The Role of the Renin-Angiotensin System in Cardiovascular Disease

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Recent findings have increased our understanding of the role of the renin-angiotensin system in cardiovascular diseases. This was possible by the progress made in molecular biology and the import of this methodology into cardiovascular research by several groups in the past few years. Studies by these groups accumulated evidence especially for the existence and physiological importance of organ-based renin-angiotensin systems and their involvement in the pathogenesis of hypertension and the accompanying disorders of the heart, the kidney, and the vessels. This Review will focus mainly on the relevance of the local renin-angiotensin systems for cardiovascular diseases and the value of transgenic animals as models to analyse these systems (*Hypertens Res* 1994; 17: 1-16).

Key Words: renin-angiotensin system, kidney, heart, antihypertensive treatment, essential hypertension

It has long been known that the renin-angiotensin system (RAS) plays an integral role in the regulation of blood pressure as well as in electrolyte and body fluid balance. In its classical definition, the RAS maintains blood pressure via the effector peptide angiotensin II (ANG II) generated within the circulation in an enzymatic cascade initiated by renin, which is secreted by the juxtaglomerular cells of the kidney. Renin cleaves angiotensinogen (AOPEN) derived from the liver yielding the inactive decapeptide ANG I, which in turn is activated proteolytically by the endothelium-associated angiotensin-converting enzyme (ACE) to yield the octapeptide ANG II. ANG II increases blood pressure in several ways, *i.e.*, vasoconstriction, stimulation of aldosterone secretion in the adrenal gland, sodium retention in the kidney and intestine, and upregulation of the sympathetic tone.

Furthermore, degradation products of ANG I and ANG II like ANG (1-7), ANG III, or ANG IV have been shown to elicit biological effects (1-3) but the *in vivo* significance of these peptides still awaits clarification. All angiotensin peptides can be produced from the sole precursor AOPEN also by enzymatic reactions beyond the classical RAS. One important example is the chymase present in human hearts partially replacing the function of ACE (4). The relevance of these metabolic pathways, however, is still enigmatic.

The importance of the RAS goes far beyond blood pressure regulation and includes the whole cardiovascular homeostasis. This is proven by the therapeutic value of drugs interfering with this system like ACE-inhibitors (CEI) and the newly developed ANG II receptor antagonists and renin in-

hibitors. Furthermore, genetic studies in rats and humans have shown that particular alleles of the AOPEN and the ACE gene are linked to hypertension (5-7) and myocardial infarction (8). It has been postulated that ANG II is active on the cellular level, exerting paracrine or even autocrine actions (9). In addition, it was demonstrated that ANG II biosynthesis can occur in the tissues themselves, leading to the concept of organ-based, local RAS (10, 11). The functionality of these systems has been established in a number of tissues such as the kidney, adrenal gland, brain, heart and vasculature. Evidence that these tissue RAS may act independently of the plasma system can be found in numerous studies investigating the chronic effects of CEI on blood pressure (12). The reports demonstrated that the antihypertensive effects of these substances were predominantly correlated to inhibition of ACE in the tissues and not in the plasma.

The investigation of the RAS in humans has limitations for practical and ethical reasons. Most of these studies have been confined to the measurement of the plasma components of the RAS and their response to physiological and pathophysiological stimuli. With the evolving concept of local RAS, the investigation of the tissue-specific gene expression of its components has become increasingly important. The opportunities to study the action of these genes in humans are rare, since viable human tissue is only accessible from surgery specimens (13) or biopsy samples (14), which provide only very small amounts of tissue. To overcome this setback, animal models with genetic hypertension have successfully been developed *e.g.* the spontaneously hypertensive rat (SHR) and

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transgenic rats like TGR (mREN2) 27 (15), which show characteristics similar to human hypertension (16). In addition, it was possible by transgenic techniques to express the human RAS in rats by the production of TGR (hREN) and TGR (hAOPEN), rats harbouring the human genes for renin and AOPEN, respectively (17). These animals for the first time allow experimental studies on the human RAS.

Local Renin-Angiotensin Systems

To support the existence of independently expressed and regulated tissue RAS it is necessary to demonstrate the local synthesis of the components, renin, AOPEN, and ACE and the presence of functioning ANG II receptors. In addition, the synthesis of these proteins should be regulated independently of the circulating RAS. These criteria are met in several tissues, *e.g.*, brain, adrenal gland, heart, and vessels, where the mRNA for renin, AOPEN, ACE, and ANG II receptors have been shown to be regulated by physiological or pharmacological effectors (18-27). The relevance of these systems is confirmed by the fact that chronic treatment with CEI failed to correlate with pretreatment plasma renin activity but did correlate with sustained inhibition of ACE-activity in the kidney (28). Thus, there is evidence that besides the circulating endocrine RAS there are several tissue RAS serving para-, auto- or intracrine functions.

Kidney

The kidney is the major renin secreting organ of the body. Bilateral nephrectomy results in the disappearance of active renin from the plasma and in a substantial decline in circulating prorenin (29). However, the demonstration of renal gene expression of renin, AOPEN and ACE points towards the existence of an intrarenal RAS regulating intrarenal hemodynamics and sodium homeostasis.

