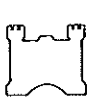
Structure and Regulation of Prorenin

ISBN 90-9012921-9, NUGI 743



Print: Offsetdrukkerij Ridderprint B.V., Ridderkerk

Structure and Regulation of Prorenin

(Structuur en Regulatie van Prorenine)

Proefschrift
ter Verkrijging van de Graad van Doctor
aan de Erasmus Universiteit Rotterdam
op Gezag van de Rector Magnificus
Prof. Dr. P.W.C. Akkermans M.A.
En volgens Besluit van het College voor Promoties

De Openbare Verdediging zal Plaatsvinden op
Woensdag 8 september 1999 om 15.45 uur
door
Jacob Deinum
Geboren te Ede

PROMOTOR: PROF. DR M.A.D.H. SCHALEKAMP

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Contents

1 Introduction

The treatment and prevention of cardiovascular disease is one of the triumphs of modern medicine but we have a long way to go before this success is completed. Heart attack and stroke are still common and, in the western world, cardiovascular disease remains the main cause of morbidity and mortality.

A major player in cardiovascular homeostasis is the renin-angiotensin system (RAS), and the growing knowledge of this system has led to the development of agents that specifically interact with components that are part of the RAS. 'Anti-RAS' drugs are now widely used in the management of hypertension, heart failure and diabetic nephropathy. However, as is true for cardiovascular medicine in general, many problems remain to be solved. Our understanding of how the RAS works and how to modify its actions is still far from complete.

One century ago Tigerstedt and Bergmann coined the name 'renin' for a hypertensive factor in rabbit kidney.[1] They showed that this factor was present in renal cortex and that it was secreted into renal venous blood. It was retained by dialysis membranes and sensitive to heat, which suggested its protein-nature. After these initial observations renin sank into oblivion for a few decades until interest flared up after the experiments by Goldblatt et al.[2] who showed that clamping a renal artery in a dog caused hypertension. They believed a humoral factor to be the hypertensive principle, which was shown to be renin by Pickering et al.[3] From then on unraveling of the structure of what nowadays is known as the RAS made steady progress, culminating in the cloning of the genes of its constituents (for a thorough review of renin's history see ref. 4).

The Renin-Angiotensin System and its Components

The RAS is a proteolytic cascade, connected to a signal-transduction system (Fig. 1) in which renin (EC 3.4.23.15) cleaves off the decapeptide angiotensin I (AngI) from the N-

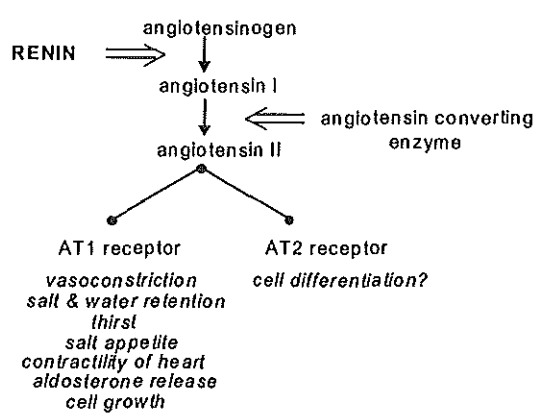


Figure 1. Schematic outline of the renin-angiotensin system. *Double arrow* indicates a proteolytic event.

terminal part of angiotensinogen. AngI is proteolytically converted to AngII by angiotensin converting enzyme (ACE). AngII acts through two kinds of receptors, the AT_1 and AT_2 receptor (AT1R and AT2R). Most effects of AngII are mediated through the AT1R. The AT2R is mainly expressed during ontogeny, and although no clear functions have been elucidated yet, it may be associated with cell differentiation and regeneration.[5,6]

Not shown in Fig. 1 is prorenin. It was discovered in 1971 when Lumbers found that amniotic fluid, left at low pH in the cold, gained renin activity.[7] Later a similar phenomenon was described in plasma by Skinner.[8] Acidification was not strictly necessary for activation, since incubation at low temperature also increased renin activity, albeit to only 15 percent of activity after acidification. Soon it was postulated that this inactive, but activatable 'big' renin -its molecular weight was 5 kD higher than that of renin- was the biosynthetic precursor of renin. Hence, it was named prorenin.[9] Only with the cloning of the renin gene in 1984,[10] prorenin was definitively proved to be the precursor of renin. In normal human plasma prorenin circulates at a level of on average 9 times that of renin. Prorenin has also been demonstrated in plasma and/or tissues of cat,[11] dog,[12] cattle,[13] pig,[13] horse,[13] sheep,[14] rabbit,[15] rat,[16] and mouse.[17]

Synthesis and Biochemistry of Renin and Prorenin

The renin gene, spanning 12 kb, is located on chromosome 1 and consists of 10 exons with a coding sequence of 1218 bases. The initial translation product of the renin gene is preprorenin, consisting of 406 amino acids. The signal, 'pre'-peptide of 23 amino acids targets the nascent protein into the endoplasmatic reticulum and is cleaved off upon entering.[18] Prorenin (M_r 47,000) enters either a regulated or a constitutive secretory pathway.[19] In the regulated pathway, which in humans has only been demonstrated in the juxtaglomerular cells of the afferent arterioles in the kidney, prorenin is converted proteolytically to renin (M_r 40,000). This process takes place in dense secretory granules where a processing enzyme cleaves off the 43 amino acid N-terminal prosegment.[19] Release of stored renin from these secretory granules occurs after appropriate stimuli (see below). In the constitutive pathway, which is present in the kidney as well as at extrarenal sites of renin gene expression, there is a continuous flow of prorenin to the cell membrane and release into the extracellular space.[19]

Renin and prorenin are glycoproteins and belong to the class of aspartic proteases. These are characterized by two aspartic residues in their active center, which is located in a cleft between two homologous lobes.[20] Aspartic proteases are secreted as zymogens, which have an N-terminal propeptide obstructing the active site-cleft, thereby inhibiting enzymatic activity. Well-known examples of aspartic proteases are pepsin, cathepsin D, chymosin and HIV protease.

Proteolysis of angiotensinogen by renin is a first-order, one-substrate reaction. Angiotensinogen concentration in human plasma is about 1500 nM,[21] which is near the Michaelis constant. This means that AngI and AngII generation are determined not only by the renin concentration but also by angiotensinogen concentration.

Prorenin is proteolytically converted to renin *in vitro* by the addition of proteases like trypsin or plasmin. In plasma the conversion is caused by endogenous kallikrein that is generated from prekallikrein after destruction of the natural inhibitors of contact activation of the coagulation cascade by exposure of plasma to low pH or to low temperature.[22] This highly interesting *in-vitro* connection between the coagulation and renin-angiotensinogen system, however, has not been demonstrated to operate *in vivo*, although patients with prekallikrein deficiency have an increased prorenin-to-renin ratio in their plasma.[23] Purified prorenin from either amniotic fluid or plasma, or recombinant prorenin may also be activated by exposure to low pH (acid-activation) or to cold (cryo-activation), but in this case the activation is non-proteolytic and rapidly reversible during incubation at neutral pH and 37 °C.[24] It is assumed that this activation is caused by a conformational change of the molecule by which the propeptide moves out of the enzymatic cleft, thus permitting entry of the scissile bond of angiotensinogen into the catalytic center.[21]

Regulation of Renin and Prorenin in Health and Disease

Renin secretion by the kidney is determined by various factors. First, there is a baroreceptor mechanism in the afferent artery that stimulates renin secretion from juxtaglomerular cells in case of low perfusion pressure.[25] Second, the delivery of sodium chloride to the distal tubule is sensed in the macula densa; low levels stimulate renin secretion, high levels inhibit.[26] The third factor is β_1-adrenoreceptor-mediated: stimulation causes renin release, inhibition decreases renin output.[27] Humoral factors may also influence renin secretion. The most important is AngII itself, which inhibits renin secretion.[28] A summary of various clinical conditions and pharmacological interventions that may change renin secretion can be found in **Table 1**.

Many of these interventions cause increases in plasma renin within minutes. Because prorenin is not stored, but secreted continuously by the kidney, it can not respond to acute stimuli.[29] Only when a stimulus persists will prorenin levels rise. Most stimuli that acutely raise renin secretion will also raise prorenin levels in the long run, probably through increased gene transcription.

Fig. 2 shows renin/total renin ratios as a function of total renin in various chronic clinical conditions. It appears that total renin is determined by the underlying stimulus and that the conversion of prorenin to renin increases with increasing total renin levels. There are some exceptions to the rule, like pregnancy and diabetes mellitus with microvascular complications. Also subjects with prekallikrein deficiency (own observation),[23] or heterozygous for a renin gene mutation causing a premature stop codon[30] have relatively high prorenin levels. There is no clear explanation for this phenomenon. One can hypothesize that as long as the renin and total renin levels conform to a curve defined by points 1 through 9 in Fig. 2, the kidney is responding physiologically to a disturbance of homeostasis, through one of the aforementioned regulatory mechanisms of renin and prorenin release. If the renin/total renin ratio is off the line, there may be some disturbance in the renin and prorenin synthesis machinery, probably at the level of extrarenal renin gene transcription or intrarenal prorenin-renin conversion.

Table 1. Clinical conditions affecting plasma renin levels.

Stimulus to juxtaglomerular cell	Decreased plasma renin	Increased plasma renin
↑or ↓ perfusion pressure	sodium loads mineralocorticoid excess liddle's syndrome Cushing's syndrome	renovascular hypertension malignant hypertension cirrhosis with ascites hemorrhage diuretics gastrointestinal loss upright posture heart failure nephrotic syndrome
↑or ↓ sodium delivery to macula densa	sodium loads	sodium restriction
β_1 stimulation or inhibition	autonomic dysfunction beta blockade adrenergic neuronal blockade	direct vasodilator therapy pheochromocytoma hypoglycemia hyperthyroidism sympathicomimetic agents
Angiotensin II		ACE inhibitor angiotensin II receptor antagonist
Unknown	hyporeninemic hypoaldosteronism low-renin hypertension	acute glomerulonephritis high-renin hypertension

Prorenin as Pro-enzyme or Prohormone

Prorenin is a most unusual precursor in that no evidence exists that it is activated to renin in the circulation. Other proteolytic enzymes also circulate in a precursor form, a good example being coagulation factors, which belong to the group of serine proteases. But in contrast with prorenin, coagulation factors are activated continuously at sites where a trigger is present. The concentration of circulating activated coagulation proteases is always very low, much lower relative to their precursor than the concentration of renin relative to that of prorenin. The activity of coagulation factors is also heavily restrained by the presence of inhibitors, whereas for renin no circulating inhibitor is known to exist. If one considers renin as a hormone and prorenin as a prohormone (see below), there is a peculiarity that contrasts with the proenzyme/enzyme relationship of circulating serine proteases. Where prorenin levels relative to renin levels appear not high enough to regard prorenin as a typical circulating protease precursor, they are not low enough for a typical prohormone. Peptide prohormones usually circulate at lower levels than their active

counterpart. For instance, insulin circulates together with its precursor proinsulin, but proinsulin concentration is at most 70% of insulin concentration. Moreover, proinsulin has some insulin-like action of its own, in contrast with prorenin, which has not (yet) been shown to possess any activity in the circulation.

Although prorenin's role as the renin precursor in the kidney is clear, its function, if any, in the circulation or at other sites remains an enigma despite an estimated one thousand[31] published papers.

Thinking on prorenin can be facilitated when we are aware of paradigms that were developed for the RAS and aspartic proteases. I shall discuss these first before elaborating on hypotheses concerning the role of prorenin.

