning of the experiments. Rats underwent induction of MI (chapter 2.2.1) or sham-operation. At 1, 4, 7, 21, or 90 days after surgery infarct and sham rats (n=6 per time point per group) were killed by ether overdose. In a second series of experiments, animals (n=3) underwent the same surgery as described above. Since highest AO mRNA levels were found at 4 days after surgery, this time point was also chosen for in situ hybridization of AO.

Tissue processing

The heart was removed and a slice of tissue parallel to the base was cut and formalin fixed overnight and paraffin embedded for determination of the infarct size (chapter 2.3.1). The rest of each heart was divided in infarcted or non-infarcted (sham) LV, SE and RV. The tissues were subsequently frozen in liquid nitrogen and stored at -70°C. In the second series of experiments, MI or sham-operated animals (n=3 per group) in these series were sacrificed with ether. The hearts were removed and cut in 2 parts parallel to the base. Both parts were formalin fixed overnight for determination of infarct size and in situ hybridization.

RNA isolation

Total RNA was extracted using the LiCl/urea method (9) as described in chapter 2.3.2.

Competitive RT-PCR for angiotensinogen

The competitive RT-PCR was used for quantification of AO mRNA. The selection of the AO primers, creation of the internal standard, the reverse transcription and the PCR is described in chapter 2.4. The competitive RT-PCR for AO consisted of a double PCR. The PCR was performed at 0.5 min. at 94°C, 0.5 min. at 62°C, 1 min. at 72°C, 20 cycles.

In situ hybridization

The rat AO PCR fragment of 482 bp, described in chapter 2.4.1, was ligated in the TA-vector. A ³⁵S-UTP-labeled antisense riboprobe was created by transcription using T7 RNA polymerase (Promega). Four µm sections, formalin fixed, paraffin embedded hearts were used for the in situ hybridization as described in chapter 2.6.

Northern hybridization analysis

ANF mRNA expression was used as an indicator for cardiac hypertrophy in the different compartments of the hearts in infarct and sham rats, 1, 7 and 90 days after surgery. This is described in chapter 5.

Statistical analysis

All data are presented as mean $\pm$ SEM. Statistical differences were calculated using the ANOVA and Bonferroni's t-test. Significance was accepted at the level of $p < 0.05$ (chapter 2.9).

6.3 RESULTS

Infarct size and ANF mRNA expression

The animals in this chapter were the same as in chapter 5. Therefore, data concerning the ANF mRNA expression in the different compartments of the heart following MI are described in chapter 5.3. In summary, ANF mRNA levels were increased in all compartments of the heart at the different time points after MI. The mean infarct size in the experimental MI group at different time points was $44 \pm 3\%$.

Competitive RT-PCR

Cardiac AO levels were extremely low. For this reason we used a double set of primers, with the second set as nested primers. After this second PCR, AO levels could be quantified. There were no differences in AO mRNA levels between the compartments of the infarcted animals and the sham animals at the different time points after operation. Mean AO mRNA levels in LV were 870 ± 250 ag/100 ng total RNA (figure 1), in SE 421 ± 167 ag/100 ng total RNA and in RV 750 ± 246 ag/100 ng total RNA. AO mRNA levels were elevated in the LV at 4 days after operation in sham animals when compared to the other time points after surgery in sham animals, indicating an activation of the AO gene by the operation itself.

In situ hybridization

Since the highest AO mRNA levels were found at 4 days after surgery, this time point was also used for the localization of the AO mRNA in the infarcted heart and compared to sham hearts. The AO mRNA density in the sham and infarcted myocardium was very low. Only in the epicardium of sham and infarcted animals specific AO labeling was detected (figure 2).

Figure 1. A) Gel electrophoresis after competitive RT-PCR of angiotensinogen in the infarcted (MI) or sham (SH) left ventricle (LV), showing the target DNA (482 bp) and internal standard DNA (372 bp). A constant amount of 500 ng total RNA was used together with increasing amounts of internal standard RNA (1 to 50 fg). **B)** Angiotensinogen (AO) mRNA amount in MI or SH rats in LV at 1, 4, 7, 21 and 90 days after MI. * $P<0.05$, significantly different from sham at day 7, 21 and 90; # $P<0.01$, significantly different from sham at day 1.

Figure 2. Photomicrograph (dark-field) of in situ hybridization on heart tissue sections, 4 days after MI, performed with a ^{35}S -labeled antisense angiotensinogen riboprobe. Arrowhead indicates site of angiotensinogen labelling. E = epicardium; bar represents 72 μm .

6.4 DISCUSSION

In the present study we found very low levels of AO mRNA in the myocardium in sham and infarcted rats. We did not observe changes in AO mRNA expression in the different compartments of the heart at the different time points after MI. Apparently increased levels of AO mRNA were observed in the infarcted left ventricles at 4 days after surgery. However, these levels were not different between the infarcted and sham rats. In situ hybridization showed that this AO mRNA was predominantly localized in the epicardium.

Experimental MI is a model that includes cardiac hypertrophy as well as infarct healing. The mean infarct size of the infarction group was 44%. The hypertrophic response of the myocardium after MI was corroborated in our studies by a significant increase of ANF mRNA expression in all compartments of the heart at 1, 7 and 90 days after MI, when compared to sham hearts, as described in chapter 5. ANF is induced in the early phase of cardiac hypertrophy and may be used as a marker for cardiac hypertrophy (7).

Lindpaintner *et al.* (5) used a RNase protection assay, and demonstrated a marginal activation (1.6-fold) of the AO gene in the non-infarcted LV at 5 days after MI. At 25 days after MI expression was no longer different from that in sham animals. Also in other models of cardiac hypertrophy, like SHR and aorta coarctation

in rats, activation of the AO gene has been demonstrated (1,11). However, in a volume-overload model no changes were observed in cardiac AO mRNA levels at 1 and 7 days after surgery (2). Our data suggest that the intracardiac synthesis of the RAS is not necessary for the development of cardiac hypertrophy following MI in the rat, since AO was not activated in the hypertrophied non-infarcted myocardium. Infarction-induced hypertrophy is a combination of pressure and volume overload hypertrophy. The difference of activation of the cardiac AO gene may be explained by the difference of the experimental models used.

In addition, ACE and renin mRNA were increased in the border zone of the infarct area following MI, but not in the hypertrophied compartments of the non-infarcted myocardium (chapter 4 and 5, respectively). We suggested a role for intracardiac ACE and renin in infarct healing in the early phase after MI. In this period after MI we observed high levels of AO mRNA in both infarcted and sham animals. The high AO mRNA levels were localized in the epicardium, suggesting an effect of the surgery in both groups of animals.

In this study cardiac AO mRNA levels were very low. However, AO may be not rate-limiting and protein levels may be sufficient to produce increased amounts of intracardiac angiotensin II (ANG II) in different pathological conditions. The ability to synthesize angiotensin I and ANG II in the isolated rat heart, without exogenous AO, but in the presence of exogenous renin, strengthens that suggestion (4).

Others have also found activation of the components of the intracardiac RAS in different models of cardiac hypertrophy. Recently, Boer *et al* (2) found a more than 40-fold increase of renin mRNA, 7 days following a volume-overload model in rats. At 1 day after surgery no increase of renin mRNA was observed. In SHR increased cardiac renin mRNA expression was demonstrated (3). In addition, increases of ACE mRNA levels, ACE activity, AT1a mRNA and angiotensin receptor density have been observed in pressure-overload induced hypertrophy (1,6,8,10).

In summary, this study demonstrates unchanged levels of AO mRNA in the non-infarcted hypertrophied rat heart, as also observed for ACE and renin mRNA in chapter 4 and 5, respectively. From these data we conclude that the synthesis of intracardiac AO does not play a dominant role in the development of cardiac hypertrophy and in the role of wound healing following MI in the rat. Furthermore these data provide further evidence that the intracardiac RAS does not play a dominant role in the development of cardiac hypertrophy after MI in the rat.