Using immunocytochemical techniques, renin protein in the kidney has been localized in afferent and efferent arteriolar juxtaglomerular cells, interlobular arteries as well as in proximal, connecting and cortical collecting tubules in lower quantities (30-32). Since these methods do not differentiate between uptake from blood and local synthesis, *in-situ*-hybridization has been applied, confirming in mature rodents and humans the juxtaglomerular apparatus as the principal locus of renal renin mRNA-expression (33, 34). A low expression of renin is detected in the mesangium, other vascular structures, and in proximal tubular cells. However, during ontogeny, renin expression varies with ongoing maturation. In tissue sections of rat fetal kidney renin mRNA is detected in the vascular pole of juxtamedullary nephrons and afferent, interlobular and arcuate arteries. In newborns, expression in the latter two locations disappears and finally in adult animals, renin mRNA is restricted to the juxtaglomerular apparatus (35). The ontogenetic pattern of renin gene expression is paralleled by a 10- to 20-fold decrease in the amount of renal renin mRNA from

fetal through newborn to adult stages (35, 36). The role of renovascular renin in ontogeny is unclear but may be related to the possible effect of ANG II on angiogenesis (37). Within the cells of the juxtaglomerular apparatus renin is stored in vesicles and may be intracellularly activated to active renin or also be sequestered as inactive prorenin.

Albeit in adulthood, renin expression is confined to the juxtaglomerular apparatus in the basal state, vascular smooth muscle cells, extending along the afferent arteriole may be "recruited" to express renin in response to various pharmacological and physiological stimuli (38, 39). Thereby, an increase in renal renin mRNA may be due to an increase in the number of renin-producing cells rather than in renin synthesis per cell as shown in isolated renal microvessels (40). Stimulation of renal renin mRNA content with recruitment of renin producing cells is induced by treatment with CEI (39, 41, 42). CEI enhance renal renin expression probably by the interruption of the negative feedback inhibition of renin synthesis exerted by ANG II (43). Different CEI strongly vary in their potency to raise renal renin mRNA-expression. In a study by Keuneke *et al.* (44) using SHR, lisinopril elicited a much higher increase in renal renin gene expression than captopril or quinapril given in equidepressor doses. This may be related to differences in tissue-availability of the individual CEI. In contrast to that, renin inhibitors such as ES 1005 are reported to suppress renal renin gene expression in marmosets (45). Other stimuli eliciting intrarenal renin synthesis comprise sodium-depletion (41, 46, 47), β -adrenoreceptor activation (48), adrenalectomy (41), dietary protein (49, 50, J.W., unpublished observations) or renal nerve stimulation and ureteral obstruction (51, 52). Although all these factors enhance renal renin expression, their effect on plasma renin activity and prorenin is differential. Sodium-depletion (53) and dietary protein (54, 55) raise plasma renin activity. In contrast, ureteral obstruction only alters prorenin but not plasma renin activity (51, 56). Only few data are available, if the increase in renal renin content is due to enhanced gene transcription, to the rate of degradation, or changes in the proportions of renin mRNA to other RNAs. Furthermore, the increase in renal renin mRNA in sodium-depletion does not correspond in a linear fashion to changes in renal renin protein or active renin secreted from the kidney into the plasma (53) indicating post-transcriptional events.

AOPEN protein has been demonstrated first in granules isolated from the kidney cortex (57) and could then be localized to the proximal tubular brush border by immunocytochemistry (58). Local synthesis was suggested by its disappearance after protein synthesis inhibition (59) and confirmed by the detection of the mRNA (46, 60-63) and the demonstration of its specific regulation by sodium (46, 64), androgens (65) but not by CEI (38). *In situ* hybridization revealed that its expression is confined to the proximal convoluted tubule (34) and it could be shown that primary cells obtained from this area are capable to produce AOPEN (66).

ACE is present in high amounts in kidney tissue (67-69) where it is located in blood vessels and in the brush border and basolateral membrane of proximal tubular cells (70-77).

Receptors for ANG II, mostly of the AT₁ subtype (78) and its mRNA (24, 25), have been described in the kidney (79, 80). They are localized on the mesangial cells and the basement membranes of the glomeruli, the tubular brush border and basolateral membranes and in the inner stripe of the outer medulla, where they are confined to the interstitial cells (81-87).

It was hypothesized that these RAS-components act together to regulate the sodium and water balance of the organism first intrarenally and only if this is not sufficient renin is released into the circulation and activates aldosterone secretion from the adrenal (88). After a decrease in the distal tubular sodium, chloride load, or fluid flow rate, the macula densa emits a signal, possibly nitric oxide (89), eliciting the release of renin from the juxtaglomerular cells into the lumen of the afferent arteriole and into the interstitium (32, 90-95). From the latter site, it diffuses to the anatomically close proximal tubular epithelium. There, high amounts of AOPEN and ACE are present and allow the generation of high local concentrations of ANG II (94, 96, 97) which is a potent stimulator of sodium absorption (98-103), hydrogen ion secretion followed by bicarbonate absorption (104, 105), and ammonia production (106, 107) in this part of the nephron. In addition, the renin released into the glomerular blood stream together with systemic AOPEN and endothelial ACE, forms ANG II, which contracts mesangial cells and the efferent arteriole thereby increasing the glomerular ultrafiltration coefficient (102, 108-113).