The Paradigm of (Pro)Renin as an Aspartic Protease

Although the structure of prorenin has not been determined directly by X-ray diffraction analysis, it is very likely from computer simulation studies that it has the geometry common to other aspartic protease zymogens (pro-enzymes).[32,33] The single polypeptide chain is arranged in two homologous lobes with a deep cleft in between, which contains the active site. This cleft is covered by a propeptide, which is the N-terminal part of the polypeptide chain and blocks the access of substrate and hence renders the molecule enzymatically inactive.[34]

Aspartic proteases have low substrate specificity, are inhibited by the statin-containing peptide pepstatin and usually have a low pH optimum (< 3). They are synthetized as zymogens and are then transported to a site with low pH. At this low pH, the propeptide

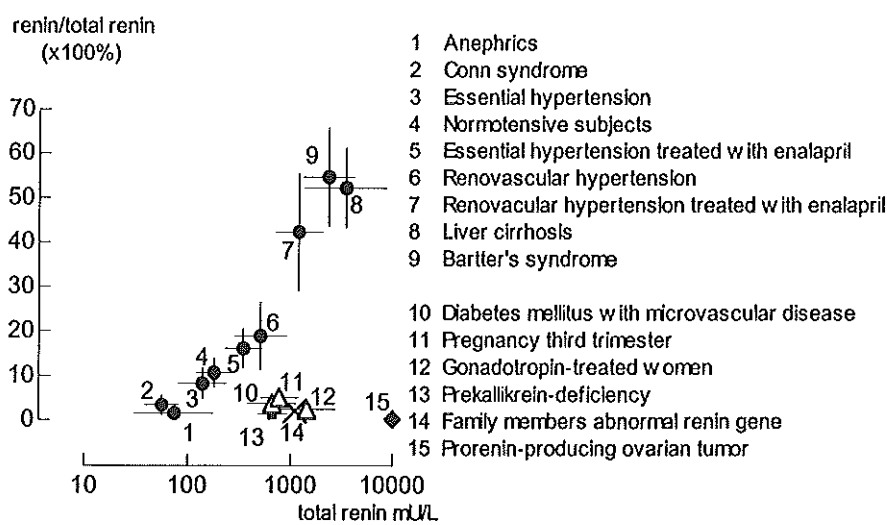


Figure 2. Relationship between renin/total renin ratio (*ordinate*, mean±SD) and total renin (=renin+prorenin) (*abscissa*, geometric mean, 95% CI) in various clinical conditions. Data kindly provided by F. Derkx, published in: Deinum et al, Textbook of Hypertension, Eds Weber&Oparil, WB Saunders, 1999, Ch. 7,with permission.

unfolds and is subsequently cleaved off by intra- or intermolecular proteolysis.[20] In view of the low substrate specificity, the secretion as zymogen, the activation at low pH and the low pH optimum can be seen as effective safeguards against inappropriate proteolysis at the physiological pH of blood. For instance, pepsinogen secreted in the stomach is only activated when it reaches the acidic environment of the stomach. Likewise, procathepsin D will not become enzymatically active until it reaches the acidic pre-lysosomes.

Despite its similar structure, prorenin does not fit in nicely with the picture of an aspartic protease. First, there is no evidence that prorenin is capable of auto-activation. This is explained by the exclusive dedication of the active site of the renin molecule to one substrate, angiotensinogen. This mono-specificity is another deviation of the general aspartic protease scheme. In humans, angiotensinogen is cleaved at the fairly high pH optimum of 5.6, with still 50% activity at pH 7.4.[35] This relatively high pH optimum is a third difference with other aspartic proteases. Furthermore, transport is not to the exterior (like pepsinogen) or to intracellular organelles like lysosomes (cathepsin D), but to the blood. Finally, part of prorenin, after its synthesis in the kidney in specialized cells, is activated in these cells to renin, before it is secreted into the blood. This is unusual for extracellular aspartic proteases and, for that matter, as mentioned above, unusual for any circulating proteolytic enzyme.

Although prorenin appears to be an exception among the aspartic protease zymogens, one wonders whether it has retained some of the typical features of this class of proteolytic enzymes. For instance, is prorenin secreted into the blood in order to be transported to its site of action, presumably outside the intravascular compartment, where it is then activated to renin? Does prorenin assume an enzymatically active conformation *in vivo* by unfolding of its prosegment, like other aspartic proteases do at low pH? Is its site of action a place where pH is closer to the renin pH optimum and where spontaneous activation may take place?

The Paradigms of Local Tissue Renin-Angiotensin Systems

Classically the renin-angiotensin system is seen as an endocrine system. Circulating renin splices AngI from angiotensinogen and AngI is converted to AngII by ACE in the pulmonary circulation. AngII is then transported to its target sites. Although *strictu sensu* renin is not a hormone, it can be considered as such, because its concentration is rate-determining in AngII generation and subject to tight control. Hence, according to this concept, the level of renin in plasma is thought to be an adequate measure of RAS activity.

In the past years another concept has emerged in which so-called local or tissue RASs are postulated. The essence of this paradigm is that these systems produce AngII outside the circulating blood and that AngII acts in a paracrine or autocrine way, *i.e.* binds to receptors on neighbor cells or on the very cell where AngII is formed. The concept is based on several observations. Pharmacologic interference with the RAS by renin inhibitor causes changes in blood pressure that in magnitude and duration are discrepant with the concentration of plasma AngII or with plasma renin activity,[36,37] which is well explained

by a selective interference of the renin inhibitor with tissue RASs. More direct evidence for the existence of RASs is provided by measurements of tissue concentrations of RAS components showing that these concentrations are too high to be accounted for by plasma contamination and by demonstration of messenger RNA of components of the RAS.[38] This evidence supports the hypothesis that a local RAS is not only present in the kidney but also in some extrarenal tissues like brain, adrenal gland and gonads.[38,39] An exhaustive summary of the evidence for local RASs can be found in ref. 40. In these organs there is evidence of local production of prorenin and possibly renin, and this raises the possibility that angiotensin formation in those tissues is regulated independently of kidney renin.

The paradigm of kidney-independent RASs has been extended to the heart and blood vessels but renin mRNA is very low in these tissues. Extensive studies in humans and in pigs by Admiraal and Danser et al.[41] using systemic infusions of radiolabeled AngI and II and measurements of the blood plasma levels of both radiolabeled and endogenous angiotensins, in different regional vascular beds, demonstrate that part of AngI in the circulation is indeed produced at tissue sites and that this locally produced AngI makes a significant contribution to the circulatory level of AngII. However, the local angiotensin production appeared to be dependent on kidney-derived renin. Similar studies in pigs, but now with measurements of tissue levels of radiolabeled and endogenous angiotensins, showed that most of the AngII present in cardiac tissue is not derived from the circulation but is produced in the tissue and this locally produced AngII is derived from AngI that is also produced in the tissues.[42] It is therefore possible that a significant proportion of the cardiac AT-receptors are activated by this *in-situ* produced AngII, rather than by AngII from the circulation. The cardiac production of angiotensins, however, depended on kidney-derived renin; after bilateral nephrectomy the tissue levels of AngI and II fell to undetectably low levels.[43] In human hearts, both under normal and pathological conditions, the tissue levels of renin were directly correlated with the levels in blood.[44]

The fact that the angiotensin formation in cardiac tissue depends on kidney-derived renin does not mean that changes in circulating AngII always lead to parallel changes in tissue AngII. There is evidence to suggest that most of locally generated tissue AngII that is derived from *in-situ* produced tissue AngI, does not enter the circulation. Experiments in pigs showed that suppressed production of plasma AngII following treatment with the ACE-inhibitor captopril, had little effect on the level of AngII in cardiac tissue.[42]

Hypotheses Concerning Prorenin

Various hypotheses have been put forward to provide a role for prorenin and, hence, to justify its existence.[31,45] Most of these hypotheses remain within the framework of the aforementioned paradigms. They are summarized in **Table 2**. Some of them are quite obvious, others are highly speculative.

What evidence do we have to prove or disprove any of these hypotheses?

Let us first consider observational data. Anephric subjects have very low, although not zero,[46] circulating levels of renin, AngI and AngII in the presence of near-normal levels of prorenin and high levels of angiotensinogen.[47] This indicates that prorenin is

Table 2. Hypotheses concerning prorenin

		Reference
1	Only the kidney processes prorenin to renin	45
2	Prorenin is a source of renin, by conversion to renin in the circulation	31
3	Prorenin could play a direct role, not mediated by renin and not necessarily related to fluid, electrolyte and blood pressure homeostasis	31
4	Prorenin can mimic the function of renin in vivo without cleavage of its prosegment	43
5	Prorenin could be the transporter of potential renin activity to various target organs, where it acts directly	31
6	Circulating prorenin is a "spill-over" from renal and (or) extrarenal sources, where renin or prorenin functions within those tissues or organs and not in the circulation	31
7	The tissue renin systems function via prorenin	43
8	Circulating renin and prorenin may bind to tissue by specific binding sites.	45
9	Prorenin may displace renin from binding sites and thus decrease local renin activity	54
10	Prorenin is an idiosyncratic by-product of renin synthesis and, in fact, redundant, and not worthy of any further study	

not activated in the circulation and, hence, hypothesis (2) in Table 2 is unlikely. Hypothesis (1) may be restated as: circulating renin is derived from the kidney.

Experimental evidence against hypothesis (2) has also been obtained from infusion studies of prorenin and from observations in transgenic animals. Short-term infusion of recombinant human prorenin in monkeys did not lead to higher renin, AngI or AngII levels.[48, 49] If any change was observed, it was a slight decrease in blood pressure. This argues against hypothesis (2). On the other hand, infusion experiments of radio-labeled prorenin showed binding of prorenin in the liver and kidney and conversion to renin.[50] This argues against hypothesis (1). Binding of prorenin to tissues or cells has been demonstrated,[51-53] although not necessarily to cells derived from kidney or liver. Activation of prorenin to renin by extrarenal cells has also been confirmed.[52] The physiological significance of renin binding was investigated in an experiment by Hu et al. who infused either renin or Ang II in rats to the extent that both regimens yielded identical plasma levels of Ang II.[54] If local renin activity after renin binding would be important renin infusion would be expected to have additional hemodynamic and hormonal effects. This appeared not to be the case. This argues against hypothesis (9). Hypothesis (9) was also challenged in a study by Muller et al. who infused human prorenin and renin in a rat, transgenic for human angiotensinogen.[55] Infusion of prorenin alone or in combination with renin did not affect blood pressure or Ang II levels.

An interesting transgenic rat model has been developed by Mullins et al.[56] They inserted the murine *Ren*2-renin gene in the genome of a rat strain. Transgenic rats developed an AngII-dependent hypertension with grossly elevated plasma prorenin levels. The renin gene was overexpressed in adrenals, but not in kidney. Although the mechanism by which hypertension is caused in these rats is still elusive, the model raises the possibility

that renin or perhaps prorenin may cause hypertension independently of the plasma RAS. On the other hand, transgenic rats, overexpressing rat prorenin in the liver, showed normal blood pressure. In spite of the absence of hypertension, however, cardiomegaly and severe renal lesions were present.[57] Plasma prorenin levels in these transgenic rats are increased 400-fold. This suggests that (circulating?) prorenin has a role in the development of cardiac and renal pathology and refutes hypothesis (10). To my knowledge, animals whose renin gene has been knocked out have not been constructed, but then, would any change observed in such an animal be due to the absence of renin or the absence of prorenin or both?

The picture that emerges is that prorenin is unlikely to play a role in the circulation, but may have a role in local RASs.

Studies that appear to refute definitively one hypothesis or the other are scarce. The main obstacle is probably the fact that it is nearly impossible to study prorenin in animals in the absence of renin. Any change in the level of prorenin is usually accompanied by a change in the level of renin . Infusion studies as mentioned above were performed with heterologous prorenin and renin, and this leads to suppression of endogenous prorenin and renin. Results may therefore not be extrapolated to the physiology of homologous, endogenous prorenin. Furthermore, in humans, prorenin and renin may only be studied in blood, whereas it may well be that tissue concentrations are more important. Osmond et al. have pointed out that another and main obstacle to further progress is the difficulty in measuring prorenin.[31] I shall now first address this issue.