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CHAPTER 7

**INTRACARDIAC AND SYSTEMIC FEEDBACK REGULATION OF THE
RENIN-ANGIOTENSIN-SYSTEM DURING EARLY CAPTOPRIL
TREATMENT FOLLOWING MYOCARDIAL INFARCTION IN THE RAT**

ABSTRACT

In the present study we investigated the regulation of the expression of components of the intracardiac and systemic renin-angiotensin-system (RAS) by interference with the RAS in rats following myocardial infarction (MI). Wistar rats underwent ligation of the left descending coronary artery, resulting in MI, or a sham-operation. In a second series of experiments animals were treated with the angiotensin-converting-enzyme-inhibitor (ACE-I), captopril (0.5 mg/kg/hr) from 0-7 days after sham or MI-surgery. In all experimental groups rats were sacrificed at 7 days after surgery (n=6 per group) and the heart was removed and divided into right ventricle (RV), septum (SE) and left ventricle (LV). Furthermore lungs, liver and kidneys were removed. Tissues were quickly frozen in liquid nitrogen. Cardiac angiotensinogen (AO), renin, angiotensin I-converting-enzyme (ACE) and renal renin mRNA levels were quantified by the competitive reverse transcriptase polymerase chain reaction (RT-PCR). Hepatic AO, pulmonary ACE and cardiac atrial natriuretic factor (ANF) mRNA expression were evaluated by the Northern hybridization analysis. At 7 days after MI we found an 8-fold increase of renin mRNA and 3-fold increase of ACE mRNA (n=6) in the infarcted LV, whereas AO mRNA levels were not changed after MI. No increases of AO, renin and ACE mRNA levels were found in the non-infarcted compartments, which were hypertrophying as judged from increased ANF mRNA expression. Hepatic AO mRNA levels were not changed following MI. However, renal renin and pulmonary ACE mRNA levels were increased respectively 3 and 1.7-fold following MI. Seven days of captopril treatment had no effect on the intracardiac expression of the RAS components neither in sham nor in infarcted rats. Captopril treatment in sham animals significantly increased renal renin mRNA levels, whereas it had no effect on hepatic AO mRNA and pulmonary ACE mRNA levels. A trend towards an increase in renal renin and hepatic AO mRNA levels was observed in captopril treated infarct animals. The increase of pulmonary ACE mRNA in infarcted animals was suppressed by captopril treatment. These data indicate an activation of the intracardiac and systemic renin and ACE genes, but not of AO following MI. Furthermore, these data suggest differential feedback regulation of the local and systemic RAS in infarcted and sham rats.

7.1 INTRODUCTION

Although activation of the intracardiac RAS in experimental heart failure has been established, little is known about its regulation in this condition. Wollert *et al.* (28) demonstrated increased cardiac ACE mRNA levels after MI. These ACE mRNA levels were not affected by ACE-I treatment for 6 weeks. More is known about the regulation of the systemic RAS in heart failure. An upregulation of renal renin mRNA and a downregulation of pulmonary ACE mRNA have been demonstrated after MI in rats (23). Renal renin mRNA was found to be further increased after treatment with the ACE-I enalapril (21). In contrast hepatic AO mRNA was not influenced by

experimental MI and treatment with an ACE-I (21).

In situations other than experimental heart failure, the regulation of the systemic RAS has been studied extensively. Stimulation of AO has been demonstrated following sodium depletion, and treatment with glucocorticoids, estrogens and interleukins (3,9,26). Administration of renin was associated with decreases in plasma AO and liver AO levels (16,27). Besides renin, also angiotensin II (ANG II) itself influences AO production. ANG II exerts a positive feedback on hepatic and renal AO gene expression and protein synthesis (11,22). In contrast ANG II has been shown to exert a negative feedback on renin gene transcription and renal renin secretion (14,22). Sodium deprivation has been demonstrated to increase renal renin mRNA expression (19). In chapter 3 we described an upregulation of renal renin mRNA after short-term captopril treatment (17). In that chapter we also discussed a differential regulation of AO mRNA in different tissues, including the atria. We suggested that a balance between the different feedbacks of ANG II and renin may regulate AO mRNA expression. Little is known about the regulation of ACE activity and even less is known about the regulation of the transcription of the ACE gene. ANG II infusion resulted in decreased pulmonary ACE mRNA and ACE activity levels (20). Similarly, increases of pulmonary ACE mRNA and activity were observed after treatment with the ACE-inhibitor quinapril (20). Furthermore, β -adrenergic stimulation glucocorticoids and androgens were found to stimulate ACE activity in different tissues (5-7,15). An increase as well as a decrease of ACE activity was observed following hypoxia (10,12).

In the present study we investigated the regulation of the intracardiac and systemic renin-angiotensin-systems after experimental MI by early treatment with the ACE-I captopril. At 7 days after MI, intracardiac renin and ACE mRNA levels were increased, whereas intracardiac AO mRNA was not. None of the intracardiac components of the RAS were influenced by 7 days treatment with captopril in sham and infarcted animals. Renal renin and pulmonary ACE, but not hepatic AO mRNA levels were increased after MI when compared to sham animals. Furthermore, we observed suppression of pulmonary ACE mRNA and a tendency towards an increase of hepatic AO mRNA levels following captopril treatment in MI animals. These data suggest a differential regulation of the intracardiac and systemic RAS after MI

7.2 MATERIALS AND METHODS

Surgery

Adult male WKY rats were used, weighing 250-300 g at the beginning of the experiments. Rats underwent induction of MI (chapter 2.2.1) or sham-surgery. At 7 days after surgery MI and sham rats (n=6 per group) were sacrificed by ether overdose. In a third and fourth group, animals underwent the same surgery as described above. In these groups infarct (MICAP) and sham animals (SHCAP) (n=6

per group) received the ACE-I captopril (0.5 mg/kg/hr) for 7 days by osmotic minipumps (chapter 2.2.4).

Tissue processing

In all animals the heart was removed and a slice of tissue parallel to the base was cut, formalin fixed overnight and paraffin embedded, for measuring infarct sizes (chapter 2.3.1). The rest of each heart was divided in infarcted or non-infarcted (sham) LV, SE and RV. Furthermore, kidneys, liver and lungs were removed. The tissues were subsequently frozen in liquid nitrogen and stored at -70°C.

RNA isolation

Total RNA was extracted using the LiCl/urea method (24) as described in chapter 2.3.2.

Competitive RT-PCR for angiotensinogen, renin and ACE

The competitive RT-PCR was used for quantification of AO, renin and ACE mRNA. The selection of the primers, synthesis of the internal standards, the reverse transcription and the PCR is described in chapter 2.4. The competitive RT-PCR for AO consisted of a double PCR. The first and second PCR of AO were performed at 0.5 min. at 94°C, 0.5 min. at 62°C, 1 min. at 72°, 20 cycles.; the PCR-cycle used for renin was 0.5 min at 94°C, 0.5 min at 65°C, 1 min at 72°, 40 cycles and the PCR-cycle used for ACE was 0.5 min. at 94°C, 0.5 min. at 65°C, 1 min. at 72°, 30 cycles.

Northern hybridization analysis

A total of 15 µg total RNA from the different compartments of the heart, lung, liver and kidney were used for Northern blotting. The probes used for hybridization were the cDNA inserts to AO (1634 bp) and 28S rRNA (2100 bp) and the PCR-generated probes for ACE and ANF. The 28S signal was used as an internal standard, and AO, ACE and ANF signals were related to this 28S signal (chapter 2.5). Blots containing the total RNA of the compartments of the heart were hybridized with the ANF probe; blots containing total RNA of the lungs and liver were hybridized with the ACE probe and the AO probe, respectively.

Statistical analysis

All data are presented as mean ± SEM. Statistical significance was calculated using Kruskal-Wallis test. Statistical significance was accepted at the level of $p < 0.05$ (chapter 2.9).

Figure 1. ANF mRNA expression in the left ventricle (LV), septum (SE) and right ventricle (RV) in sham (SH), myocardial infarction (MI), sham treated with captopril (SHCAP) and in myocardial infarction treated with captopril (MICAP). The ANF/28S ratio of sham values was set to the value of 1. * $P < 0.05$, significantly different from sham.