Furthermore, ANG II increases sodium reabsorption by a reduction of renal papillary blood flow probably elicited via contraction of the medullary interstitial cells (87, 114). The systemic circulation is protected from these fluctuations of ANG II concentrations by a very efficient clearing mechanism exerted by the kidney itself (115-117) leading to ANG II levels in the renal vein which are not higher than in the extrarenal plasma.

Locally produced ANG II may also be involved in adaptive processes during hypertension, heart failure and renal diseases accompanied by a reduction of renal mass. It induces hypertrophy of proximal tubular cells (118, 119), renal vascular smooth muscle and interstitial cells, accompanied by an increase in collagen deposition (120, 121). If being elicited inappropriately, these beneficial effects may injure the kidney leading to vascular lesions, interstitial fibrosis, and finally glomerular sclerosis. Evidence for these mechanisms is provided by experiments showing the positive effects of CEI and ANG II receptor antagonists on the progression of these diseases in animal models (122, 123) and patients, e.g. with diabetic nephropathy (124-127). The deleterious implications of a high protein diet in some renal diseases might also be transmitted via an increased activity of the intrarenal RAS (50, 128).

Furthermore, the chronic state of heart failure is associated with changes in renal function. It has been demonstrated that rats with mild heart failure do not respond to volume loading with an increased urinary excretion. Ichikawa *et al.* (129) found a decreased glomerular filtration rate in single nephrons and an increased perfusion resistance in rats suffering from heart failure. Although plasma level of renin and ANG II were within normal levels perfusion resistance and single nephron GFR were normalized after local administration of CEI into the renal artery, whereas in control rats no change of renal function was observed following administration of CEI. It has also been demonstrated that renal AOPEN mRNA levels were increased in rats with heart failure following experimental myocardial infarction (130). There was a positive correlation between the size of the infarction, which reflects the severity of heart failure and renal AOPEN mRNA, whereas transcript levels in the liver representing the main site of AOPEN production remained unchanged. The decrease in blood pressure after chronic administration of CEI correlated with renal ACE inhibition and not with ACE activity in the plasma (28, 131, 132). It was also shown that an increase of renal AOPEN mRNA in rats with heart failure was prevented by CEI (28).

Involvement of renal renin in hypertension: renal artery stenosis is the most pertinent example of renin-dependent secondary hypertension. In two kidney, one clip hypertension renal renin mRNA levels rise 4- to 6-fold in the clipped kidney whereas in the contralateral kidney renin mRNA is suppressed (133, 134). In the early phase of Goldblatt hypertension the increase in renal renin mRNA is paralleled by an elevation in plasma active renin, whereas in the chronic phase conflicting results are reported showing high or normal levels of renal renin and plasma renin and angiotensin levels (133, 135). The activation of local renin synthesis in the clipped kidney raises glomerular capillary hydraulic pressure and maintains glomerular filtration. This explains the episodes of acute renal failure induced by CEI in patients with bilateral renal artery stenosis or solitary kidneys (136). Intrarenal "Goldblatt" mechanisms with induction of renal renin synthesis due to structural vascular alterations and local ischemia have been implicated in the high prevalence of hypertension in renoparenchymatous diseases. In other forms of hypertension, which are not characterized by elevated plasma active renin levels, renin gene expression is also modulated in the kidneys. SHR exhibit a decrease in renal renin mRNA with the development of hypertension in comparison to age-matched Wistar-Kyoto (WKY) controls (137). However, stimulation by sodium-depletion or CEI caused a greater increase in renal renin gene expression in SHR than in WKY-rats even though no differences in plasma renin activity were noted (138). How these abnormalities in renal renin gene expression relate to the pathogenesis of hypertension in this models has not been clarified yet. Transgenic animal studies using gene constructs suitable to activate or deactivate local RAS in a

tissue-specific manner may be required to delineate the contribution of renal renin to hypertension.

Heart

The heart is one of the most sensitive target organs to the actions of ANG II. As the local production of AOPEN (22, 60, 139-141), renin (22, 142-144) and ACE (14, 23) mRNA, as well as the presence of ANG II receptors (145-148) was confirmed in rat and human myocardium, the existence of a local RAS in the heart was postulated (149-151).

The positive inotropic effect of ANG II was reported by several investigators (152). To distinguish between direct and indirect effects, which are primarily due to the modulation of the sympathetic nervous system, investigations applying β -adrenoceptor blockers (152-155), extrinsic cardiac denervation (147, 156), isolated cardiomyocyte preparations (157, 158), or isolated perfused hearts (159, 160) were performed. On the molecular level the inotropic effects of ANG II are apparently mediated by its influence of calcium homeostasis and transmembranous conductance. Several studies (155, 161) showed that ANG II changed the plateau phase of the cardiac action potential increasing its height and duration and leading to a rise in peak developed tension.