Measurements of Renin and Prorenin

Measurement of prorenin is intimately linked to how renin is measured. In contrast with many other proteins, there is no generally accepted standard method to determine renin. It is therefore no surprise that the question of which method to use for renin measurement has been subject to hot debate,[58] and it still is.

The most widely employed way to measure renin is through its enzymatic activity. This indirect way of quantification is performed by incubating renin with its substrate. AngI is then quantitated by radioimmunoassay and this gives an estimate of the amount of renin.

Many groups advocate the use of the plasma renin activity (PRA) assay which uses the endogenous angiotensinogen, present in the plasma sample, as substrate. In most plasma samples the concentration of endogenous angiotensinogen is close to the K_m of the renin-substrate reaction, so that results of the PRA assy are determined by the plasma concentration of renin as well as by that of angiotensinogen. Only when the concentration of endogenous angiotensinogen is elevated, as is the case in pregnancy, following the use of oral contraceptives or a high dose of glucocorticoid, and in hyperthyroidism, PRA results are mainly determined by the plasma concentration of renin. PRA usually correlates well with the asssay of PRC (plasma renin concentration), in which exogenous angiotensinogen is added in order to obtain zero-order kinetics of AngI generation. Both in the PRA and PRC assays, the AngI-to-AngII converting activity and the AngI-

and AngII-degrading activity have to be removed from the plasma sample before the AngI-generating step. This is done by methods that destroy these enzymes or remove them from plasma or by the addition of the appropriate enzyme inhibitors.

Prorenin cannot be measured via its enzymatic activity, but must be converted to renin by limited proteolysis. This can be achieved by either trypsin or by acidification of plasma to pH 3.3 and subsequent incubation at 4°C and pH 7.4. The difference in enzymatic activity before and after activation is then a measure of prorenin concentration. These enzymatic methods have several drawbacks. First, proteolytic activation may not be complete, which leads to underestimation of prorenin. Second, the proteolysis may be too extensive and lead to destruction of renin and prorenin and hence to underestimation of prorenin. Third, in some assays, acidification of plasma has been used to destroy angiotensin-degrading enzyme activity. At the time such assays were performed, it was not known that plasma contained prorenin and that prorenin was activated by acidification. The use of an acidification step in assays of plasma renin leads to the co-measurement of prorenin, and this has been the cause of much confusion. For instance, there has been controversy about whether or not plasma renin is reduced following treatment of hypertension with b-adrenoceptor blocker, whether or not renin suppression contributes to the decrease in blood pressure. Eventually, with the use of better assays that do not co-measure prorenin, it has been established that beta-blocker treatment lowers plasma renin effectively, whereas prorenin is unaltered or elevated.[59] Finally, it has been described that, during the proteolytic activation step in rat plasma, trypsin may splice the N-terminal 14 amino acid-peptide from angiotensinogen, which then acts as a substrate for renin.[60]

Recently, direct immunoradiometric assays (IRMAs) specific for human renin have become available.[61, 62] These offer speed and reliability and can be routinely standardized. These IRMAs, however, are somewhat less sensitive and probably less suitable in low renin states.[58] Another problem of the IRMAs may be that a small percentage of prorenin is co-measured as renin in these assays. Because of the relatively high prorenin levels, this may influence the results, especially in plasmas with a low renin level.[62] These assays, however, are well suited for most clinical purposes.[62] Prorenin can also be measured by these IRMAs after the proteolytic conversion of prorenin to renin. This, however, is associated with the same potential problems as encountered in the assays that rely on renin's enzymatic activity.

It has been argued, probably rightly so, that the lack of standardization is one of the reasons why still so much remains to be discovered about the renin-angiotensin system.[#] [63] A WHO renin standard is available,[64] established in 1974, derived from human kidney

This has been pointed out eloquently by Sir George Pickering. At a meeting in 1963 he said: ..Another method is the hillbilly method. You take the unknown in one hand and put stones in a bucket in the other hand until the bucket feels the same weight as the unknown. Then you guess the weight of the stones. Sometimes the methods used to assay renin and angiotensin seem to me to be very much like the hillbilly method. Even today we have no agreed unit for the assay of renin. Many workers do not even use a standard. You cannot compare results from day to day, from one laboratory to another, from country to country...

and defined by its hypertensive action in a bioassay. It seems that efforts have been made to establish a purer standard,[65] based on recombinant human renin. However, little progress has been made, and it is impossible or at least hazardous at present to compare renin values obtained in one laboratory with those from another.

Scope of the Thesis

As pointed out above, it is still very difficult to verify or falsify hypotheses concerning prorenin's physiology by rigorous experiments. We have to rely on observational studies. This thesis is no exception to the rule and in a largely phenomenologic approach we will adress three issues, namely reversible activation of prorenin and its implications for measurement methodology, deranged plasma levels of prorenin in diabetes mellitus and finally tissue levels of prorenin.

Chapter 2 is concerned with structural aspects of prorenin during its non-proteolytic activation. What are the characteristics of prorenin activation *in vitro* and can we generate from these characteristics a hypothesis of how prorenin activation occurs *in vivo*? Chapter 3 is concerned with the methodology of renin and prorenin measurements. The observations described in chapter 2 led to new assays of renin and prorenin, which lack some of the drawbacks of older methods, and may set a new, so much needed, standard in renin and prorenin measurement methodology.

Certain pathological states in which prorenin regulation is altered, may provide clues to the physiology of prorenin. Diabetes mellitus, complicated by microvascular complications is such a condition (Fig. 2). In chapter 4 we studied how plasma prorenin is related in time to the development of microalbuminuria (chapter 4.1). Since the development of albuminuria in diabetes mellitus probably is also determined by genetic factors we investigated whether renin gene variants are associated with either the development of nephropathy or plasma prorenin levels (chapter 4.2). Thirdly, we studied prorenin in tissues. Observations in pathological human eye fluids (chapter 5.1) were corroborated by prorenin and renin measurements in normal bovine eyes (chapter 5.2).

References

1 Tigerstedt R, Bergmann PG. Niere und kreislauf. Scand Arch Physiol 1898;8:223-71.
2 Goldblatt H, Lynch J, Hanzal RF et al. Studies on experimental hypertension. I. The production of persistent elevation of systolic blood pressure by means of renal ischemia. J Exp Med 1934;59:347-79.
3 Pickering GW, Prinzmetal M. Some observations on renin, a pressor substance contained in normal kidney, together with a method for its biological assay. Clin Sci 1938;3:211-27.
4 Peart WS. Evolution of renin. Hypertension 1991;18[suppl III]:III-100-III-108.
5 Unger T, Chung O, Csikos T, Culman J, Gallinat S, Gohlke P, Hohle S, Meffert S, Stoll M, Stroth U, Zhu YZ. Angiotensin receptors. J Hypertension Suppl 1996;14:S95-S103.
6 Griendling K, Lassègue B, Alexander W. Angiotensin receptors and their therapeutic implications. Annu Rev Pharmacol Toxicol 1996;36:281-306.
7 Lumbers ER. Activation of renin in amniotic fluid by low pH. Enzymologia 1971;40:329-36.
8 Skinner SL, Cran EJ, Gibson R, Taylor R, Walters WA, Catt KJ. Angiotensin I and II, active and inactive renin, renin substrate, renin activity and angiotensinase in human liquor amnii and plasma. Am J Obstetr Gynaecol 1975;121: 626-30.
9 Sealey JE, Laragh JH. "Prorenin" in human plasma? Circ Res 1975;36 Suppl I:I-10-I-16.

10 Hobart PM, Fogliano M, O'Connor BA, Schaefer IM, Chirgwin JM. Human renin gene: structure and sequence analysis. Proc. Natrl. Acad. Sci USA 1984;81:5026-30.
11 Glorioso N, Troffa C, Laragh JH, Atlas SA, Marion D, Sealey JE. The cat: an animal model for studies of inactive renin. Am J Physiol 1987;252:E509-18.
12 Wilczynski EA, Osmond DH. Plasma prorenin in humans and dogs. Species differences and furtherevidence of a systemic activation cascade. Hypertension 1983;5:277-85.
13 Nielsen AH, Gotfredsen P, Nielsen PB, Hyttel P, Poulsen K. Measurement of renin and prorenin in cattle, hog and horse. Comp Biochem Physiol A 1991;100:127-31.
14 Stanley JR, Giammattei CE, Sheikh AU, Green JL, Zehnder T, Rose JC. Effects of chroniv infusion of angiotensin II on renin and blood pressure in the late-gestation fetal sheep. Am J Obstet Gynecol 1997;176: 931-37.
15 Osmond DH, Cooper RM. Cryoactivation and tryptic activation of blood 'prorenin' in normal man and animals.Can J Physiol Pharmacol 1978 ;56:792-805.
16 Barrett JD, Eggena P, Sambhi MP. Activation of rat plasma renin. Endocrinology 1981 108:778-85.
17 Morris BJ. Molecular weights of renin and inactive renin in mouse plasma. J Hypertens 1987;5:633-5.
18 Morris BJ. Molecular biology of renin. I: gene and protein structure, synthesis and processing. J Hypertens 1992;10:209-14.
19 Hsueh WA, Baxter JD. Human Prorenin. Hypertension 1991;17:469-79.
20 Szecsi PB. The aspartic proteases. Scand J Clin Lab Invest 1992;52 (Suppl 210):5-22.
21 Derkx FHM, Stuenkel C, Schalekamp MPA Visser W, Huisveld IH, Schalekamp MA. Immunoreactive renin, prorenin and enzymatically active renin in plasma during pregnancy, and in women taking contraceptives. J Clin Endocrinol Metab 1986;63:1008-15.
22 Derkx FHM, Bouma BN, Schalekamp MADH. Prorenin-renin conversion by the contact-activation system in plasma: role of plasma protease inhibitors. J Lab Clin Med 1984;103:560-67.
23 Leckie BJ, Morton JJ Relation between renin and prorenin in plasma from hypertensive patients and normal people: evidence for different renin:prorenin ratios. J Hum Hypertens 1995;9:493-6.
24 Derkx FHM Schalekamp MPA, Schalekamp MADH. Prorenin-renin conversion. Isolation of an intermediary form of activated prorenin. J Biol Chem 1987;262:2472-2477.
25 Skinner SI, McCubbin JW, Page IH. Renal baroreceptor control of acute renin release in normotensive, nephrogenic and neurogenic hypertensive dogs. Circ Res 1964;15:522-631.
26 Vander AJ. Control of renin release. Physiol Rev 1967;47:359-82.
27 Vander AJ. Effect of catecholamines and the renal nerves on renin secretion in anesthetized dogs. Am J Physiol 1965;209:659-62.
28 Vander AJ, Geelhoed GW. Inhibition of renin secretion by angiotensin II. Proc Soc Exp Biol Med 1965;120:399-403.
29 Katz SA, Malvin RL. Renin secretion:control, pathways and glycosylation. In Robertson JIS, Nicholls MG (Eds): The renin-angiotensin system, vol I, p24.10. London. Gower Medical Publishing, 1993.
30 Villard E, Lalau JD, van Hooft IS, Derkx FH, Houot AM, Pinet F, Corvol P, Soubrier F. A mutant renin gene in familial elevation of prorenin. J Biol Chem 1994;269:30307-12.
31 Osmond DH, Sealey JE, McKenzie JK. Activation and function of prorenin: different viewpoints. Can J Physiol Pharmacol 1991;69:1308-14.
32 Shiratori Y, Nakagawa S, Hori H, Murakami K, Umeyama H. Protein modeling of human prorenin using the molecular dynamics method. J. Mol. Graophics 1990;8:163-68.
33 James MNG, Sielecki AR. Molecular structure of an aspartic proteinase zymogen, porcine pepsinogen, at 1.8 Å resolution. Nature 1986;319:33-8.
34 Tang J, Wong RNS. Evolution in the structure and function of aspartic proteases. J Cell Biochem 1987;33:53-63.
35 Derkx FHM, Tan-Tjiong HL, Wenting GJ, Boomsma F, Man in't Veld AJ, Schalekamp MADH. Asynchronous changes in prorenin and renin secretion after captopril in patients with renal artery stenosis. Hypertension 1983;5:244-56.