7.3 RESULTS

Infarct size and ANF mRNA expression

There was no difference between the mean infarct sizes in the two MI groups. In the untreated MI group mean infarct size was $43 \pm 5\%$ ($n=6$), whereas mean infarct size in the captopril treated group was $40 \pm 3\%$ ($n=6$). As a marker for cardiac hypertrophy we determined ANF mRNA expression, which increased 2.4-fold in the infarcted LV at 7 days after MI. A 1.8 fold increase of ANF mRNA expression was found in the SE and a 1.9 fold increase in RV, as compared to the ANF mRNA expression in the different compartments of the sham group. In the MI animals there was a tendency towards decreasing ANF mRNA levels in the different compartments after treatment with captopril, but this was not significant (figure 1).

Angiotensinogen

Cardiac: AO mRNA was extremely low in sham and infarcted hearts. AO mRNA levels were 438 ± 104 ag/100 ng total RNA in the sham LV, 412 ± 131 ag/100 ng total RNA in the sham SE and 284 ± 89 ag/100 ng total RNA in sham RV. No differences in AO mRNA levels were found between sham hearts and infarcted

A

B

C

Figure 2. Angiotensinogen (AO) mRNA amount in the left ventricle (A), septum (B) and right ventricle (C) in sham (SH), myocardial infarction (MI), sham treated with captopril (SHCAP) and in myocardial infarction treated with captopril (MICAP).

Figure 3. Hepatic angiotensinogen (AO) mRNA expression in sham (SH), myocardial infarction (MI), sham treated with captopril (SHCAP) and in myocardial infarction treated with captopril (MICAP). The AO/28S ratio of sham values was set to the value of 1.

hearts in any of the compartments. Early captopril treatment in the sham animals as well as the infarcted animals did not affect AO mRNA levels in any of the compartments (figure 2A, B and C).

Hepatic: The ratio of the densitometric values of AO and 28S of sham animals was set to the value of 1. AO levels of the MI as well as the SHCAP group were not different from the sham group. However, a trend towards increase of hepatic AO mRNA levels was observed in the MICAP group (2.3-fold), as compared to the MI group (figure 3).

Renin

Cardiac. Renin mRNA expression was very low in all compartments of the sham-hearts at 7 days after surgery. In this group, renin mRNA levels were above the level of accurate quantification in only 2 out of 6 left ventricles at 7 days after sham surgery. Reducing the amounts of internal standard did not solve this problem. Mean renin mRNA levels were 2.7 fg/100 ng total RNA in the sham LV. In the MI group renin mRNA levels were high in the infarcted left ventricles. Mean renin mRNA levels in the 7 day infarcted LV were 22 ± 6 fg/100 ng total RNA, indicating a more than 8-fold increase as compared to sham. In the RV and SE of MI animals

Figure 4: Renin mRNA amount in the left ventricle in sham (SH), myocardial infarction (MI), sham treated with captopril (SHCAP) and in myocardial infarction treated with captopril (MICAP). * $P < 0.05$, significantly different from sham.

Figure 5: Renal renin mRNA amount in sham (SH), myocardial infarction (MI), sham treated with captopril (SHCAP) and in myocardial infarction treated with captopril (MICAP). * $P < 0.05$; # $P < 0.01$, significantly different from sham.

renin mRNA levels were below the level of accurate quantification. Also in the SHCAP group renin mRNA levels were not above the level of accurate quantification in any of the compartments. However, in the infarcted LV of animals in the MICAP group renin mRNA levels were slightly higher (30 ± 9 fg/100 ng total RNA) than renin mRNA levels in the LV of the MI group, although these levels were not significantly different (figure 4).

Renal: Renal renin mRNA levels were 129 ± 68 fg/100 ng total RNA in the sham group, which indicated that renal renin mRNA levels were at least 50-fold higher when compared to renin mRNA levels in sham LV. In the MI group renal renin mRNA levels increased 3.1-fold as compared to the sham group. In the SHCAP there was a 3.2-fold increase in renal renin mRNA as compared to the untreated sham group. Renal renin mRNA levels in the MICAP group were slightly higher than in the untreated MI group (1.4-fold increase), although this increase was not significantly different (figure 5).

ACE

Cardiac: In sham-operated rats the mean value of ACE mRNA expression was 383 ± 130 fg/100 ng total RNA in the LV, 310 ± 193 fg/100 ng total RNA in SE and 567 ± 240 fg/100 ng total RNA in the RV. At 7 days after MI ACE mRNA expression increased 3-fold in the infarcted LV when compared to the non-infarcted LV in the sham group. In the infarcted animals no significant change in ACE mRNA expression was observed in the SE and RV. Captopril treatment did not affect ACE mRNA levels in the different compartments of the sham or infarcted rats (figure 6A, B and C).

Pulmonary: The ratio of the densitometric values of ACE and 28S of sham animals was set to the value of 1. In the MI group pulmonary ACE mRNA levels were 1.7-fold higher than in the untreated sham rats. Captopril treatment in the sham group did not affect pulmonary ACE mRNA levels. The increase of pulmonary ACE mRNA levels in the MI group was suppressed after captopril treatment (figure 7).

7.4 DISCUSSION

The data presented in this study show a difference in the regulation of the expression of components of the cardiac and systemic RAS after captopril treatment in infarcted and sham-operated rats. Intracardiac renin and ACE mRNA Levels were increased after MI, whereas intracardiac AO was not. None of the intracardiac components of the RAS were affected by captopril treatment in sham and infarcted animals. Renal renin and pulmonary ACE mRNA were increased following MI. Hepatic AO mRNA expression was not changed following MI. Captopril treatment resulted in increased renal renin mRNA levels in the sham-group, but had no effect

A **B** **C**

Figure 6. ACE mRNA amount in the left ventricle (A), septum (B) and right ventricle (C) in sham (SH), myocardial infarction (MI), sham treated with captopril (SHCAP) and in myocardial infarction treated with captopril (MICAP). * $P < 0.05$, significantly different from sham.

Figure 7. Pulmonary ACE mRNA expression in sham (SH), myocardial infarction (MI), sham treated with captopril (SHCAP) and in myocardial infarction treated with captopril (MICAP). The ACE/28S ratio of sham values was set to the value of 1. * $P < 0.05$, significantly different from sham.

on hepatic AO and pulmonary ACE mRNA levels. Captopril following MI resulted in suppression of pulmonary ACE mRNA and a trend towards an increase for hepatic AO. The renal renin mRNA expression was not affected in captopril treated infarcted animals.

The unchanged levels of cardiac ACE and hepatic AO mRNA and the increased levels of renal renin mRNA after captopril treatment in infarct or sham animals, respectively, are in accordance with data from other studies. Recently, cardiac ACE mRNA expression was studied after treatment with an ACE-I in infarcted rats. In that study no difference was observed after 6 weeks of treatment with the ACE-I (28). Schunkert *et al.* (21) found unchanged hepatic AO and renal renin mRNA levels at 12 weeks after MI. After 6 weeks of treatment with the ACE-I enalapril, starting at 6 weeks after MI, renal renin mRNA levels increased 2.5-fold, whereas hepatic AO mRNA levels remained unchanged. After 6 weeks of severe heart failure (infarct sizes $\geq 40\%$) increased levels of renal renin mRNA (1.7-fold) were found (23). Pulmonary ACE mRNA and activity has been demonstrated to decrease after severe MI in the rat (8). In the same study increased levels of plasma ACE activity were observed.

The mechanism for the differential regulation of the intracardiac and systemic RAS in normal and pathophysiological conditions is unclear. Several possibilities have to be considered.

- 1) Renal renin mRNA levels were elevated after captopril treatment in the sham animals, indicating that low ANG II plasma levels stimulate renal renin gene expression. This feedback was not observed for the cardiac renin gene.

However, cardiac renin mRNA levels were extremely low and were not detectable in all hearts. Therefore, we can not totally exclude an upregulation of cardiac renin mRNA after captopril treatment, resulting in cardiac renin mRNA levels, which may be still undetectable for our methods. Because cardiac renin mRNA levels could be quantified in some of the sham animals, but not in the captopril treated sham animals, this possibility is highly unlikely.