Moreover, ANG II may be involved in the development of cardiac hypertrophy. CEI prevent or decrease cardiac hypertrophy in animals (162, 163) and humans (164, 165) but these effects are accompanied by a change in the systemic hemodynamics. Several authors have studied the regulation of the cardiac RAS in left ventricular hypertrophy due to aortic banding. It has been demonstrated that aortic coarctation results in a significant upregulation of AOPEN and renin mRNA in the left ventricle, whereas no correlation existed with plasma renin levels which were only transiently increased after the constriction (166, 167). In addition, it has been demonstrated that ACE mRNA and the intracardiac conversion of ANG I to ANG II are increased in the presence of established pressure overload hypertrophy of the left ventricle of rat hearts (168).

Several studies have been performed in order to test the effect of CEI on left ventricular hypertrophy. ACE inhibition in experimental aortic banding significantly reduces and causes regression of left ventricular hypertrophy (169), even at subdepressor doses (170). In addition, after enalapril treatment, an upregulation of AOPEN and renin gene expression has been reported (166) suggesting a negative feedback of ANG II on the pathway. Furthermore, other models of left ventricular hypertrophy, like SHR or isoproterenol-infused rats, respond very sensitive to CEI by a reduction in left ventricular weight accompanied by a regression in myocardial fibrosis (171-174). These changes were not correlated with blood pressure reduction or plasma ANG II levels but with a fall in cardiac ANG II concentrations (173, 174).

The possible implication of the cardiac RAS has also been studied in experimental heart failure. Some authors have reported a transient increase of

AOPEN mRNA in myocardial infarction and failure (175). A doubling of AOPEN gene expression as compared to control rats has been described in ventricular myocardium during cardiac chronic tachypacing (176). In chronic heart failure induced by ligation of the left coronary artery in rats an enhancement of ACE mRNA was found, which correlated with the cardiac ACE activity, but not with its activity in other organs (177).

Therefore, the cardioprotective effects of CEI in the acutely ischemic or infarcted myocardium are not surprising. Studies on isolated heart preparations (159) demonstrated the prevention of post-ischemic dysrhythmias as well as improvement of coronary flow and ventricular function by administration of CEI. In this experimental model, intracardiac hormonal systems such as the RAS and the bradykinin system can be studied exclusively. Moreover, data demonstrating a reduction in infarction size after coronary artery ligation in dogs, treated with CEI (178), show that the area at risk and the infarcted region were smaller than expected from the finding that CEI improve the coronary flow. This effect may be due to an interaction with the myocardial angiotensin metabolism but also bradykinin seems to be involved, as this reduction was abolished by a bradykinin antagonist (160).

Several studies (159, 160, 179-182) demonstrated that CEI reduce the incidence and mean duration of malignant arrhythmias. This effect could be blunted by ANG II and bradykinin inhibitors, but not by ANG I. In addition, the observed reduction of norepinephrine overflow in ischemic hearts may be the result of an interaction between ANG II and the sympathetic nervous system.

An additional pathophysiological aspect in the postischemic phase of acute ischemia is the "stunned" myocardium. This phenomenon is defined as a transient contractile dysfunction in the peri-infarction area. Morphological changes appear in the extracellular collagen matrix connecting the cardiac myocytes. These may be elicited by collagenases activated by oxygen-derived free radicals, which are increasingly produced during reperfusion. The preservation of the intercellular collagen by some CEI may be caused by the free radical scavenging properties of the sulfhydryl group of some of these compounds (183, 184).

The pathomorphological changes after myocardial infarction are described in the term "ventricular remodeling" leading to progressive ventricular dilatation and eventually to pump failure. This dilatation is not necessarily associated with an increase of filling pressure, but frequently accompanied by a rightshift of the ventricular pressure-volume curve characteristic of a volume overload hypertrophy (185, 186). Histologically the ventricular remodeling appears as hypertrophy accompanied with a side-to-side slippage of the myocytes, which leads to an increase of the ventricular volume and a maintained or even reduced wall thickness (187, 188). In addition, myocardial fibrosis with changes of the extracellular matrix is reported (189). The beneficial effects of CEI consist of a decrease or prevention of

ventricular remodeling and leads to an improvement of the functional status as well as a reduction of the mortality in animals and in humans (190–192). The fact that CEI, in contrast to other vasodilators, prevent ventricular remodeling (192) may be caused by their direct influence on the local tissue RAS.

The results of the animal studies may not necessarily be applicable to the human system, *e.g.*, because of the presence of chymase, an ANG II generating enzyme not influenced by CEI (see above). Clinical trials of CEI enrolling patients with left ventricular dysfunction mostly after myocardial infarction gave controversial results (191, 193–198). Probably, the benefit of ACE inhibitors is dependent on the onset of treatment (197) and on the particular drug used as captopril with its sulfhydryl group in contrast to enalapril seems to have effects additional to the inhibition of the RAS, *i.e.* the scavenging of free radicals (see above). Early functions of systemic or locally produced ANG II in healing and adaptive growth response processes after myocardial infarction may be beneficial but if acting longterm may promote ventricular dilatation (191).

However, the importance of ACE already in the etiology of myocardial infarction is corroborated by a genetic study revealing specific alleles of this gene as risk factors for the disease in humans (8). The physiological causes for that, however, are still elusive and might imply the bradykinin system besides the local or the systemic RAS.