36 Meiracker AH, Admiraal PJJ, Man in't Veld AJ, Derkx FH, Ritsema van Eck HJ, Mulder P, van Brummelen P, Schalekamp MADH. Prolonged blood pressure reduction by orally active renin inhibitor RO 42-5892 in essential hypertension. Br Med J 1990;301:205-10.

37 Derkx FHM, van den Meiracker AH, Fischli W, Admiraal PJ, Man in't Veld AJ, van Brummelen P, Schalekamp MADH. Nonparallel effects of renin inhibitor treatment on plasma renin activity and angiotensins I and II in hypertensive subjects. An assay-related artifact. Am J Hypertens 1991;4:602-9.

38 Deschepper CF, Mellon SH, Cumin F, Baxter JD, Ganong WF. Analysis by immunocytochemistry and in situ hybridization of renin and its mRNA in kidney, testis, adrenal and pituitary of the rat. Proc Natl Acad Sci USA 1986;83:7552-56.

39 Wagner J, Danser AHJ, Derkx FHM, de Jong PTVM, Mullins JJ, Schalekamp MADH, Ganten D. Demonstration of renin mRNA, angiotensinogen mRNA and angiotensin converting enzyme mRNA expression in the human eye: evidence for an intraocualr renin-angiotensin system. Br J Ophthalmol 1996;80:159-63.

40 Robertson JIS, Nicholls MG. The renin-angiotensin system. Gower Medical Publishing. London 1993. Chapters 41-48.

41 Admiraal PJJ, Danser J, Jong MS, Pieterman H, Derkx FHM, Schalekamp MADH. Regional angiotensin II production in essential hypertension and renal artery stenosis. Hypertension 1993;21:173-84.

42 Van Kats JP, Danser AHJ, Van Meegen JR, Sassen LM, Verdouw PD, Schalekamp MADH. Uptake and production of angiotensins by the heart. A quantitative study in pigs with the use of radiolabeled angiotensin infusions. Circulation 1998;98:73-81.

43 Danser AH, van Kats JP, Admiraal PJ, Derkx FH, Lamers JM, Verdouw PD, Saxena PR, Schalekamp MADH. Cardiac renin and angiotensins. Uptake from plasma versus in situ synthesis. Hypertension 1994;24:37-48.

44 Danser AHJ, van Kesteren CAM, Bax WA, Tavenier M, Derkx FHM, Saxena PR, Schalekamp MADH. Prorenin, renin, angiotensinogen, and angiotensin-converting enzyme in normal and failing human hearts. Evidence for renin binding. Circulation 1997;96:220-6.

45 Sealey JE, Rubattu S. Prorenin and renin as separate mediators of tissue and circulating systems. Am J Hypertens 1989;2:358-66.

46 Campbell DJ, Kladis A, Skinner SL, Whitworth JA. Characterization of angiotensin peptides in plasma of anephric man. J Hypertension 1991; 9:265-74.

47 Sealey JE, White RP, Laragh JH, Rubin AL. Plasma prorenin and renin in anephric patients. Circ Res 1977;41:17-21.

48 Lenz T, Sealey JE, Maack T. Half-life, hemodynamic, renal and hormonal effects of prorenin in cynomolgus monkeys. Am J Physiol 1991;260:R804-R810.

49 Hosoi M, Kim S, Takada T, Suzuki F, Murakami K, Yamamoto K. Effects of prorenin on blood pressure and plasma renin concentratrions in stroke-prone spontaneously hypertensive rats. Am J Physiol 1992;262: E234-39.

50 Kim S, Hosoi M, Ikemoto F, Murakami K, Ishizuka Y, Yamamoto K. Conversion to renin of exogenously administered recombinant human prorenin in liver and kidneys of monkeys. Am J Physiol 1990;258: E451-8.

51 Sealey JE, Catanzaro DF, Lavin TN, Gahnem F, Pitarresi T, Hu LF, Laragh JH. Specific prorenin/renin binding (ProBP). Identification and characterization of a novel membrane site. Am J Hypertens 1996;9:491-502.

52 van Kesteren CAM, Danser AHJ, Derkx FHM, Dekkers DHW, Lamers JMJ, Saxena PR, Schalekamp MADH. Mannose 6-phophate receptor-mediated internalization and activation of prorenin by cultured neonatal rat cardiac myocytes and fibroblasts. Hypertension 1997; 30:1389-96.

53 Nguyen G, Delarue F, Berrou J, Rondeau E, Sraer JD. Specific receptor binding of renin on human mesangial cells in culture increases plasminogen activator inhibitor-1 antigen. Kidney Int 1996; 50:1897-1903.

54 Hu L, Catanzaro DF, Pitarresi T, Laragh JH, Sealey JE. Identical hemodynamic and hormonal responses to 14-day infusions of renin or angiotensin II in conscious rats. J Hypertens 1998;16:1285-98.

55 Muller DN, Hilgers KF, Mathews S, Breu V, Fischli W, Uhlmann R, Luft FC. Effects of human prorenin in rats transgenic for human angiotensinogen. Hypertension 1999; 33:312-17.

56 Mullins JJ, Peters J, Ganten D: Fulminant hypertension in transgenic rats harboring the mouse Ren-2 gene. Nature 1990;344:541-4.

57 Veniant M, Menard J, Bruneval P, Morley S, Gonzalez MF, Mullins J. Vascular damage without hypertension in transgenic rats expressing prorenin exclusively in the liver. J Clin Invest 1996;98:1966-70.
58 Sealey JE, Trenkwalder P, Gahnem F Catanzaro D, Laragh JH. Commentary: Plasma renin methodology: inadequate sensitivity and accuracy of direct renin asay for clinical applications compared with the traditional enzymatic plasma renin activity assay. J. Hypertens 1995;13: 27-30. See also J. Hypertens 1995;13:19-26, 31, 367-369 and 371.
59 Derkx FHM, van Gool JM, Wenting GJ, Verhoeven RP, Man in 't Veld AJ, Schalekamp MADH. Inactive renin in human plasma. Lancet 1976;7984:496-9.
60 Johannessen A, Nielsen AH, Jacobsen J, Poulsen K On the measurement of inactive renin in rat plasma: activation by trypsin generates interfering tetradecapeptide-like material and destroys angiotensinogen. J Hypertens 1989;7:395-402.
61 Ménard J, Guyenne T, Corvol P, Pau B, Simon D, Roncucci R. Direct immunometric assay of active renin in human plasma. J. Hypertens 1985;3(suppl 3): S275-8.
62 F.H.M. Derkx, R.A. de Bruin, J.M.G. van Gool, M.-J. van den Hoek, C.C.M. Beerendonk, F. Rosmalen, P. Haima, M.A.D.H. Schalekamp, Clinical validation of renin monoclonal antibody-based sandwich assays of renin and prorenin, and use of renin inhibitor to enhance prorenin immunoreactivity, Clin. Chemistry 1996;42:1051-63.
63 Robertson JIS, Nicholls MG. Appendix II. Standardization and standards. *In* The renin-angiotensin system, Robertson JIS, Nicholls MG, *eds*. Gower medical publishing, London 1993, A.7-A.9.
64 Bangham DR, Robertson I, Robertson JIS, Robinson CJ, Tree M. An international collaborative study of renin assay: Establishment of the international reference preparation of human renin. Clin Sci Mol Med 1975; 48(suppl):135-159.
65 Poole S. Renin and angiotensin standards. *In* The renin-angiotensin system, Robertson JIS, Nicholls MG, *eds*. Gower medical publishing, London 1993, 17.1-17.5.

Chapter 2 Structure of Prorenin

2.1 Non-proteolytic 'Activation' of Prorenin by Active Site-Directed Renin Inhibitors as Demonstrated by Renin-Specific Monoclonal Antibody

Summary

Incubation of human plasma prorenin (PR), the enzymatically inactive precursor of renin, with a number of non-peptide high-affinity active site-directed renin inhibitors induces a conformational change in PR, which was detected by a monoclonal antibody that reacts with active renin but not with native inactive PR. This conformational change also occurred when inactive PR was activated during exposure to low pH. Non-proteolytically acid-activated PR, and inhibitor-'activated' PR, as well as native PR, were retained on a blue Sepharose column, in contrast to proteolytically activated PR. Kinetic analysis of the activation of plasma prorenin by renin inhibitor (INH) indicated that native plasma contains an open intermediary form of prorenin, PR_{oi}, in which the active site is exposed and which is in rapid equilibrium with the inactive closed form, PR_c. PR_{oi} reacts with inhibitor to form a reversible complex, PR_{oi}·INH, which undergoes a conformational change resulting in a tight complex of a modified open form of prorenin, PR_o, and the inhibitor, PR_{oi}·INH→PR_o·INH. The PR_{oi}-to-PR_o conversion leads to the expression of an epitope on the renin part of the molecule that is recognized by a renin-specific monoclonal antibody. Presumably, PR_o corresponds to the enzymatically active form of PR that is formed during exposure to low pH. Thus, it seems that the propeptide of PR interacts with the renin part of the molecule not only at or near the enzyme's active site but also at some distance from the active site. Interference with the first interaction by renin inhibitor leads to destabilization of the propeptide, by which the second interaction is disrupted and the enzyme assumes its active conformation. The results of this study may provide a model for substrate-mediated prorenin activation and increase the likelihood that enzymatically active prorenin is formed in vitro.

Introduction

The conversion, by limited proteolysis, of the inactive proenzyme form of serine proteinases into their active counterpart has been extensively studied. Prorenin, the enzymatically inactive proenzyme form of the aspartic proteinase renin is also activated by limited proteolysis, which causes the prosegment to be cleaved from the prorenin polypeptide chain.[1-5] The spatial conformation of the folded renin polypeptide chain shows a bilobar structure, the two lobes being separated by a cleft containing the enzyme's active site.[6] Presumably, in prorenin the cleft is covered by the prosegment (closed form of prorenin).[7,8]

Prorenin can also be activated without proteolysis, by exposure to low pH or low temperature.[9-13] Probably the non-proteolytic activation of prorenin involves a conformational change by which the cleft is uncovered (open form of prorenin), so that the scissile peptide bond of angiotensinogen can get access to the active site of the enyme. It is generally assumed that prorenin's physiological function depends on its conversion to renin. However, since studies with purified recombinant human prorenin

provided some evidence for its spontaneous activation, the possibility arises that apart from renin, prorenin itself may have some physiological role.[7,14]

Here we report on evidence that the native closed form of prorenin, without any pretreatment, is in rapid equilibrium with an open form that is capable of reacting with a number of high-affinity active site-directed renin inhibitors. This reaction leads to a conformational change of the prorenin molecule, which is recognized by a monoclonal antibody that binds to renin but not to the closed form of prorenin. These *in-vitro* observations raise the possibility that an enzymatically active form of prorenin may exist in vivo.

Materials and Methods

Chemicals

Two monoclonal antirenin antibodies were used. They were obtained from Pasteur Diagnostics, Marnes de Coquette, France. Their characteristics have been described in detail.[15-17]

Monoclonal antibody 3E8 (mAb3E8) binds prorenin as well as renin, and does not inhibit renin's enzymatic activity. This antibody was coupled to polyacrylamide-agarose beads (diameter, 60-170 μm) containing ferrous oxide (Magnogel, Diagnostics Pasteur, Marnes La Coquette, France), as described elsewhere.[18] The beads with coupled antibody, 1.3 mg of antibody per of Magnogel, were obtained from Pasteur Diagnostics. The second monoclonal antibody, mAb4G1, which was labeled with ^{125}I (925 kBeq/μg antibody), recognizes an epitope of renin and inhibits renin's enzymatic activivity but does not cross-react with inactive prorenin.[15]

Human kidney renin standard, MRC 68/356, was obtained from the Medical Research Council Institute for Biological Standards and Control, Potters' Hill, Hertfordshire, United Kingdom. Plasmin (EC 3.4.21.7) was obtained from Kabivitrum. Soybean trypsin inhibitor (SBTI)[19] was obtained from Sigma.