- 2) In the captopril-treated group following MI pulmonary ACE mRNA levels are suppressed, whereas cardiac ACE mRNA levels are still elevated. The increase of ACE mRNA levels in the LV and lungs may result from a difference in controlling ACE mRNA levels. Increased wall tension, stretch, or the direct effect of cell death, releasing stimulating substances, may control mRNA levels of the intracardiac ACE, whereas hemodynamics may play an important role in controlling pulmonary ACE mRNA levels. After MI the controlling mechanism for regulating mRNA levels of the cardiac RAS may overrule the mechanism for controlling the systemic RAS.

The differential regulation of the renin-angiotensin-systems may indicate that the intracardiac and systemic RAS have different functions. Whereas the systemic RAS is thought to be involved in regulation of hemodynamic homeostasis, an activated intracardiac RAS after MI may be involved in wound healing (18) and remodelling of the heart (13,25), as discussed in chapters 4 to 6 of this thesis.

Although cardiac mRNA levels of the components of the RAS are not suppressed after ACE-I treatment, ACE-inhibition most probably results in lower cardiac ANG II levels in the captopril treated than in the untreated MI group. First the dose of the ACE-I used in this study results in a 17-fold shift in the pressor dose-response curve to angiotensin I, indicating effective ACE-inhibition (25). Captopril is able to penetrate into the heart and it therefore is unlikely that intracardiac ANG II levels are not suppressed after captopril treatment (4). Moreover, in chapter 3 we observed a downregulation of atrial AO mRNA after short-term captopril treatment, suggesting effective cardiac ACE-inhibition.

The mean Infarct sizes in the different groups were 43% and 40% in the untreated and treated MI group, respectively. Infarction from this size results in hypertrophy of the non-infarcted myocardium (1,2), as was also shown by the increased levels of ANF, a marker for cardiac hypertrophy. Captopril treatment resulted in suppression of ANF mRNA, although this effect was not significant. In our study captopril treatment may have been too short to completely suppress ANF mRNA in infarcted rat hearts. Wollert *et al.* (28) observed a downregulation of cardiac ANF mRNA expression in infarcted rats, 6 weeks after ACE-I treatment. However, also in that study ANF mRNA expression was not completely suppressed to sham levels (28).

In summary, we found an activation of the intracardiac and systemic renin and ACE after MI. Whereas captopril treatment did not affect the intracardiac RAS mRNA expression, the systemic RAS was affected by the captopril treatment. These

data indicate differential regulation of the intracardiac and systemic RAS in normal and pathological conditions.

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CHAPTER 8

GENERAL DISCUSSION

8.1 INTRODUCTION

In this thesis we studied the role of the intracardiac renin-angiotensin-system (RAS) in the development of cardiac hypertrophy. Furthermore we studied its regulation by applying different interventions in the RAS. We found:

- A) No activation of the different components of the intracardiac RAS in the hypertrophied myocardium following myocardial infarction (MI), indicating that the intracardiac RAS does not play a dominant role in the development of cardiac hypertrophy following MI.
- B) An activation of the intracardiac renin gene and angiotensin I-converting-enzyme (ACE) gene, protein and activity in the border zone of the infarcted area, suggesting a role for the intracardiac RAS in wound healing.
- C) A differential regulation of the RAS in different tissues, and in normal and pathophysiological conditions, suggesting a tissue-specific regulation of the RAS.

8.2 THE EXISTENCE OF AN INTRACARDIAC RAS

As discussed in chapter 1, the existence of all components of the intracardiac RAS has been detected (4,5,12,16,21,28,35). A prerequisite for the existence of an intracardiac RAS is the presence of mRNA expression of the different components in the heart. In normal conditions we found a low expression of components of the RAS in the heart. Using conventional techniques, like Northern hybridization analysis, intracardiac mRNA expression of the components of the RAS was not detectable. Therefore, we used a competitive RT-PCR for quantifying low levels of mRNA of these components of the intracardiac RAS (chapter 4-7). Thus this thesis confirms the existence of an intracardiac RAS. Of these components, angiotensinogen (AO) and renin displayed the lowest levels of mRNA.

8.3 THE INTRACARDIAC RAS VERSUS THE SYSTEMIC RAS

Physiological conditions

Circulating components of the RAS may contribute to the intracardiac levels of angiotensin II (ANG II) and vice versa. In sham-operated hearts we observed low mRNA expression of components of the RAS. Although overall expression of ACE was low, it was localized in specific cells (endothelium) and displayed measurable activity. Levels of AO and renin mRNA were low; immunocytochemical localization was impossible, suggesting similarly low protein levels. However, since these components are considered to be able to diffuse into the heart (12) they can, in combination with relatively high intracardiac ACE-activity, contribute to intracardiac formation of ANG II. Thus, as is illustrated in figure 1, under normal

Interactions between the systemic and intracardiac RAS

Figure 1. Interactions between the systemic (blood) and intracardiac (heart) renin-angiotensin-system (RAS) under physiological and pathophysiological conditions. AO = angiotensinogen; ACE = angiotensin I-converting-enzyme; ANG I = angiotensin I; ANG II = angiotensin II.

conditions interaction between the circulating and intracardiac RAS may contribute to intracardiac ANG II-formation, with emphasis on the circulating RAS.

Pathophysiological conditions

In chapters 3 to 6 we studied the activation of the intracardiac RAS in pathophysiological situations, like MI and aorta coarctation. A downregulation of atrial AO mRNA was observed following aorta coarctation (chapter 3) whereas no ventricular AO mRNA could be detected by Northern hybridization analysis. Localized activation of the renin gene (chapter 5) and the ACE gene and protein (chapter 4) were found in the early phase following MI in the border zone of the infarcted area, whereas no activation of the cardiac AO gene was found (chapter 6).

Other studies have demonstrated activation of the intracardiac RAS in pathophysiological conditions, like MI (11,14,29) and pressure- and volume-overload hypertrophy (1,3,40). However activation of the intracardiac renin mRNA following MI has not been demonstrated before. Furthermore, time, site and function of activation of the intracardiac RAS are not clear.

Plasma ANG II (pANG II) levels and plasma renin activity (PRA) are elevated

after acute MI or aorta coarctation. This activation of the systemic RAS occurs shortly after MI or aorta coarctation. In most studies pANG II levels and PRA are back to base line levels within 5 days (chapter 3), however, in severe heart failure pANG II and PRA may also remain elevated for several weeks (1,20,36,42).

The cardiac AO gene is not activated after MI. This does, however, not rule out increased ANG II-formation. First, cardiac AO levels may be sufficient and not rate-limiting for the formation of angiotensin I (ANG I), in spite of the activation of the cardiac renin gene. Second, if cardiac AO becomes rate-limiting, it may be completely or partly derived from the circulation.

After MI, we demonstrated a clear activation of the renin gene, which is regarded as the rate limiting enzyme. Intracardiac renin mRNA levels after MI were only 3-fold lower than renal renin mRNA levels in sham animals (chapter 7), suggesting that the intracardiac renin levels may be high enough for substantial production of ANG I, independently of systemic renin.

As discussed above, we suggested that in sham animals intracardiac ACE activity may be sufficient for the formation of intracardiac ANG II. In the infarcted left ventricle ACE mRNA and activity were 3-fold higher than sham levels. The above argued increase of ACE substrate, ANG I, by the activation of the renin gene, may, in fact, induce activation of the ACE gene and protein. With increased ANG I levels, ACE may become rate-limiting. By the activation of ACE, the increased ANG I levels can translate into increased ANG II levels. In summary, in pathophysiological situations, like MI, except for the possible diffusion of circulating AO into the heart, the formation of intracardiac ANG II by the other components may be greatly independent on the systemic RAS (figure 1).

8.4 REGULATION OF THE INTRACARDIAC RAS AND THE SYSTEMIC RAS

Little is known about the regulation of the intracardiac RAS, in contrast what is known about the regulation of the systemic RAS. The most obvious findings were obtained with steroids (6,13), sodium deprivation, or with injections/infusions of components of the RAS itself, like ANG II and renin (2,19,22). ANG II has been shown to exert a stimulatory effect on AO synthesis and release, and an inhibitory effect on renin and ACE release or activity (41,43). Renin has been demonstrated to exert a negative feedback on AO production (31,51).