Vasculature

It is well known that chronic hypertension induces secondary structural changes of the vascular wall leading to an enhanced vasoconstrictor response to vasoactive substances such as ANG II. The activation of vascular smooth muscle cells by ANG II leads to the formation of two second messengers, inositol triphosphate (IP₃) and diacylglycerol. IP₃ rapidly mobilizes free calcium and thus initiates the calcium dependent and calmodulin activated phosphorylation of the myosin light chain resulting in cellular contraction, whereas diacylglycerol via protein kinase C is responsible for sustained tonic effects of vascular smooth muscle cells (199).

Since several autocrine- paracrine acting substances are generated in the vessel wall such as endothelin-1 or nitric oxide it was suggested that vascular ANG II may also exert paracrine functions. Biochemical and immunohistochemical studies revealed renin, AOPEN and ACE in the vasculature. Renin-like activity was found early in vessel homogenates (200). Interest in vascular renin was revived when renin-like activity in some resistance vessels was shown to persist up to 12 days after bilateral nephrectomy in dialyzed dogs (201). Vascular renin even increased in these nephrectomized animals following hemorrhage, as did plasma renin. Others found that renin in aortic wall has a much more prolonged half-life than plasma renin but decreases after total nephrectomy (202). However the presence of renin mRNA in vascular tissue is still a matter of debate. One report indicated the expres-

sion of the renin gene in aortic tissue (21), while this was not confirmed in other studies (203, 204).

The mRNA for AOPEN was detected in the media and adventitia of the rat aorta (205, 206). ACE mRNA was found in human aorta, saphenous vein as well as in the umbilical vein (23). In SHR, ACE activity in aorta, brachial and mesenteric vessels as well as in the portal vein was higher as compared to normotensive WKY, whereas plasma ACE activity in SHR was lower than in WKY (207). In cut strips of arteries from dogs contraction was induced following administration of ANG I. This effect was suppressed by CEI or ANG II antagonists (208). In addition, Andre *et al.* demonstrated that aortic smooth muscle cells are able to convert ANG I to ANG II (209). ANG II receptors are also expressed in the vasculature, and recent data have established the existence of the ANG II type 1 receptor mRNA in this tissue (25).

The components of the vascular RAS interact to form ANG I and II: Isolated perfused vascular beds release considerable amounts of these peptides (210–212). This release is greatly inhibited by CEI and stimulated by the perfusion with AOPEN or renin (212, 213). Angiotensin release and metabolism may be under regulatory influence of β -adrenergic tone (214) and adenosine (215) and may vary in different models of experimental hypertension (213, 216). More than half of ANG I during a single pass through a bloodless perfusion system is converted to ANG II of which about 50% is degraded or taken up (212). Furthermore, comparable results were obtained in CEI-treated humans infused with radiolabelled ANG I. At least half of the venous ANG I level was produced in the vasculature of these volunteers. Again, about 50% of arterial ANG I was taken up or degraded in the vasculature of the lower limbs during one circulation (217).

These findings confirm the hypothesis of an autocrine-paracrine regulated RAS, which may be involved in the regulation of vessel tone as well as in the development of adaptive changes secondary to hypertension. What is the evidence that the RAS has such functions in the vasculature *in vivo*? The trophic actions of ANG II were first described by Schelling *et al.* (218), who found that ANG II increased the cell number of cultured mouse fibroblasts. The effect was dose-dependent and could be blocked by the ANG II receptor antagonist, saralasin. These findings were later confirmed and expanded by other authors (for review see 219, 220). The stimulation of smooth muscle cells in culture by ANG II, however, had somewhat controversial effects. Whereas some authors also described hyperplastic effects of ANG II on vascular smooth muscle cells (221), other studies detected a hypertrophic response only (222, 223). This apparent discrepancy can possibly be explained by species variability and differences in the experimental conditions.

Further evidence that ANG II is involved in growth phenomena can be derived from experiments showing its stimulatory effect on proto-oncogenes in vascular smooth muscle cells. These

genes play a role in the development of the cardiovascular structure and are normally not expressed in mature cells but can be expressed again under pathological conditions such as cardiovascular hypertrophy (224). Indeed, several investigators (205, 225-228) were able to demonstrate that the peptide increased the expression of the proto-oncogenes *c-fos*, *c-myc*, *c-jun* as well as of the platelet derived growth factor (PDGF) in a dose-dependent manner. The direct link between ANG II and the hypertrophic changes was clearly demonstrated by experiments showing that antisense inhibition of *c-fos* (229) or PDGF (230) was able to block the stimulating effects of ANG II on DNA and protein synthesis in smooth muscle cells. Despite this, it should be pointed out that the studies were performed in cultured cells, and it remains to be determined whether or not there is an interaction between ANG II and proto-oncogene activation *in vivo* and in hypertension. The growth-promoting action is further supported by the recent finding that ACE-inhibition and ANG II receptor blockade blunts and ANG II infusion stimulates vascular smooth muscle proliferation after myointimal injury with a balloon catheter (231-236). However, a controversy exists which ANG II receptor subtype mediates these actions: While the growth-promoting effects on cultured vascular smooth muscle cells are inhibited by the AT₁-antagonist DuP753 (losartan) (237, 238), the neointima formation is blocked by the AT₂-specific drug CG42112A (236). The relevance of vascular ANG II production for the vascular hypertrophy accompanying hypertension and the mechanisms involved is still a matter of intensive research (239-241).