The following active site-directed renin inhibitors were studied: remikiren (Hoffmann-La Roche, Basel, Switzerland),[20] A-64662 (enalkiren, Abbott Laboratories, Abbott Park, IL),[21] and CGP 38560A (Ciba Geigy, Basel, Switzerland).[22]

A plasma pool from healthy subjects (n = 14) was used as a source of renin and prorenin. Blood from these subjects was collected into polystyrene tubes containing trisodium citrate solution (0.2 mL in 10 mL blood, final citrate concentration 13 mM), and immediately centrifuged for 10 min at 3,000 x g and room temperature. The plasma was stored at -70 °C.

Binding of Prorenin to Gel Beads by Immunoadsorption. mAb3E8 coupled to Magnogel beads was used to trap renin and prorenin from plasma. The beads were suspended in 0.2 mM imidazole-HCI buffer containing 0.25% gelatin and 15 mm sodium azide. The suspension (250 μL), which contained 4% Magnogel, was added to plasma (250 μL), and the mixture was incubated for 2 h at room temperature, while the tubes containing the mixture were placed in a horizontal rack shaker to keep the beads in suspension. At the end of the incubation period, 2 mL of phosphate-buffered saline (PBS, 0.1 M sodium phosphate buffer pH 7.4, containing 0,05 M NaCl) was added, and

the tubes were shaken for 2 min. The beads were then separated from the buffer solution, without removing them from the tubes, by means of a magnetic ruler placed in close contact with the tubes, and the supernatant was discarded. Again 2 mL of PBS was added, and after 2 min of shaking, the separation procedure was repeated, and the washed beads were resuspended in PBS.

Proteolytic Activation of Gel-bound Prorenin by Incubation with Plasmin. Magnogel-mAb3E8 beads with bound prorenin from 250 μL of plasma were suspended in 250 μL of PBS containing 0.2 μM plasmin, and incubated for 24 h at 4 °C, while the tubes were continuously shaken. This procedure caused complete activation of prorenin, as was demonstrated by measuring the enzymatic activity of the beads and by comparing the results with those from our routine enzyme-kinetic assay of renin and prorenin.[23,24] Magnogel-mAb3E8 beads with bound renin from the MRC human kidney renin standard did not show a change in enzymatic activity after treatment with plasmin. Complete activation of prorenin by plasmin and the absence of any effect of the plasmin treatment on the MRC human kidney renin standard was also demonstrated with the immunoradiometric assay, in which the washed plasmin-treated beads were incubated in a buffer containing ^{125}I-labeled mAb4G1 and the quantity of antibody bound to the beads was measured after incubation, by γ counting (immunoradiometric assay, see below).

Non-proteolytic 'Activation' of Gel-bound Prorenin by Incubation with Renin Inhibitor. Magnogel-mAb3E8 beads with bound prorenin from 250 μL of plasma were suspended in 250 μL of PBS containing renin inhibitor in concentrations ranging from 10^{-8} to 10^{-4} M. The mixture was incubated for periods ranging from 30 min to 24 h, while the mixture containing the beads was continuously shaken. During this procedure the prorenin molecule undergoes a conformational change, by which it becomes recognizable by the antibody mAb4G1. Because this antibody recognizes renin but not inactive prorenin, the effect of renin inhibitor on the binding of prorenin to the antibody is referred to as 'activation'. The quantity of ^{125}I-labeled mAb4G1 bound to the beads after incubation with this antibody was used as a measure of the activation of prorenin by renin inhibitor (immunoradiometric assay, see below).

Non-proteolytic Activation of Prorenin in the Fluid Phase in Complete Plasma by Incubation with Renin Inhibitor or by Acidification. To study the effect of renin inhibitor on prorenin in complete plasma, normal plasma pool (6 mL) to which 60 μL of the serine protease inhibitor SBTI (final concentration, 50 μM) and either PBS alone or PBS containing remikiren (60 μL) had been added was incubated for 24 h at 4 °C, at room temperature, or at 37 °C. The final concentration of remikiren was 10 μM. Samples were taken at different time intervals for immunoradiometric assay of renin (see below).

It is known that prorenin is reversibly activated after acidification.[11,12] After restoration of pH prorenin again assumes its inactive form. To study the effect of renin inhibitor on the inactivation of acid-activated plasma prorenin, plasma was diluted with an equal volume of 0.05 M glycine HCl buffer, pH 2.5, containing 0.1 M NaCl, and dialyzed at 4 °C for 24 h against 0.05 M glycine HCl buffer, pH 3.3, containing 0.1 M NaCl.[13] The plasma was then neutralized by adding an equal volume of 0.1 M PBS, pH 8.0, containing 0.05 M NaCl. Fine adjustment of pH to 7.4 was made by adding 1 M NaOH or 1 M

HCl. After pH adjustment, which took less than 5 min, either PBS alone or PBS containing remikiren was added (10 μL in 1 mL of pH-adjusted plasma). The final concentration of remikiren was 10 μM. Renin was measured by immunoradiometric assay (see below) either immediately after pH adjustment or after a 1-h incubation 37 °C.

Immunoradiometric Assay of Proteolytically Activated and Renin Inhibitor-Activated Gel-bound Prorenin. The assay was carried out according to Refs. 16 and 17. Magnogel-mAb3E8 beads with bound prorenin from 250 μL of plasma were treated with plasmin or renin inhibitor, as described above. The beads were then washed in PBS and resuspended in 250 μL of 0.05 M Tris-HCl buffer, pH 7.4, containing ^{125}I-labeled mAb4G1 (approximately 100,000 cpm), 10% horse serum, and 15 mM sodium azide. The mixture was incubated for 3h at room temperature, while the tubes containing the beads were continuously shaken. After incubation the beads were washed three times with PBS, and radioactivity was counted. Nonspecific binding of radiolabeled antibody was less than 0.2%. Results are expressed as ng/liter, using highly purified human renin as the standard.[15]

Enzyme-kinetic Assay of Gel-bound Renin and Proteolytically Activated Gel-bound Prorenin. To measure the binding of renin and prorenin to the Magnogel-mAb3E8 beads, the enzymatic activity of the beads was determined as the quantity of AngI that formed during incubation with sheep angiotensinogen (1 μM) at 37 °C for up to 1 h. The beads with bound renin and prorenin were incubated with angiotensinogen before and after treatment with plasmin 4 °C, in order to convert prorenin into renin. AngI generation rates by the beads were compared with the results of our routine enzyme-kinetic assays of renin and prorenin in plasma, which were carried out under comparable conditions and which also made use of sheep angiotensinogen as the substrate.[22] It appeared that more than 90% of the renin and prorenin present in plasma was trapped by the Magnogel-mAb3E8 beads.

In order to measure K_i of the renin inhibitor remikiren, Magnogel beads with bound kidney renin from MRC standard 38/356 were also incubated with sheep angiotensinogen (1 μM), in the presence of the renin inhibitor in concentrations ranging from 10^{-10} to 10^{-5} M. K_m for the reaction of gel-bound kidney renin with sheep angiotensinogen was also measured. It was 0,24 μM (mean of triplicate measuremen which is similar to K_m (0.21-28 μM) for fluid phase renin.[23] A plot of $V_o/(V_i - 1)$ against inhibitor concentration was made (V_o and V_i are the enzymatic activities in the absence and presence of inhibitor, respectively. The slope of this line equals $1/K_{i(app)}$. K_i was calculated according to the equation $K_i = K_{i(app)}/(1 + [S]/K_m)$, in which [S] is the angiotensinogen concentration. K_i of remikiren appeared to be 8 nM (mean of triplicate measurement).

Separation of Proteolytically Activated and Renin Inhibitor-Activated Prorenin by Dye Ligand Chromatography. Plasma was treated with Sepharose-bound trypsin as described by Derkx et al.[22] or by incubation with the renin inhibitor remikiren (final concentration, 50 μM) for 48 h at 4 °C. Native plasma or trypsin- or inhibitor-treated plasma (2 mL) was applied to a 1 x 16-cm column of blue Sepharose (Pharmacia LKB Biotechnology Inc.). The column had been equilibrated with 20 mM sodium phosphate buffer, pH 7.2. Elution was performed in two steps, *i.e.* with the equilibration buffer and

same buffer containing 1.4 M NaCl.[13] The flow rate was 60 mL/h and 2.0-mL fractions were collected. Equilibration and elution were carried out at room temperature. The renin content of the fractions was determined with the immunoradiometric assay, both before and after incubation of the fractions with plasmin, 0.2 μM final concentration, for 24 h at 4 °C.

Kinetic Analysis of Renin Inhibitor-Induced Activation of Prorenin. We assumed the renin inhibitor to interact with prorenin in the manner of active site-directed irreversible inhibitors[24] as follows.

$$E + \mathrm{INH} \underset{\text{reversible complex}}{\overset{K_i}{\rightleftharpoons}} \xrightarrow{k_{max}} \text{irreversible complex}$$

The first step is the formation of the reversible E·INH complex that is in rapid equilibrium with E and INH, K_i being defined as follows.

$$K_i = [E]\cdot[INH]/[E\cdot INH]$$

The second step is the formation of the irreversible E·INH complex, which is a first order reaction with the rate constant k_{max}. This first order reaction is the rate-limiting step. This reaction sequence implies that the inhibition rate is saturable with respect to the concentration of enzyme-inhibitor complexes.

We further assumed the existence of closed and open forms of prorenin. In the closed form, PR_c, the cleft is covered by the prosegment so that the inhibitor cannot reach the active site of prorenin. Our results (see later) indicated that the irreversible E·INH complex but not the reversible E·INH complex, could be recognized by the monoclonal renin antibody mAb4G1. This implies the existence of two open forms of prorenin. Both can bind active-site ligands but one form, PR_o is recognized by the antibody, whereas the other, an intermediary open form, PR_{oi}, is not. PR_{oi} is assumed to be in rapid equilibrium with PR_c. The anticipated reaction sequence is then as follows.

$$\begin{array}{ccccc} & & \overset{K_i}{\rightleftharpoons} & & \overset{k_{max}}{\longrightarrow} \\ PR_{oi} & + \mathrm{INH} & & PR_{oi}\cdot\mathrm{INH} & \longrightarrow PR_o\cdot\mathrm{INH} \\ K_a \uparrow\downarrow & & & & \\ PR_c & & & & \end{array}$$

K_i and K_a are defined as follows.

$$K_i = [PR_{oi}][INH]/[PR_{oi} \cdot INH] \quad (1)$$

$$K_a = [PR_{oi}]/[PR_c] \quad (2)$$

$[PR_c]$, $[PR_{oi}]$, [INH] and $[PR_{oi} \cdot INH]$ are the concentrations of PR_c, PR_{oi}, INH, and $PR_{oi} \cdot INH$ complex. The anticipated reaction scheme leads to the following.

$$-d[PR]/dt = [PR_{oi} \cdot INH] \cdot k_{max} \quad (3)$$

[PR] is defined as follows.

$$[PR] = [PR_c] + [PR_{oi}] + [PR_{oi} \cdot INH] \quad (4)$$

On the basis of equations 1, 2, and 4, $[PR_{oi} \cdot INH]$ can be expressed as a function of [PR], [INH], K_i and K_a as follows.

$$[PR_{oi} \cdot INH] = \frac{[INH]/K_i}{1 + 1/K_a + [INH]/K_i} \cdot [PR] \quad (5)$$

The solution to equation 3 is

$$[PR]_t = [PR]_0 e^{-A \times k_{max} \times t} \quad (6)$$

in which

$$A = \frac{[INH]/K_i}{1 + 1/K_a + [INH]/K_i} \quad (7)$$

and $[PR]_t$ and $[PR]_0$ are the concentrations of PR at t = t and t = 0. From equations 6 and 7 it follows that

$$k_{obs} = \frac{[INH]/K_i}{1 + 1/K_a + [INH]/K_i} \cdot k_{max} \quad (8)$$

$$\frac{1}{k_{obs}} = \frac{K_i(1 + 1/K_a)}{k_{max}} \cdot \frac{1}{[INH]} + \frac{1}{k_{max}} \quad (9)$$

in which k_{obs} is the first-order rate constant for $PR_o \cdot INH$ complex formation.