We observed a differential regulation of AO mRNA in different tissues following interventions in the RAS, like short-term captopril treatment, nephrectomy or aorta coarctation. In chapter 3 it was suggested that a balance between plasma ANG II levels and renin activity may regulate tissue AO mRNA levels, especially in the atria, brain and liver. In addition, renal renin mRNA was upregulated after short-term

Figure 2. The regulation of the tissue and systemic renin-angiotensin-system following interventions in the renin-angiotensin-system or following myocardial infarction. AO = angiotensinogen; ANG I = angiotensin I; ANG II = angiotensin II; δ = differences; + = stimulation; - = inhibition.

captopril treatment. This upregulation of renal renin mRNA was also observed in chapter 7, where seven days of treatment with captopril resulted in an upregulation of renal renin mRNA expression. These data demonstrate that in the normal situation the systemic and some tissue renin-angiotensin-systems, like in atria and brain, may be regulated by the plasma components of the RAS (figure 2). The purpose for this regulation may be to control hemodynamic homeostasis. The feedback on renal renin mRNA expression following captopril treatment is the most effective feedback for restoring ANG II levels, since renin is viewed as the rate-limiting enzyme of the RAS cascade. Levels of the components of the RAS in the ventricles are not affected by captopril treatment in the normal situation. In paragraph 8.3 we already discussed that, under physiological conditions, the intracardiac RAS is dependent on the systemic RAS. Therefore, levels of the components of the cardiac RAS in this situation may be greatly dependent on the regulation of the systemic RAS.

After MI, renal renin and pulmonary ACE mRNA levels were increased (chapter 7). The increase of pulmonary ACE mRNA levels after MI, may be a result of the increase of renal renin mRNA levels. In that situation ANG I formation may no longer be the rate-limiting step. In response to the increased ANG I levels, pulmonary ACE

may be upregulated. Captopril treatment in infarcted animals results in a suppression of pulmonary ACE mRNA levels (chapter 7, figure 2). As discussed in chapter 8.3, the intracardiac RAS after MI, may be greatly independent of the systemic RAS. The intracardiac mRNA levels of components of the RAS are not changed after seven days of captopril treatment in infarcted rats (chapter 7). Renin and ACE genes are still activated, whereas AO mRNA levels are still low. These data indicate a differential regulation between the intracardiac and systemic RAS and may furthermore indicate differential functions of the intracardiac and systemic RAS.

The possibility exists that the observed lack of regulation of the components of the intracardiac RAS depends upon a lack of inhibition of intracardiac ACE-activity. This is, however, not likely, because the dose of the ACE-I used in this thesis causes a 17-fold shift of the pressor-dose response curve to ANG I, indicating effective inhibition of the ACE-I (46). Furthermore, captopril is able to diffuse into the heart (10), as also suggested by the inhibition of atrial AO mRNA expression following short-term captopril treatment (chapter 3).

8.5 POSSIBLE FUNCTIONS OF THE INTRACARDIAC RAS AFTER MYOCARDIAL INFARCTION

Although the intracardiac RAS is activated in models of cardiac hypertrophy, and angiotensin II (ANG II) itself is seen as a growth factor, the role of the intracardiac RAS in the development of cardiac hypertrophy may not be its most important role. Localized activation of renin and ACE around the infarct, but not in the hypertrophied non-infarcted compartments of the heart supports this hypothesis. Also others have observed the most pronounced effects on the activity of the components of the RAS in the infarcted left ventricle. However, in these studies increased activity of the components of the RAS was also found in the hypertrophied non-infarcted myocardium. Activation of the intracardiac RAS in the other compartments of the heart by others may be explained by the use of less accurate techniques than the competitive RT-PCR, different preparation of the heart tissue, or differences in the experimental protocols. First, a semi-quantitative PCR as performed by Hirsch *et al.* (20), makes it difficult to observe differences in mRNA expression due to the introduction of a higher variance in the results by this technique. For instance, observed differences may be explained by differences in efficiency of amplification during PCR. Second, because activation of the intracardiac RAS occurs around the infarcted zone it is possible that large infarctions expand to the right ventricle or septum, thereby increasing the mRNA levels of the RAS genes in these tissues. Third, ACE binding has been demonstrated to be associated with reparative fibrosis, which occurs around the necrotic area of the infarct (49). Reparative fibrosis may also occur in the non-infarcted myocardium. However, we never observed reparative fibrosis in the non-infarcted myocardium. These differences may depend upon ventilation of the animals during surgery. Lack

of ventilation during surgery may cause small focal ischemic areas in the "non-infarcted" septum and right ventricle, resulting in reparative fibrosis. This may explain the increased ACE binding in the non-infarcted myocardium in studies, in which ventilation during surgery did not take place. In this thesis we concluded that the intracardiac RAS does not play a dominant role in the development of cardiac hypertrophy after MI. However, we can not exclude that the activated components of the intracardiac RAS, leading to increased levels of ANG II, may, by diffusion or in combination with the circulating RAS, still play a part in the hypertrophic response of the non-infarcted myocardium.

So, if the intracardiac RAS does not play a dominant role in the development of cardiac hypertrophy after MI, what, if any, is then the function of the intracardiac RAS? The site of activation of the components of the intracardiac RAS around the necrotic area after MI may provide an answer to this question. Renin mRNA was found in close association with the infarcted area, while expression in the non-infarcted myocardium was very low (chapter 5). Although ACE mRNA was also found around the infarct, ACE mRNA expression could be localized predominantly in endothelial cells (chapter 4). It has been demonstrated that all components of the RAS are expressed in the different cells of the heart, like myocytes, fibroblasts and endothelial cells. In this thesis, none of the components of the RAS displayed mRNA expression in the cardiomyocytes. Activation of both these components of the intracardiac RAS were found to be activated in the same area, around the vicinity of the infarct area. This suggests that the intracardiac RAS acts in a paracrine or autocrine way, leading to the formation of intracardiac ANG II. ACE, the final enzyme of the RAS cascade, is predominantly localized in endothelial cells, which suggests that the formation of ANG II mainly occurs on the endothelial cells. ANG II formation on or in the vicinity of the endothelial cells may result in proliferation of the endothelial cells. Proliferation of endothelial cells indicates the formation or outgrowth of blood vessels, *i.e.* neovascularization and angiogenesis. Stoll *et al.* (48) demonstrated an antiproliferative action of ANG II, mediated via the AT₂ receptor, in cultured coronary endothelial cells. However, an angiogenic effect of ANG II has been described in the chorioallantoic membrane (26,27). Furthermore also in the kidney and cornea ANG II was able to induce formation of new blood vessels (15). Recently, Nelissen-Vrancken *et al.* (32) demonstrated a complete normalization of maximal coronary flow, an indicator for capillary capacity, in the infarcted left ventricle, within 35 days after MI, suggesting vessel growth or dilatation of pre-existing vessels in the region adjacent to the infarcted zone. This response could be inhibited by captopril treatment (Nelissen-Vrancken, submitted).

In addition to its effect on endothelial cells ANG II may also influence other cells, like (myo)fibroblasts. The activation of the renin and ACE genes may occur in these (myo)fibroblasts (chapter 4 and 5). Stimulation of fibroblasts by ANG II results in proliferation of these fibroblasts and synthesis of extracellular matrix components (9,39). Appearance of (myo)fibroblasts around the necrotic area has been described in human infarcts (55). Components of the ECM, like collagen I and III and

fibronectin are activated in the infarcted left ventricle in the early phase after MI (8,44). In addition ACE-I results in suppression of the increased deposition of ECM components after MI (25). This suggests that the intracardiac RAS may also be involved in formation of fibrous tissue in the infarcted left ventricle, leading to cardiac stiffening and depressed cardiac function (53).

Furthermore, the intracardiac RAS may stimulate the synthesis of ECM components in fibroblasts as a part of the wound healing process. In addition to the role of ECM components in cardiac fibrosis, ECM components also play an important role in wound healing. Growth factors, like PDGF and TGF β stimulate the production of components of the ECM (17,30). Additionally, TGF β has been found around the infarct area (52,54). ANG II has been found to activate the expression of these growth factors in different cells, including fibroblasts. Therefore, an activated intracardiac RAS may activate the production of ECM components directly, or indirectly via growth factors in fibroblasts, and thereby playing a role in the process of wound healing.