Transgenic Animals

A way to discriminate the effects caused by the RAS from those by other peptide systems is to modulate its activity exclusively by incorporating the gene of one of its components into the genome of a normal animal, *i.e.*, to produce a transgenic mouse or rat with one of the genes of the RAS. These animals enable investigators to study the effects of a single additional gene via a dysregulated RAS on the cardiovascular system. This is especially interesting when the gene is derived from humans in whom studies about the physiological effects of the RAS are difficult to perform. In addition, these animal model systems allow the testing of drugs that specifically interfere with the RAS like ANG II receptor antagonists and renin inhibitors, which are currently under intensive investigation. Two recently developed transgenic rat strains are described in the following sections.

TGR (mREN2) 27

The introduction of the *Ren-2* gene of the DBA/2 mouse into the genome of rats by microinjection techniques has led to the generation of the transgenic rat model TGR (mREN2) 27 (15). These rats develop fulminant hypertension, beginning at 4 weeks and reaching a maximum at 9 weeks of age.

The *Ren-2* transgene in TGR (mREN2) 27 is expressed most abundantly in adrenal gland followed by thymus, intestine, fat, female and male genital tract, kidney, brain, and pituitary (242-244). In adrenal gland, the expression pattern resembled that of female DBA/2 mice (245) as renin mRNA was found in two separate layers, the innermost region of the zona glomerulosa together with the outer zona fasciculata and patches of cells in the zona reticularis bordering the medulla (246). In a non-transgenic rat adrenal gland, renin gene expression is barely detectable indicating that the signal in TGR (mREN2) 27 is mainly derived from *Ren-2* transcripts. Tissue renin protein concentrations correspond to the mRNA measurements in being low in kidney and enhanced in adrenal gland indicating that the transgene mRNA is accurately translated (15).

These striking peculiarities of transgene expression have consequences for the physiology of TGR (mREN2) 27. Having in mind the decreased renin gene expression in the kidney, it is not surprising, that active renin concentration in the plasma of TGR (mREN2) 27 is lower than in control animals (15, 247). The same was true for nearly all other parameters of the RAS, like ANG I, ANG II and AOPEN, but not for prorenin. This precursor is highly enriched in plasma reaching more than 20-fold higher levels than in Sprague-Dawley (SD)-rats. Most probably, it is mainly derived from the adrenal glands as its concentration is lowered down to about 20% by bilateral adrenalectomy (246). In addition, it could be shown that isolated adrenal cells from TGR (mREN2) 27 secrete considerable amounts of active renin and prorenin (248). The physiological relevance of the high plasma prorenin levels especially in the development of hypertension is still subject of research. The fact, that blood pressure was reduced to nearly normotensive values after adrenalectomy (246) does not prove a causal relationship of transgene expression and prorenin secretion in the adrenal gland with the high blood pressure, as it can also be explained by the total elimination of steroid production.

ANG II is well known as stimulator of aldosterone synthesis in the adrenal gland (249, 250). With the concept of local acting tissue RAS also in the adrenal gland (251-258), the enhanced renin gene expression and activity in this organ of the transgenic rats may lead to an increased level of ANG II and in turn to an elevation of steroid, especially aldosterone, secretion. While adult hypertensive animals did not corroborate this hypothesis exhibiting only insignificantly increased urinary steroid concentrations (259) the excretion of deoxycorticosterone, corticosterone, and aldosterone was elevated in young animals during the development of hypertension 1.5 to 2.5-fold compared with SD-rats (259). When the animals were treated with adrenocorticotrophic hormone (ACTH), their urinary corticosterone excretion increased more pronounced than in SD-rats (259). In addition, the already very abundant plasma prorenin was further enhanced about 10-fold by this treatment (259). These data

indicate that there is an intimate interplay between ACTH, the intraadrenal RAS and steroid production by the adrenocortical cells in TGR (mREN2) 27. These interactions were studied in more detail *in vitro* with adrenal cells isolated from transgenic rat adrenals (248). They may well be relevant for the development of hypertension, however not, as one would expect, by the elevated mineralocorticoid levels, as the mineralocorticoid receptor antagonist spironolactone did not lower blood pressure in young TGR (mREN2) 27 (243). But the stimulated adrenal RAS is most probably involved in this process, as high dose dexamethasone treatment inhibiting ACTH and thereby adrenal steroid production blocks blood pressure elevation in young TGR (mREN2) 27 significantly (260). In contrast, the same dose of dexamethasone has a marked hypertensinogenic effect in adult SD-rats. In addition and in agreement with the ACTH experiment described above, prorenin in the plasma of TGR (mREN2) 27 is markedly downregulated by this treatment.

Taken together these data argue in favor of a causal effect of a stimulated adrenal RAS in the development of hypertension, however, via an up to now unknown mechanism including prorenin or steroids lacking mineralocorticoid action.