By plotting $1/k_{obs}$ on the *y*-axis against $1/[INH]$ on the *x*-axis, a straight line is obtained intersecting the *x*-axis at $-1/[K_i(1 + 1/K_a)]$ and the *y*-axis at $1/k_{max}$. Assuming K_i for prorenin to be the same as for renin, K_a can be calculated.

Results

Proteolytic Activation of Gel-bound Plasma Prorenin by Plasmin and Non-proteolytic Activation by Renin Inhibitors. Incubation of gel-bound kidney renin with plasmin or with the renin inhibitors remikiren, A 64662, or CGP 38-560A for 24 h at 4 °C did not alter the binding of renin to the radiolabeled monoclonal antibody mAb4GI, an antibody that specifically reacts with renin and not with native inactive prorenin. In contrast, not only plasmin but also the renin inhibitors increased the binding of plasma prorenin to this antibody (Table 1 and Fig. 1). When gel-bound plasma prorenin was activated with plasmin and then incubated with the renin inhibitors, the binding of the activated prorenin to the antibody was not altered by the renin inhibitors (data not shown).

Apparently, complex formation with the renin inhibitors is associated with a conformational change in prorenin, by which it becomes recognizable by the antibody. Because this conformational change also occurs when inactive prorenin is converted by plasmin to enzymatically active renin, the effect of the renin inhibitors on the binding of prorenin to the renin-specific monoclonal antibody is designated here as activation. The activated prorenin, which is detected with the reninspecific monoclonal antibody mAb4G1, cannot be detected via its enzymatic activity, because of the presence of renin inhibitor.

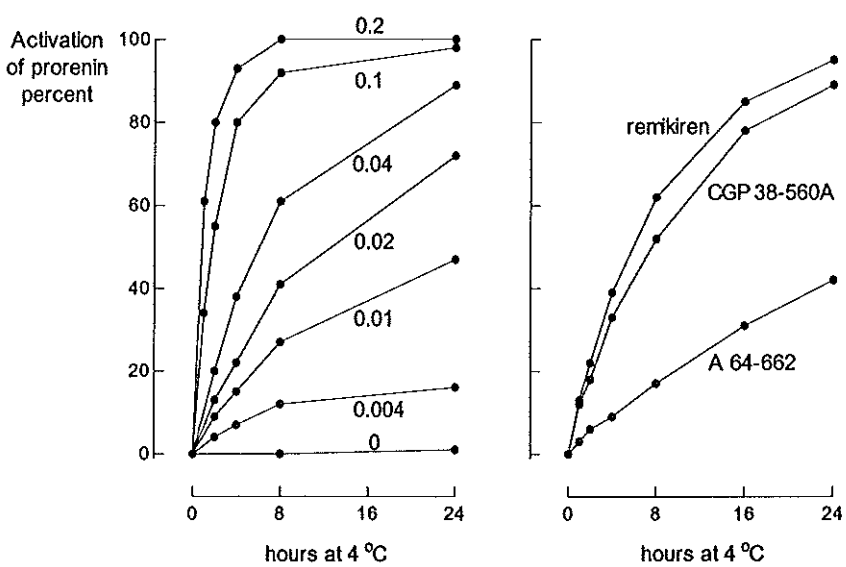


Figure 1. Proteolytic activation of gel-bound plasma prorenin by plasmin (0.004-0.2 µM) (A) and nonproteolytic activation by various renin inhibitors (10 µM) (B) at 4 °C. Activated prorenin was measured with the immunoradiometric assay using the monoclonal antibody mAb4G1 that recognizes renin but not native inactive prorenin. Results (mean, n = 3) are expressed as percentage of maximal activation by plasmin.

Table 1. Proteolytic activation of gel-bound prorenin by plasmin and nonproteolytic activation by renin inhibitors

	Renin and activated prorenin expressed as renin equivalents			
	Immunoradiometric assay		Enzyme-kinetic assay	
	Kidney renin	Plasma	Kidney renin	Plasma
	ng/liter			
PBS	29±2	31±3	33±3	34±2
Plasmin, 0.2 μM	32±2	312±18	30±3	304±19
Remikiren, 10 μM	32±1	301±14	<0.25	<0.25
A 64-662, 10 μM	29±3	149±11	<0.25	<0.25
CGP 38-560A, 10 μM	32±2	298±3	<0.25	<0.25

Results (mean±SD, n=5) of both assays are expressed as renin equivalents (ng/liter)

In a separate series of experiments we added to the renin inhibitor solution, EDTA (final concentration, 5 mM) or the serine proteinase inhibitors phenylmethanesulfonyl fluoride (1.4 mM), or SBTI (50 μM). These additions had no effect on the activation of prorenin by the renin inhibitors. A serine proteinase appears not to be involved.

Fig. 2 shows the activation at 4 °C of gel-bound plasma prorenin over time at different concentrations of the renin inhibitor remikiren. The reaction of gel-bound prorenin with the inhibitor followed first-order kinetics. The data are in good agreement with the anticipated reaction sequence. They support a rapid equilibrium between PR_c and PR_{oi}

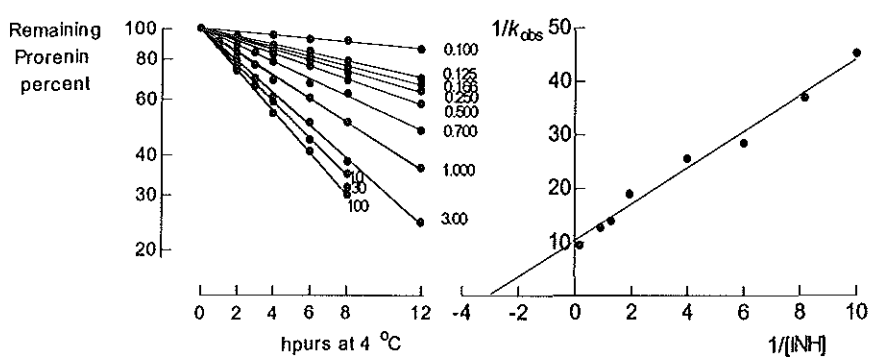


Figure 2. Kinetics of activation of gel-bound plasma prorenin by the renin inhibitor remikiren at 4 °C. Activated prorenin was measured with the immunoradiometric assay using the monoclonal antibody mAb4G1 that recognizes renin but not native inactive prorenin. Results are expressed as percentage of the total prorenin concentration as measured after activation by plasmin. The observed first-orde rate constant, k_{obs} (expressed in h^{-1}), at various concentrations of the inhibitor (0.1-100 μM), are given by the slopes of the lines (A). The reciprocal of k_{obs} plotted against the reciprocal of the inhibitor concentrations (expressed in μM) gives a straight line (B). The line is the least-square fit for $1/k_{obs}$ at concentrations of the renin inhibitor ranging from 0.100 to 3 μM. Results for the concentrations 10, 30, and 100 μM were close to the intercept with the y-axis, which is at $1/k_{max}$. The intercept with the x-axis is at $-1/[K_i(1+1/K_a)]$.

and between PR_{oi}, inhibitor and a complex of PR_{oi} with inhibitor. The subsequent slow conversion of PR_{oi} to PR_o in this complex is associated with a conformational change that is recognized by the monoclonal renin antibody mAb4G1. That the first step, $PR_c \rightarrow PR_{oi}$, is not rate-limiting, is also supported by experiments in which gel-bound plasma prorenin or prorenin in whole plasma was incubated for up to 24 h at 4 °C (in the presence of 50 μM SBTI to prevent proteolytic activation) prior to the addition of the renin inhibitor remikiren (10 μM). These experiments demonstrated that the preincubation did not modify the rate of prorenin activation. As shown in Fig. 2, the rate of prorenin activation by remikiren increases with increasing concentrations of this inhibitor until a maximum was reached. These findings are compatible with the assumption that irreversible binding of the inhibitor to prorenin does not take place directly but via the formation of a reversible complex.

Kinetic analysis according to Equation (9) gave a value of 0.1 h^{-1} for k_{max}, and a value of 330 nM for $K_i(1 + 1/K_2)$. K_i of remikiren for gel-bound kidney renin was 8 nM. If K_i for gel-bound prorenin is the same as for gel-bound renin, K_2 equals 40, which would mean that 2.5% of native prorenin is in the open form.

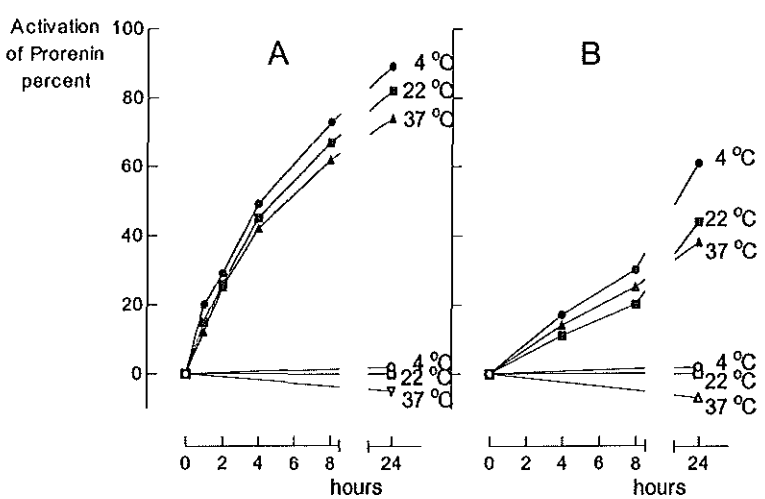


FIG. 3. Influence of temperature on the nonproteolytic activation of gel-bound plasma prorenin (A) and of prorenin in the fluid phase in whole plasma (B) by the renin inhibitor remikiren (10 μM). Activated prorenin was measured with the immunoradiometric assay using the monoclonal antibody mAb4G1 that recognizes renin but not native inactive prorenin. Results for control incubations without renin inhibitor are indicated by open symbols. Results (mean, n=3) are expressed as percentage of maximal activation by plasmin (for gel-bound plasma prorenin) or by Sepharose-bound trypsin (for plasma) (22, 23).

Fig. 3 shows that prorenin activation by renin inhibitor not only occurs at 4 °C but also at room temperature and at 37 °C. In fact, the rate of activation of gel-bound plasma prorenin these higher temperatures was hardly different from the rate at 4 °C.

Non-proteolytic Activation of Prorenin in the Fluid Phase in Whole Plasma by Renin Inhibitor or by Acid and Prevention of Inactivation of Acid-activated Prorenin by Renin Inhibitor. Prorenin activation by the renin inhibitors, as detected with the monoclonal antibody mAb4GI, could not only be demonstrated for gel-bound plasma prorenin but also in the fluid-phase in whole plasma. Fig. 3 shows the activation by the renin inhibitor remikiren. The rate of activation was lower than for gel-bound plasma prorenin. This is probably due to the fact that more than 90% of the inhibitor is bound to plasma protein,[25] so that the effective inhibitor concentration is lower in plasma than in the buffer solution in which the gel beads are suspended. Activation in whole plasma occurred at 4 °C and also at room temperature and at 37 °C.