8.6 THE TRIGGER FOR THE ACTIVATION OF THE INTRACARDIAC RAS AFTER MYOCARDIAL INFARCTION

Although the activation of the intracardiac RAS after MI is now established, it is not clear what the trigger is for activation of the intracardiac RAS after MI. Several factors, like increased left ventricular end diastolic pressure (LVEDP), stretch, hormonal factors or myocyte necrosis have been suggested to trigger the intracardiac RAS (29,37). In chapter 4 and 5 we demonstrated the localized increase of renin and ACE mRNA's around the infarcted area. This specific localization suggests that a circulating substance is not a trigger for the activation of the intracardiac RAS, since than a more homogeneous activation of the intracardiac RAS would be expected. That a circulating factor, like ANG II, does not play an important role in the activation of the intracardiac RAS is supported by the findings in chapter 7. Captopril treatment, which reduces ANG II levels, did not result in suppression of the activated RAS.

MI results in thinning of the left ventricular wall by the necrotic area and the side to side slippage of the myocytes. In addition, LVEDP is increased after MI. Therefore, accordance to LaPlace's law, wall tension can be expected to be higher in the infarcted left ventricle. Furthermore, the loss of viable cells in MI is associated with an increased risk of aneurysm (the outward bulging of the thinned myocardium), increasing the stretch of cells surrounding the necrotic area. In accordance, stretched myocytes *in vitro* released ANG II (37).

The activation of the intracardiac RAS around the necrotic area also suggests that the RAS may be triggered by a direct effect of the necrotic area, like the release of different stimuli from dead myocytes or hypoxia. Not only activation of the RAS has been demonstrated following hypoxia (24), but hypoxia also induces vascular growth (45). It is not known whether this response is mediated via ANG II.

8.7 CONCLUSIONS AND FUTURE OUTLOOK

Experimental and clinical studies demonstrated the beneficial effects of ACE-I treatment in cardiovascular diseases. Decreased morbidity and mortality rates were evident after ACE-I treatment in these studies (18,23,33,34,47). However, ACE-I treatment in the early phase after MI resulted in less consistent data when compared with delayed ACE-I treatment (38,50), although recent large clinical studies demonstrated a decrease of the morbidity rate even after early captopril treatment in patients with MI (7,23). We observed an activated intracardiac and systemic RAS in the early phase after MI (chapter 4-7). Although an activated RAS in MI may be a compensatory response to improve the circulation, in the end it may stimulate further progression of heart failure.

Captopril treatment resulted in suppression of the systemic RAS, but it had no effect on the activated cardiac RAS in infarcted rats. However, this does not exclude suppression of intracardiac ANG II levels after captopril treatment; since captopril is able to penetrate cardiac tissue (10), most probably cardiac ANG II levels are lower in captopril treated infarcted animals than in untreated infarcted animals. Detrimental effects observed after early ACE-I treatment may be explained by interference with the infarct healing process, through decreased cardiac ANG II levels. Alternatively, if intracardiac ANG II levels are not decreased after captopril treatment, structural and functional effects observed after ACE-I treatment, as discussed in the introduction (chapter 1), may be the result of inhibition of the systemic RAS.

In conclusion, the intracardiac RAS does not play a predominant role in the development of cardiac hypertrophy after MI. Furthermore, it may play a role in wound healing in infarcted rats. The increased systemic RAS may be responsible for the hypertrophic response of the myocardium after MI. Therefore, assuming that the intracardiac RAS has a beneficial role after MI, a possible treatment of patients after MI, may be a combination of suppressing the systemic RAS, but not the intracardiac RAS, thereby suppressing cardiac hypertrophy and leaving the wound healing process intact. Cushman *et al.* (10) demonstrated that differences in physico-chemical properties of ACE-inhibitors results in different patterns of inhibition of plasma- and tissue-ACE (including cardiac ACE). Thus, the outcome of the treatment may depend upon the choice of the ACE-I. It is difficult to predict differences from Cushman's data, because he only studied the effects of single injections. Our hypothesis does, however, warrant more extensive characterization of cardiac ACE-activity during chronic ACE-I treatment.

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SUMMARY

Cardiac hypertrophy is a risk factor for cardiovascular complications and results in a higher morbidity and mortality. Myocardial infarction (MI) or hypertension are important determinants for the development of cardiac hypertrophy. The intracardiac renin-angiotensin-system (RAS) is thought to play a role in the development of cardiac hypertrophy. In this thesis, experiments were performed to study the intracardiac RAS in the development of cardiac hypertrophy. Furthermore the regulation of the RAS was studied in physiological and pathophysiological conditions.

In **chapter 3** the regulation of the mRNA of angiotensinogen (AO) and renin, two components of the RAS, was studied in different tissues, following aorta coarctation (7 days), bilateral nephrectomy (24 hours) and captopril treatment (24 hours) in Wistar Kyoto (WKY) rats. These different experimental groups were chosen, because of their different effects on the systemic RAS. Each experiment resulted in different plasma levels of renin and angiotensin II (ANG II), two important modulators in the regulation of AO and renin production. Furthermore, aorta coarctation results in cardiac hypertrophy and therefore the role of intracardiac AO and renin in the development of cardiac hypertrophy following aorta coarctation could be studied. Nephrectomy significantly increased AO mRNA in the liver and the brain, whereas it had no effect on other tissues examined. Renin mRNA expression following nephrectomy was not affected in any of the tissues examined. Both aorta coarctation and captopril treatment resulted in a downregulation of atrial AO mRNA. However, no difference of AO mRNA expression was observed in the other tissues following aorta coarctation or captopril treatment. Again renin mRNA expression was not affected in any of the tissues examined following aorta coarctation. In addition, renal renin mRNA expression was increased after captopril treatment. From these data, we concluded that AO and renin mRNA are differently regulated in the tissues examined. AO mRNA in the liver and brain appears to be tonically inhibited by renin, whereas AO mRNA in the atria is not. A negative feedback of renin mRNA expression by ANG II was observed in the kidney, but not in other tissues. Additionally we concluded that myocardial hypertrophy is not necessarily associated with increased AO and renin mRNA expression.

In **chapter 4** Wistar rats underwent sham-surgery or an induction of MI, a model for studying the development of cardiac hypertrophy and wound healing. At different times (1-90 days) after surgery the heart was removed and divided into the left ventricle (LV), septum (SE) and right ventricle (RV). Cardiac angiotensin I-converting-enzyme (ACE) mRNA expression was quantified and localized in the infarcted rat heart. Furthermore, ACE-activity and ACE-immunoreactivity was studied following experimental MI. Since cardiac ACE mRNA levels are low, we used a sensitive competitive reverse transcriptase polymerase chain reaction (RT-PCR) for quantifying ACE mRNA levels. We demonstrated a 3-fold increase of ACE mRNA expression in the infarcted LV at 4 and 7 days after MI. However, in the non-

infarcted myocardium ACE mRNA levels were not different from those in sham animals. At 4 days after MI, ACE mRNA and ACE-immunoreactivity were localized by in situ hybridization (ISH) and immunohistochemistry, respectively. High density of ACE mRNA as well as ACE-immunoreactivity were observed in the border zone of the infarct area, predominantly in endothelial cells, lining capillaries. In the non-infarcted and sham animals ACE mRNA and ACE-immunoreactivity were observed in the endothelial cells of larger vessels. Cardiac ACE-activity, measured at 7 and 90 days after MI, was increased 3-fold at these times in the infarcted LV, but not in the SE and RV, when compared to sham animals. From these data we concluded that intracardiac ACE does not play a dominant role in the development of cardiac hypertrophy following MI in the rat. Furthermore, we suggested that intracardiac ACE may play a role in wound healing following MI.