Interestingly, TGR (mREN2) 27 exhibit pronounced morphological alterations in kidney, heart, and vessels in the adult stage (246). In kidney, glomerular damage is evident which finally culminates in glomerulosclerosis being often the cause of early death especially in homozygous animals. The observed pathologies resemble tightly other experimental hypertension models and human disease states. Thus, TGR (mREN2) 27 represents a valid model for the secondary effects of sustained hypertension on the kidney morphology.

The same holds true for the cardiovascular alterations. The hearts and particularly their left ventricles are hypertrophied and focal perivascular fibrosis of the coronary arteries could be observed (246, 247). These and other muscular type arteries of the body including aorta exhibited an increased thickness of the tunica media based on the hypertrophy of vascular myocytes and a massive neogenesis of connective tissue matrix (247). As vessels exhibited detectable *Ren-2* gene expression in TGR (mREN2) 27 (247) an activated local tissue RAS in the vascular wall (see above) could be responsible for the hypertrophic effects. In support of this, it was observed that isolated perfused hindlimbs of the transgenic rats produce significant amounts of active renin (261).

As hypertension in TGR (mREN2) 27 is based on the expression of an additional renin gene in several tissues, CEI should have a marked anti-hypertensive effect. Indeed, Captopril (10 mg/kg body weight per day) lowered blood pressure of heterozygous (15) and homozygous (243) animals significantly after two weeks of treatment. Lisinopril turned out to be even more active in decreasing blood pressure markedly already at a dose of 0.5 mg/kg per day and normalising it with 2 and 10 mg/kg per day (262). This decrease in mean arterial

pressure is accompanied by an increase in active plasma renin and renin gene expression in kidney affecting both, the *Ren-2* transgene and the rat renin gene (262). This, again, shows that the depressed renin gene expression in the kidney is caused by either the high blood pressure or a negative feedback of ANG II based on an overactive intrarenal RAS. Preliminary data showing that the vasodilator Hydralazin reduces blood pressure without affecting renin gene expression in the kidney argue in favor of the latter hypothesis (M.L., unpublished observations).

CEI also influence the bradykinin system. In order to convincingly show that their inhibitory effect on ANG II synthesis is decisive, we treated the animals with the ANG II receptor type I antagonist losartan (243). Already 0.5 mg/kg per day had a slight blood pressure lowering effect and 10 mg/kg per day normalised it after 4 weeks of treatment. Again, plasma renin activity increased several fold revealing the stimulating effect of the treatment on kidney renin gene expression. Plasma prorenin concentration was not changed indicating that tissue RAS, especially in the adrenal gland might be regulated differently.

In conclusion, these treatments show that ANG II plays a crucial role in the hypertensinogenic process in TGR (mREN2) 27. Additionally, all available data exclude the plasma RAS as the relevant source of ANG II, but support local RAS in the adrenal gland and possibly other tissues as causative factors for the elevation of blood pressure and the accompanying phenomena. Therefore, TGR (mREN2) 27 is a valuable model to study the specific regulation of these tissue RAS and their role in blood pressure homeostasis, which seems to be more important also for human hypertension than previously thought as it might explain the long-term therapeutic effects of CEI on hypertensive patients (263). In addition, these transgenic rats allow the investigation of the sexual dimorphism in blood pressure observed also in humans. Finally and more basically, TGR (mREN2) 27 can be employed to study the tissue-specific regulation of renin genes.

TGR (hREN) and TGR (hAOPEN)

The particular species-specificity of the human renin substrate reaction prevents enzymatical cross-reaction of rat renin with human AOPEN or human renin with rat AOPEN. As a consequence, even though rats are unique models for the investigation of cardiovascular regulation, they cannot be used for the development and testing of drugs interfering specifically with the human RAS, e.g. renin inhibitors.

In order to study the gene expression of human renin and AOPEN and the species-specific kinetics of the human RAS as a model system for the testing of renin inhibitors, the entire human renin gene containing 3 kb of the 5'-flanking sequence, 10 exons, 9 introns, and 1.2 kb of the 3'-flanking sequence was microinjected into fertilized oocytes of SD-rats (17). From 87 rat eggs implanted, 15 progeny were obtained, of which 5 were found to carry

the renin transgene. Two of these founder animals, TGR (hREN) 1936 and TGR (hREN) 1988, transmitted the transgene to their offspring, and the line TGR (hREN) 1936 has been bred to homozygosity.

Human renin and prorenin, measured using a human-specific anti-renin antibody, was detected in the plasma of both TGR (hREN) lines. The enzymatic activity of the human renin in rat plasma as measured with a human substrate amounted to 18 ng ANG I/mlxh in TGR (hREN) 1988; this compares with about 1.5 ng ANG I/mlxh in human plasma. Rat prorenin, rat renin, ANG I, ANG II, and AOPEN levels were unchanged in TGR (hREN) animals as compared with SD rats, indicating that the human renin did not react with rat angiotensinogen to generate angiotensins. As could be expected, the mean systolic blood pressure in both TGR (hREN) lines was normal (133 ± 3 mmHg).