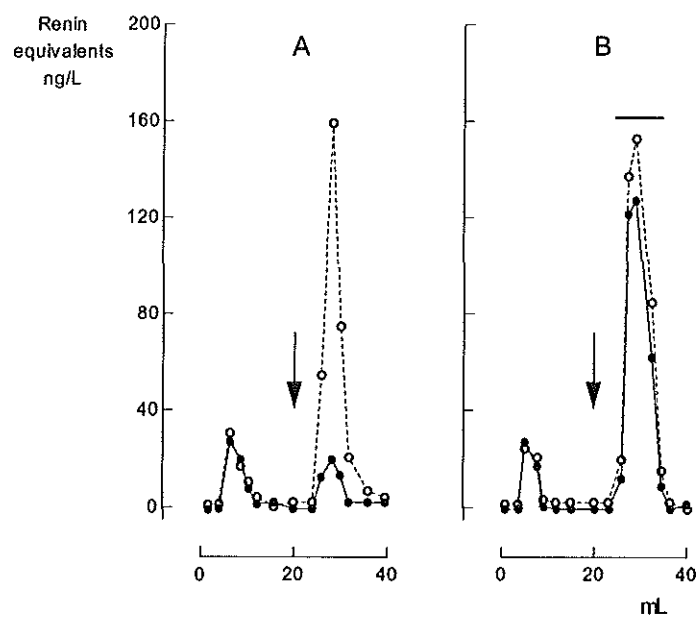


FIG. 4. Separation by blue Sepharose column chromatography of naturally occurring plasma renin and renin inhibitor-activated plasma prorenin. A, native plasma; B, plasma after incubation with the renin inhibitor remikiren (10 μM) for 24 h at 4 °C. Elution buffer: 20 mM sodium phosphate buffer, pH 7.2. The arrow indicates change of buffer: 20 mM sodium phosphate buffer, pH 7.2, containing 1.4 M NaCl. Native renin, native inactive prorenin, and renin inhibitor-activated prorenin were measured, before (closed circles) and after (open circles) plasmin treatment of the eluate, with the immunoradiometric assay using the monoclonal antibody mAb4G1 that recognizes renin but not native inactive prorenin.

Treatment of plasma at pH 3.3 at 4 °C for 24 h is known to cause reversible activation of prorenin; the acid-activated prorenin is inactivated after restoration of pH.[11, 12] We found that this reversibly acid-activated form of prorenin was not only detected by the kinetic assay but also by the direct radiometric assay, which measures the binding to the monoclonal antibody mAb4G1 that recognizes renin but not native prorenin. With this direct assay we observed that treatment of the normal plasma pool at pH 3.3 at 4 °C for 24 h caused 94% (mean, n = 3) activation of prorenin, which was not different from results obtained with the indirect kinetic assay. After rapid restoration of pH to 7.4 and incubation at 37 °C for 1 h at this pH (in the presence of 50 μM SBTI to prevent proteolytic activation), most of the activated prorenin was inactivated. The immunoradiometric assay demonstrated that only 8% (mean, n = 3) of prorenin was in the activated form, and again similar results were obtained with the kinetic assay. By using the radiometric assay it could be demonstrated that the inactivation during 1-h incubation at pH 7.4 at

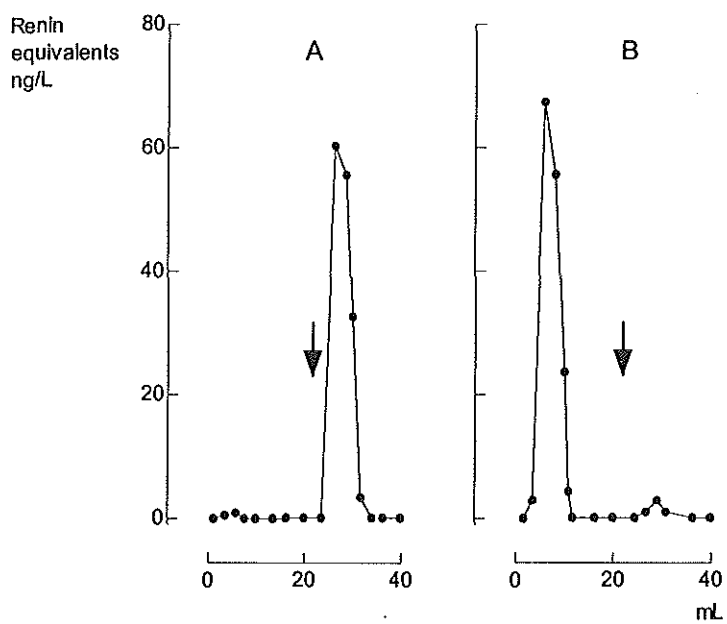


Fig. 5. Change in chromatographic behavior of renin inhibitor-activated plasma prorenin after proteolytic conversion to renin by plasmin. The second peak of Fig. 4 indicated by the horizontal line in B, was divided into two portions. One portion was rechromatographed on a blue Sepharose column without prior treatment with plasmin (A). The other portion was rechromatographed after treatment with plasmin, 0.2 μM, for 24 h at 4 °C (B). Elution buffer: 20 mM sodium phosphate buffer, pH 7.2. The arrow indicates change of buffer: 20 mM sodium phosphate buffer, pH 7.2, containing 1.4 M NaCl. Both forms of activated prorenin were measured with the immunoradiometric assay using the monoclonal antibody mAb4G1 that recognizes renin but not native inactive prorenin.

37 °C was completely prevented by the addition of the renin inhibitor remikiren (final concentration in incubate 10 μM); after incubation in the presence of remikiren, 91% (mean, n = 3) was still in the activated form.

Separation of Plasmin-activated and Renin Inhibitor-activated Prorenin. Naturally occurring renin and prorenin can be readily separated by blue Sepharose column chromatogra-phy. It is known that, under the conditions we used (20 mM sodium phosphate buffer, pH 7.2), naturally occurring plasma renin appears in the pass-through fractions, whereas native prorenin or reversibly acid-activated prorenin are retained and can be eluted by adding 1.4 M NaCl to the elution buffer.[13] **Fig. 4** shows the separation of inhibitor-activated prorenin and naturally occurring renin, when plasma is subjected to chromatography after it has been incubated with the renin inhibitor remikiren (final concentration, 10 μM) for 24 h at 4 °C. Like native prorenin and reversibly acid-activated pro-renin, renin inhibitor-activated prorenin was retained on the column and was eluted at 1.4 M NaCl.

The fractions containing naturally occurring renin, native prorenin, and renin inhibitor-activated prorenin were incubated with plasmin (0.2 μM) for 24 h at 4 °C. Rechromatography after plasmin treatment showed that renin inhibitor-activated prorenin was not bound to blue Sepharose anymore; it appeared in the pass-through fractions, as did plasmin-treated renin and plasmin-activated prorenin (**Fig. 5**).

Discussion

This study describes a specific interaction of enzymatically inactive prorenin with a number of nonpeptide high-affinity active site-directed renin inhibitors. Because this interaction was observed in fresh plasma without pretreatment, we assume that native plasma contains an open form of prorenin, in which the active site is exposed. Our kinetic analysis supports the hypothesis that this open form of prorenin, PR_{oi}, is in rapid equilibrium with the closed form, PR_c.

PR_{oi} differs from the open form of prorenin that is generated by exposing PR_c to low pH (non-proteolytic acid activation), because acid-activated prorenin is slowly formed and slowly returns to the closed form when pH is restored.[11,12] Moreover, non-proteolytically acid-activated prorenin is recognized by the monoclonal renin antibody mAb4G1, whereas PR_{oi} is not.

Our kinetic analysis implies that PR_{oi} rapidly binds to renin inhibitor to form a reversible complex, PR_{oi}·INH, and that this is followed by a slow conformational change resulting in a tight complex of the inhibitor with a modified open form of prorenin, PR_{oi}·INH→PR_o·INH. This slow conformational change is associated with the expression of an epitope on the renin part of the molecule that is recognized by the mAb4G1 antibody. Binding studies with the nonpeptide active site-directed renin inhibitor U-71038 also provided evidence for the slow formation of a tight prorenin-inhibitor complex.[7] This may be comparable to the slow PR_{oi}·INH-to-PR_o·INH conversion we describe here.

We found that both PR_{oi} and non-proteolytically acid-activated prorenin reacted with the monoclonal renin antibody mAb4G1. Moreover, both the PR_o·INH complex (with

the renin inhibitor remikiren) and the enzymatically active form of prorenin generated at low pH were bound to a blue Sepharose column, in contrast to proteolytically activated prorenin, and both forms of non-proteolytically activated prorenin could be eluted with 1.4 M NaCl. The binding of the PR_o·INH complex to the column was probably not due to the presence of remikiren, because the chromatographic behavior of naturally occurring renin or plasmin-activated prorenin was not altered by this inhibitor. We therefore tentatively assume that PR_o corresponds to the enzymatically active open form of prorenin that is generated during exposure to low pH.

Our findings suggest that one or more interactions of the propeptide with the renin part of the molecule, at sites different from the active site, are essential for keeping prorenin enzymatically inactive. Studies with peptides containing amino acid sequences of prorenin's prosegment have shown that the sequence from -p32-Phe to -p27-Pro of the propeptide in human prorenin is capable of inhibiting human renin.[1] Cleavage of the prosegment of human prorenin between residues -p34-Arg to -p30-Lys yields an active enzyme.[7] Site-directed mutagenesis of human prorenin has demonstrated that positively charged residues located near the amino terminus, -p34-Arg, -p29-Arg, and -p24-Arg, rather than -p12-Arg and -p1-Arg, of the propeptide contribute to its correct folding and to the blocking of prorenin's enzymatic activity.[2] Since it is thought that a positively charged residue located near the carboxyl terminus rather than the amino terminus is interacting with Asp residues of the enzyme's active site,[3] the studies cited above also support the view that interactions of the propeptide with the renin part of the prorenin molecule at some distance from the active site are essential for keeping the molecule enzymatically inactive.

If this is true, how then to explain the effects we observed on prorenin of low-molecular weight ligands that are specifically directed toward renin's active site? Apparently, an additional interaction of the propeptide with the renin part of the prorenin molecule near or, in fact, at the active site also contributes to the stabilization of prorenin in the inactive form. By interfering with this interaction, the active site-directed ligands may trigger further destabilization of the propeptide, so that the prorenin molecule will assume its active configuration.

Sealey[14] has argued that the presence in extrarenal tissues of *in situ* synthesized renin, as opposed to prorenin, has not been unequivocally proven. She made the suggestion that local angiotensin production in these tissues might be due to the fact that part of the *in situ* synthesized prorenin is in an open and enzymatically active conformation. Our results would then indicate that the $PR_c \rightarrow PR_{oi} \rightarrow PR_o$ reaction sequence, as described in this paper, is responsible for this local production of angiotensins in extrarenal tissues.

Transgenic rats harboring the mouse *Ren*-2 renin gene develop severe hypertension, which can effectively be treated with angiotensin-converting enzyme inhibitor. The hypertension is thought to be due to the high expression of the *Ren*-2 gene in the adrenal gland.[4] The plasma levels of renin and AngII are low in these animals but plasma prorenin is grossly elevated, presumably due to hypersecretion of prorenin by the adrenals. The present study may help to explain these surprising observations. Our results may provide

a model for substrate-mediated activation of prorenin *in vivo*, and lend support to the hypothesis that prorenin, apart from renin, may have some (patho)physiological role.

References

1 Derkx FH, Bouma BN, Schalekamp MP, Schalekamp MADH. An intrinsic factor XII- prekallikrein-dependent pathway activates the human plasma renin-angiotensin system. Nature 1979;280:315-6.

2 Sealey JE, Atlas SA, Laragh JH, Silverberg M, Kaplan. Initiation of plasma prorenin activation by Hageman factor-dependent conversion of plasma prekallikrein to kallikrein. Proc Natl Acad Sci US A 1979;76:5914-8.