In **chapter 5** we studied the expression and localization of renin mRNA in the rat heart at different times (1-90 days) after MI. WKY rats underwent sham- or MI-surgery. By competitive RT-PCR cardiac renin mRNA levels were quantified. Atrial natriuretic factor (ANF), a marker for cardiac hypertrophy, was determined by Northern hybridization analysis, in the different compartments of the heart at 1, 7 and 90 days following surgery. In sham animals cardiac renin mRNA levels were extremely low and could only be quantified in some of the animals. However, renin mRNA levels were higher in the infarcted LV. At 2, 4 and 7 days after MI, renin mRNA levels were respectively 4, 17 and 8-fold higher in the infarcted LV. In the non-infarcted hypertrophied myocardium renin mRNA expression was extremely low and indistinguishable from renin mRNA levels in sham animals. At other times after MI there was no difference in renin mRNA expression when compared to sham animals. Renin mRNA was localized in the heart by ISH at 4 days after MI, the time with the highest renin mRNA levels (*vide supra*). A high density of renin mRNA was observed in the border zone of the infarct area. In this area renin mRNA was most probably localized in (myo)fibroblasts, but not in cardiomyocytes nor in endothelial cells. In the non-infarcted myocardium renin mRNA expression was very low and indistinguishable from the background labeling. ANF mRNA expression increased 2-fold in all compartments of the heart at 1, 7 and 90 days after MI. The low amounts of renin mRNA in the non-infarcted hypertrophied myocardium indicate that the intracardiac synthesis of renin does not play a important role in the development of cardiac hypertrophy in the rat heart following MI. However, the increased renin mRNA expression in the border zone of the infarct area suggests a role for intracardiac synthesized renin in wound healing.

In **chapter 6** the expression and localization of AO mRNA was studied in WKY rats in the infarcted rat heart. As ACE and renin, AO mRNA was quantified and localized at different times in the different compartments of the heart after sham- or MI-surgery. Since these were the same animals as in chapter 5, ANF mRNA expression was increased in the different compartments of the heart, following MI (*vide supra*) By competitive RT-PCR we demonstrated that AO mRNA levels were

extremely low and that those were not changed following MI in the different compartments of the heart at any time after surgery. ISH revealed an overall low density of AO mRNA in the myocardium, with a somewhat higher density of AO mRNA labeling in the epicardium. These data demonstrate that intracardiac AO gene is not activated after MI in the rat heart, suggesting that intracardiac synthesis of AO does not play a dominant role in the development of cardiac hypertrophy or infarct healing in the rat heart after MI.

In **chapter 7** we investigated the feedback regulation of the expression of the components of the intracardiac and systemic RAS by interference with the RAS in WKY rats following MI, with or without treatment with the ACE-inhibitor captopril. At 7 days after surgery rats were sacrificed and the different compartments of the heart, lungs, liver and kidneys were removed. Cardiac AO, renin and ACE mRNA, and renal renin mRNA were quantified by the competitive RT-PCR. Hepatic angiotensinogen, pulmonary ACE and cardiac ANF mRNA expression were quantified by Northern hybridization analysis. At 7 days after MI, we found an 8-fold increase of renin mRNA expression and a 3-fold increase of ACE mRNA expression in the infarcted LV. However, as discussed in chapter 6 we did not find a difference in cardiac angiotensinogen mRNA expression between infarcted and sham animals. Hepatic AO mRNA levels were not changed following MI. Renal renin and pulmonary ACE mRNA expression increased respectively 3 and 1.7 fold following MI. Seven days of captopril treatment had no effect on the intracardiac expression of the components of the RAS. In sham animals captopril treatment resulted in an increase of renal renin mRNA, whereas it had no effect on hepatic AO and pulmonary ACE mRNA levels. The increase of pulmonary ACE in infarcted animals was suppressed after treatment with captopril. ANF mRNA expression was increased in all compartments of the heart after MI. However, captopril treatment of the MI-animals resulted did not completely suppress ANF mRNA expression in the different compartments. These data indicate an activation of the intracardiac and the systemic renin and ACE genes, but not of AO following MI. Furthermore these data suggest a differential feedback regulation of the intracardiac and systemic RAS in infarcted and sham animals.

In summary, the data presented in this thesis show that activation of the components of the intracardiac RAS does not occur in the non-infarcted hypertrophied myocardium, indicating that the intracardiac RAS does not play a dominant role in the development of cardiac hypertrophy following MI in the rat. However, the intracardiac activation of the renin and ACE genes in the border zone of the infarct area suggests a role for the intracardiac RAS in wound healing. Furthermore, the differential effects of captopril treatment on the intracardiac and systemic RAS in infarcted rats, suggests that the intracardiac RAS in the infarcted rat is less sensitive for ACE-inhibition than the systemic RAS.

SAMENVATTING

Cardiale hypertrofie (het toenemen van de massa van de hartspier) leidt tot een verhoogde morbiditeit en mortaliteit. Hypertensie en myocard infarct (MI) zijn bijvoorbeeld belangrijke oorzaken van cardiale hypertrofie. Er zijn aanwijzingen dat het renine-angiotensine-systeem (RAS) in het hart (intracardiaal) een belangrijke rol speelt in de ontwikkeling van cardiale hypertrofie. In dit proefschrift zijn experimenten gedaan om het belang van het intracardiale RAS in de ontwikkeling van cardiale hypertrofie te bestuderen. Verder zijn experimenten uitgevoerd om het RAS te bestuderen in fysiologische en pathofysiologische omstandigheden.

In **hoofdstuk 3** is de regulatie van angiotensinogeen (AO) en renine, twee componenten van het RAS, bestudeerd in verschillende weefsels, 7 dagen na het afbinden van de aorta (aorta coarctatie), 24 uur na het verwijderen van de 2 nieren (bilaterale nefrectomie) en 24 uur na behandeling met een angiotensin I-convertering-enzyme (ACE)-remmer (captopril) in Wistar Kyoto (WKY) ratten. De opzet van deze experimenten is zo gekozen, omdat ze een verschillend effect hebben op het systemische (circulerende) RAS. Elk afzonderlijk experiment resulteerde in verschillende plasma angiotensine II (ANG II) en plasma renine spiegels, twee belangrijke modulators in de regulatie van AO en renine productie. Verder leidt aorta coarctatie tot cardiale hypertrofie, waardoor het mogelijk is om de rol van AO en renine in de ontwikkeling van cardiale hypertrofie te bestuderen. Nefrectomie leidde tot een significante verhoging van AO mRNA hoeveelheden in de lever en in de hersenen, terwijl AO mRNA hoeveelheden in de andere onderzochte weefsels niet beïnvloed werden. Renine mRNA expressie werd niet beïnvloed na nefrectomie in de onderzochte weefsels. Zowel aorta coarctatie als captopril behandeling resulteerden in een verlaagde AO mRNA expressie in de atria van het hart. Geen verschil in AO mRNA expressie werd waargenomen in de andere onderzochte weefsels noch na aorta coarctatie noch na captopril behandeling. Opnieuw was ook na aorta coarctatie geen verschil waarneembaar in renine mRNA expressie in de verschillende weefsels. Renale renine mRNA expressie was verhoogd na captopril behandeling. We concludeerden dat er een verschillende regulatie van AO en renine mRNA bestaat in de verscheidene bestudeerde weefsels. AO mRNA in de hersenen en de lever blijkt tonisch geremd te worden door renine, terwijl dat niet het geval is in de atria. In de nier werd een negatieve feedback van renine mRNA expressie door ANG II waargenomen, maar niet in de andere bestudeerde weefsels. Verder concludeerden we dat cardiale hypertrofie, zoals waargenomen na aorta coarctatie, niet noodzakelijk geassocieerd is met een verhoogde AO en renine mRNA expressie.