To demonstrate the response of human renin to physiological stimuli, TGR (hREN) 1936 animals were sodium depleted, raising their human active renin concentration from 4.8 pg/ml to 58.3 pg/ml. The rat renin was also stimulated, but there was no difference between animals with or without the transgene. These data clearly indicate that the transgene is transcribed and translated and the protein activated and secreted into the plasma in both lines. The specificity of human renin prevents reaction with the endogenous rat AOPEN, leaving the plasma ANG I and ANG II levels unchanged.

Transgene expression was studied by a human renin-specific RNase-protection assay. A high expression of human renin was found in the kidney, restricted to the juxtaglomerular cells followed by lung, thymus, and gastrointestinal tract in both transgenic lines.

In addition, transgenic rats were produced carrying the human AOPEN gene comprising 1.1 kb of 5'-flanking sequences, five exons, four introns and 2.4 kb of 3'-flanking sequences with an overall size of 16.3 kb (17, 264). From the founder animals which transmitted the transgene to their progeny, one line named TGR (hAOPEN) 1663 expressed and secreted human AOPEN into the plasma at a concentration of 465.3 ± 44.9 g/ml as measured by enzyme-linked immunosorbent assay using a human-specific monoclonal antibody. This exceeds the normal AOPEN levels measured in human plasma (265) as well as in rat plasma by approximately 7 times. The production of human AOPEN in the transgenic rats did not interfere with the level of rat plasma AOPEN, since no significant difference was found between transgenic and non-transgenic littermates.

As a reflection of the species-specificity of human AOPEN, similar levels of ANG II were demonstrated in TGR (hAOPEN) and control animals, indicating that rat renin does not cross-react with human AOPEN even in the presence of excess human substrate to form ANG I. Consequently, TGR (hAOPEN) are normotensive compared to transgene-negative controls.

However, the infusion of recombinant human renin rapidly raised the blood pressure in conscious,

unrestrained TGR (hAOPEN) by 38% from normotensive values up to 198 mmHg systolic. The blood pressure response was paralleled by a corresponding increase in ANG II levels. In the presence of the human-specific renin inhibitor, RO 425892, the hypertensive response to human renin infusions as well as the rise in plasma ANG II could be completely inhibited. This indicates that (a) the interaction of human renin with human AOPEN is the sole cause of ANG II formation, (b) the transgenic human AOPEN gene product shows functional integrity, and (c) human renin does not interact with rat AOPEN. When rat renin is infused in TGR (hAOPEN), eliciting an equipressor response compared with i.v. human renin, the addition of RO 425892 does not lower the blood pressure. Thus, in this case, the rise in blood pressure is due to the interaction of rat renin with rat AOPEN.

The TGR (hREN) and TGR (hAOPEN) are valid models for the investigation of the regulation of human RAS genes *in vivo*. Furthermore, since these transgenic rats are accessible to hemodynamic or endocrinological investigations, human-specific drugs may be tested without the limitations of a primate model.

Conclusions

The role of the RAS in hypertension and other cardiovascular diseases is becoming increasingly important. One of the most relevant aspects in this respect was the verification of local synthesis of its components in several organs using molecular biological techniques, which suggested the possibility of paracrine-autocrine functions of local RAS besides the classical hormonal RAS acting on an endocrine level. *In vitro* studies on isolated tissues such as vascular smooth muscle cells or cardiomyocytes demonstrated conversion of ANG I to ANG II and activation of intracellular signal transduction pathways confirming the hypothesis of paracrine functions. The local interaction with other neurosecretory systems, in particular the sympathetic nervous system represents an important aspect of the function of tissue RAS. ANG II also has potent direct effects on myocardial contractility and may have deleterious effects during ischemia and reperfusion. These findings support the view that structural changes occurring during the chronic state of hypertension such as cardiovascular hypertrophy are possibly mediated by local RAS. The possible physiological significance of locally generated ANG II was suggested indirectly by the antihypertensive and cardioprotective effect of CEI, which strongly supports the view that the RAS is substantially involved in the pathogenesis of cardiovascular disease.

The regulation of the genes of the RAS has been shown to be altered in several models of primary hypertension. However, the underlying mechanisms of the pathogenesis remained to be elucidated. The established transgenic animal model systems will be of great value to investigate the cardiovascular effects of the RAS in further detail. To achieve this goal, new model systems will be necessary, such as

transgenic rats with other genes of the RAS or animals, which overproduce genes of the RAS only in specific tissues. In addition, it will be useful to "switch off" the activity of these genes in animals via antisense inhibition (266) or homologous recombination in embryonic stem cells (267) in order to create new animal models which lack RAS activity partly or totally.

Despite this increasing importance, the RAS is only one of many systems involved in cardiovascular regulation and hypertension. The genetic studies are far from being complete, so that we may expect new interesting candidate genes, which point to other systems also involved in these processes. To evaluate their relevance for hypertension, one has to develop model systems, e.g. transgenic animals, for other peptides, such as endothelin, vasopressin or the atrial natriuretic factor (268).

However, one has to be very careful in transferring the results obtained in animals and cell cultures to the human situation since species differences are especially marked in cardiovascular regulation. If this is kept in mind, the described experimental approaches will be valuable tools in the investigation and treatment of primary hypertension.

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