In **hoofdstuk 4** ondergingen Wistar ratten een sham- of een MI-operatie, een model geschikt voor het bestuderen van cardiale hypertrofie en wondgenezing. Op verschillende tijdstippen na de operatie werd het hart verwijderd en onderverdeeld in linker ventrikel (LV), septum (SE) en rechter ventrikel (RV). We bestudeerden de activatie van het angiotensin I-convertering-enzyme (ACE) gen (ACE mRNA) en eiwit

(ACE-activiteit) in het geïnfarceerde rattehart. Verder werd ook de lokalisatie van ACE mRNA en het eiwit (ACE-immunoreactiviteit) bestudeerd. Vanwege het feit dat cardiale ACE mRNA hoeveelheden laag zijn, hebben we gebruik gemaakt van een zeer gevoelige competitieve reverse transcriptase polymerase chain reaction (RT-PCR) voor het kwantificeren van ACE mRNA hoeveelheden. Een 3-voudig verhoging van ACE mRNA werd gevonden in de geïnfarceerde LV, 4 en 7 dagen na een MI. In het niet-geïnfarceerde myocard was er geen verschil in ACE mRNA hoeveelheden, vergeleken met de sham dieren. ACE mRNA en ACE-immunoreactiviteit werd op 4 dagen na sham of MI-operatie gelokaliseerd door middel van respectievelijk in situ hybridisatie (ISH) en immunohistochemie. Een hoge ACE mRNA densiteit en een hoge ACE-immunoreactiviteit werd voornamelijk in endotheelcellen waargenomen in het randgebied van het infarct. In het niet-geïnfarceerde hart en het sham hart werd ACE mRNA en ACE-immunoreactiviteit waargenomen in endotheelcellen van grotere vaten. De ACE-activiteit in het hart, gemeten op 7 en 90 dagen na de operatie, was 3-voudig verhoogd op beide tijdstippen in de geïnfarceerde LV, maar was onveranderd in het SE en RV, vergeleken met de sham dieren. Hieruit concludeerden we dat het intracardiale ACE geen belangrijke rol speelt in de ontwikkeling van hypertrofie na een myocard infarct. Daarentegen suggereerden we dat het intracardiale ACE een rol zou kunnen spelen in de wondgenezing na een MI.

In **hoofdstuk 5** bestudeerden we de expressie en lokalisatie van renine mRNA in het rattehart na een MI op verschillende tijdstippen. WKY ratten ondergingen een sham- of een MI-operatie. Door middel van competitieve RT-PCR werden renine mRNA hoeveelheden in het hart gekwantificeerd. Atrial natriuretic factor (ANF), een indicator voor cardiale hypertrofie, werd bepaald door middel van Northern hybridisatie analyse, in de verschillende compartimenten van het hart op 1, 7 en 90 dagen na operatie. In sham dieren waren cardiale renine mRNA hoeveelheden zeer laag. Echter, renine mRNA hoeveelheden in de geïnfarceerde LV waren aanzienlijk hoger. Op 2, 4, en 7 dagen na een MI werd respectievelijk een 4, 17 en 8-voudig verhoging van renine mRNA hoeveelheden gevonden in de geïnfarceerde LV. Renine mRNA hoeveelheden in het niet geïnfarceerde hypertrofe hart waren zeer laag en niet te onderscheiden van renine mRNA hoeveelheden in het hart van sham dieren. ANF mRNA expressie was 2-voudig verhoogd in alle compartimenten op 1, 7 en 90 dagen na MI. Renine mRNA werd gelocaliseerd in het hart door middel van ISH op 4 dagen na MI, het tijdstip met de hoogste renine mRNA hoeveelheden (*vide supra*). Een hoge renine mRNA densiteit werd waargenomen in het randgebied van het infarct. In dit gebied was renine mRNA waarschijnlijk gelocaliseerd in (myo)fibroblasten maar niet in cardiomyocyten en endotheelcellen. In het niet-geïnfarceerde hypertrofe myocard was de renine mRNA densiteit zeer laag en niet te onderscheiden van die in het hart van de sham dieren. De lage hoeveelheden van renine mRNA in het hart in het niet-geïnfarceerde myocard toont aan dat de intracardiale synthese van renine geen belangrijke rol speelt in de ontwikkeling tot cardiale hypertrofie na een MI in de rat. Echter, de verhoogde renine mRNA expres-

sie in het randgebied van het infarct doet vermoeden dat het intracardiaal gesynthetiseerde renine een role speelt in de wondgenezing na een MI in de rat.

In **hoofdstuk 6** werd de expressie en lokalisatie van AO mRNA bestudeerd in WKY ratten na een sham of MI operatie. Aangezien dezelfde dieren waren gebruikt als in hoofdstuk 5, was de ANF mRNA expressie verhoogd in de compartimenten van het hart na MI (*vide supra*). Net als ACE en renine werd AO mRNA gekwantificeerd op verschillende tijdstippen na de operatie in de verschillende compartimenten van het hart door middel van competitieve RT-PCR. Er was geen verschil in AO mRNA expressie in de verschillende compartimenten van het hart tussen geinfarceerde en sham dieren op de verschillende tijdstippen na de operatie. ISH toonde een algemeen lage AO mRNA densiteit in het myocard, met een wat hogere AO mRNA densiteit in het epicard. Deze data tonen aan dat intracardiale synthese van AO geen belangrijke rol speelt in de ontwikkeling van cardiale hypertrofie of wondgenezing in het rattehart na een MI.

In **hoofdstuk 7** onderzochten we de feedback regulatie van de expressie van de componenten van het intracardiale en het systemische RAS door interventie van het RAS in WKY ratten na een MI, met of zonder behandeling met de ACE-remmer captopril. Zeven dagen na de operatie werden de dieren opgeofferd en de verschillende compartimenten van het hart, de longen, lever en nieren werden verwijderd. Cardiale AO, renine en ACE mRNA en renale renine mRNA werden gekwantificeerd door middel van de competitieve RT-PCR. Hepatische AO, pulmonaire ACE en cardiale ANF mRNA expressies werden gekwantificeerd door middel van Northern hybridisatie analyse. 7 dagen na een MI, vonden we een 8-voudige verhoging van renine mRNA expressie en een 3-voudige verhoging van ACE mRNA expressie in de geinfarceerde linker ventrikel. Echter, zoals bediscussieerd in hoofdstuk 6 vonden we geen verschil in cardiale AO mRNA expressie tussen infarct en sham dieren. Hepatische AO mRNA was eveneens niet verhoogd na een MI. Renale renine en pulmonaire ACE mRNA expressie waren respectievelijk 3 en 1.7-voudig verhoogd na een MI. Zeven dagen captopril behandeling had geen effect op de intracardiale expressie van de componenten van het RAS. In sham dieren resulteerde captopril behandeling in een verhoogde renale renine mRNA expressie, terwijl dit geen effect had op de hepatische AO en pulmonaire ACE mRNA hoeveelheden. De verhoogde pulmonaire ACE mRNA hoeveelheden werden teruggebracht naar het basale niveau na captopril behandeling. ANF mRNA expressie was verhoogd in alle compartimenten van het hart na MI. Echter, captopril behandeling van de MI-dieren resulteerde niet in een complete suppressie van de verhoogde ANF mRNA expressie in het hart. Deze data tonen de activatie van de intracardiale en systemische renine en ACE genen aan na een MI, maar niet van AO. Verder wordt er aangetoond dat er een verschillende feedback regulatie bestaat van het intracardiale en systemische RAS in geinfarceerde en sham dieren.

Samenvattend, in dit proefschrift wordt aangetoond dat er geen activatie plaats vindt van het intracardiale RAS in het hypertrofe hart, na een MI. Hieruit conclude-

ren we dat het intracardiale RAS geen belangrijke rol speelt in de ontwikkeling van cardiale hypertrofie na een MI in het rattehart. Echter intracardiale activatie van renine en ACE genen in het randgebied van het infarct in de vroege fase na een infarct, suggereren dat het intracardiale RAS betrokken is bij de wondgenezing na een MI. Verder, de verschillende effecten van captopril behandeling op het intracardiale en systemische RAS in geinfarceerde dieren, suggereert dat het intracardiale RAS in de geinfarceerde rat ongevoeliger is voor ACE-remming dan het systemische RAS.

CURRICULUM VITAE

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- 30 september 1967 Geboren te Bergen op Zoom.
- 1979-1986 Gymnasium het Juvenaat H. Hart te Bergen op Zoom met als eindexamenvakken: Nederlands, Engels, Grieks, Wiskunde, Natuurkunde, Scheikunde en Biologie.
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