3 Inagami T, Okamoto H, Ohtsuki K, Shimamoto K, Chao J, Margolius HS. Human plasma inactive renin: purification and activation by proteases. J Clin Endocrinol Metab 1982;55:619-27.

4 Hirose S, Kim S, Miyazaki H, Park YS, Murakami K. In vitro biosynthesis of human renin and identification of plasma inactive renin as an activation intermediate. J Biol Chem 1985;260:16400-5.

5 Shinagawa T, Do YS, Baxter JD, Carilli C, Schilling J, Hsueh WA. Identification of an enzyme in human kidney that correctly processes prorenin.. Proc Natl Acad Sci US A 1990;87:1927-31.

6 Sielecki AR, Hayakawa K, Fujinaga M, Murphy ME, Fraser M, Muir AK, Carilli CT, Lewicki JA, Baxter JD, James MN. Structure of recombinant human renin, a target for cardiovascular-active drugs, at 2.5 Å resolution. Science 1989;243:1346-51.

7 Heinrikson RL, Hui J, Zurcher-Neely H, Poorman RA. A structural model to explain the partial catalytic activity of human prorenin. Am J Hypertens 1989;2:367-80.

8 Hsueh WA, Baxter JD. Human prorenin. Hypertension 1991;4:469-77.

9 Osmond DH, Ross LJ, Scaiff KD. Increased renin activity after cold storage of human plasma. Can J Physiol Pharmacol 1973;51:705-8.

10 Sealey JE, Moon C, Laragh JH, Alderman M. Plasma prorenin: cryoactivation and relationship to renin substrate in normal subjects. Am J Med 1976;61:731-8.

11 Leckie BJ, McGhee NK. Reversible activation-inactivation of renin in human plasma. Nature 1980;288:702-5.

12 Hsueh WA, Carlson EJ, Israel-Hagman M. Mechanism of acid-activation of renin: role of kallikrein in renin activation. Hypertension 1981;3 (Suppl I):I22-9.

13 Derkx FH, Schalekamp MPA, Schalekamp MADH. Two-step prorenin-renin conversion. Isolation of an intermediary form of activated prorenin. J Biol Chem 1987;262:2472-7.

14 Sealey JE, Rubattu S. Prorenin and renin as separate mediators of tissue and circulating systems. Am J Hypertens 1989;2:358-66.

15 Galen FX, Devaux C, Atlas S, Guyenne T, Menard J, Corvol P, Simon D, Cazaubon C, Richer P, Badouaille G, Richard JP, Gros P, Pau B. New monoclonal antibodies directed against human renin. Powerful tools for the investigation of the renin system. J Clin Invest 1984;74:723-35.

16 Simon D, Badouaille G, Pau B, Guyene TT, Corvol P, Menard. Measurement of active renin by the 4G1 anti-human renin monoclonal antibody. J Clin Exp Hypertens [A] 1987;9:1333-40.

17 Menard J, Guyenne TT, Corvol P, Pau B, Simon D, Roncucci R. Direct immunometric assay of active renin in human plasma. J Hypertens 1985;3(Suppl 3):S275-8.

18 Guesdon JL, Avrameas S. Magnetic solid-phase enzyme immunoassay for the quantitation of antigens and antibodies: application to human immunoglobulin E. Methods Enzymol 1981;73:471-82.

19 Fischli W, Clozel JP, el Amrani K, Wostl W, Neidhart W, Stadler H, Branca Q. Ro 42-5892 is a potent orally active renin inhibitor in primates. Hypertension 1991;18:22-31.

20 Kleinert HD, Martin D, Chekal MA, Kadam J, Luly JR, Plattner JJ, Perun TJ, Luther RR. Effects of the renin inhibitor A-64662 in monkeys and rats with varying baseline plasma renin activity. Hypertension 1988;11:613-9.

21 de Gasparo M, Cumin F, Nussberger J, Guyenne TT, Wood JM, Menard J. Pharmacological investigations of a new renin inhibitor in normal sodium-unrestricted volunteers. Br J Clin Pharmacol 1989;27:587-96.

22 Derkx FHM, Tan-Tjiong HL, Wenting GJ, Boomsma F, Man in 't Veld AJ, Schalekamp MADH. Asynchronous changes in prorenin and renin secretion after captopril in patients with renal artery stenosis. Hypertension 1983;5:244-56.
23 Derkx FHM, Stuenkel C, Schalekamp MPA, Visser W, Huisveld IH, Schalekamp MADH. Immunoreactive renin, prorenin, and enzymatically active renin in plasma during pregnancy and in women taking oral contraceptives. J Clin Endocrinol Metab 1986;63:1008-15.
24 Kitz R, Wilson IB. Esters of methanesulfonic acid as irreversible inhibitors of acetylcholinesterase. J Biol Chem 1962;237:3245-9.
25 Derkx FHM, van den Meiracker AH, Fischli W, Admiraal PJ, Man in't Veld AJ, van Brummelen P, Schalekamp MADH. Nonparallel effects of renin inhibitor treatment on plasma renin activity and angiotensins I and II in hypertensive subjects. An assay-related artifact. Am J Hypertens 1991;4:602-9.
26 Cumin F, Evin G, Fehrentz JA, Seyer R, Castro B, Menard J, Corvol P. Inhibition of human renin by synthetic peptides derived from its prosegment. J Biol Chem 1985;260:9154-7.
27 Yamauchi T, Nagahama M, Watanabe T, Ishizuka Y, Hori H, Murakami K. Site-directed mutagenesis of human prorenin. Substitution of three arginine residues in the propeptide with glutamine residues yields active prorenin. J Biochem (Tokyo) 1990;107:27-31.
28 Heinrikson RL, and Poorman RA. in *Hypertension: pathophysiology and management* (Laragh, JH, and Brenner BN, eds) pp 1179-96, Raven Press new York
29 Mullins JJ, Peters J, Ganten D. Fulminant hypertension in transgenic rats harbouring the mouse Ren-2 gene. Nature 1990;344:541-4.

2.2 Probing Epitopes on Human Prorenin During Its Proteolytic and Non-Proteolytic Activation

Summary

The conformational changes of prorenin (PR) that are associated with its reversible non-proteolytic activation and irreversible proteolytic activation were monitored with immunoradiometric assays, using antibodies against epitopes belonging to the propeptide or the renin-part of PR. Binding of PR to the renin inhibitor remikiren or protonation of PR resulted in the slowly progressive and simultaneous expression ($t_{1/2} \cong 3.5$-5.0 hours at 4°C) of epitopes of the N-terminal and C-terminal halves of the propeptide and an epitope that is manifest on renin but not on native non-activated PR. During reversible PR activation-inactivation, expression and disappearance of these epitopes coincided with the appearance and disappearance of enzyme activity. Cleavage of the propeptide from the renin-part of PR by plasmin, as demonstrated by the failure of remikiren to unmask the N-terminal and C-terminal propeptide epitopes, was, with some time lag, followed by the simultaneous expression ($t_{1/2} \cong 60$ minutes at 4°C) of the renin-specific epitope and enzymatic activity. Based on these findings we propose a model for the non-proteolytic activation of PR that involves the formation of an intermediary form of activated PR with the following properties: 1) the covalently bound proptide has moved out of the active-site cleft, so that binding sites are exposed to active-site ligands, 2) the propeptide is still not in the 'relaxed'conformation that is characteristic for fully, non-proteolytically, activated PR, and 3) the N-terminal part of the renin polypeptide chain has not yet attained the proper location that is required for enzymatic activity.

Introduction

Prorenin is the enzymatically inactive biosynthetic precursor of the aspartic protease renin (EC 3.4.23.15), which serves important functions in blood pressure regulation and body fluid homeostasis. Prorenin is converted to renin by cleavage of the N-terminal propeptide. Inactive prorenin is also activated non-proteolytically during exposure to low pH (acid-activation)[1,2] or low temperature (cryoactivation).[3,4] Activation also occurs when prorenin reacts with active-site-directed ligands.[5] Despite the fact that the complexes of activated prorenin with the ligands are devoid of enzymatic activity, the term 'activation' is used here to indicate that the ligands induce a conformational change of prorenin that also occurs during acid-activation and cryoactivation. This change in conformation is slowly reversible, so that prorenin remains active for some time after pH or temperature have been restored.

Aspartic proteases like renin have a bilobal three-dimensional structure with a deep cleft between the lobes, in which the active site is located. In the enzymatically inactive aspartic protease zymogen pepsinogen the propeptide is in the cleft, so that substrates cannot reach the active site. Acid-activation of pepsinogen is initiated by a reversible conformational change of the propeptide, by which the enzyme's active site becomes exposed.[6] The active site then takes part in the intra- and intermolecular proteolytic

reactions by which the propeptide is removed from the pepsin-part of the molecule. The initial reversible phase of pepsinogen activation may well be analogous to the reversible non-proteolytic activation of prorenin.

It has been reported that acid-activated prorenin reacts with antibodies directed against epitopes of the propeptide, whereas native prorenin does not.[7,8] We therefore decided to use monoclonal antibodies directed against epitopes of the propeptide to monitor the conformational changes that occur during acid-activation and cryoactivation and during activation with an active-site-directed renin inhibitor. We also used a monoclonal antibody directed against an epitope that is present on the renin-part of prorenin but is not manifest on native, enzymatically inactive, prorenin. The rate of expression of these epitopes during activation was determined and compared with the increase in enzymatic activity over time.

Materials

Antibodies. Five mouse anti-human (pro)renin monoclonal antibodies were used. These antibodies (mAbs) were >98% pure, as determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. mAbs R3-36-16 and R3-27-5 were a gift from Drs. J. Wood and M. de Gasparo (Novartis, Basle, Switzerland). Biotinylated mAb R3-36-16 and ^{125}I-labeled mAb R1-20-5 were purchased as part of a commercial kit from Nichols Institute Diagnostics, Wijchen, The Netherlands. mAbs F257-16-2C3 and F258-37-B1 were kindly provided by Dr. S. Mathews (Novartis, Basle, Switzerland). Some characteristics of these mAbs are presented in Table 1.[9] mAbs R3-36-16 and R 3-27-5 do not interfere with each other's binding to renin or prorenin and mAb R 1-20-5 does not inhibit the binding of R3-36-16 to renin. mAb F257-16-2C3 was raised against a synthetic peptide with the same sequence as the 23 amino acid N-terminal fragment of the propeptide of prorenin (-p43-Leu to -p21-Leu). mAb F258-37-B1 was raised against

Table 1. Characteristics of monoclonal anti-human renin and prorenin monoclonal antibodies

Monoclonal Antibody	Localization of binding epitope	Inhibition of Ang I-generating activity of renin
R3-36-16	on renin and native prorenin	yes[9]
R3-27-5	on renin and native prorenin	yes,partial*[9]
R1-20-5	on renin but not manifest on native prorenin (renin-specific epitope)	yes[9]
F257-16-2C3	on propeptide of prorenin, N-terminal part (prorenin-specific epitope)	no
F258-37-B1	on propeptide of prorenin,C-terminal part (prorenin-specific epitope)	no

* R3-27-5 inhibits enzymatic activity of renin to a fixed degree (30%). This is probably caused by partial steric hindrance or by an allosteric effect, which in both cases would result in a reduction of the substrate turnover.[9]

a synthetic peptide, consisting of the -p24-Arg to -p1-Arg sequence of prorenin, i.e. the C-terminal part of the propeptide.

Renin and Prorenin. Renin is the WHO human kidney renin standard, lot 68/356 (National Institute for Biologic Standards and Control, Potters Bar, Hertfordshire, UK). Recombinant human prorenin was a gift by Dr. Fischli (Hoffmann-La Roche, Basle, Switzerland). It was produced in CHO cells transfected with a vector containing human prorenin cDNA. It was partially purified from culture medium by chromatography on blue Sepharose (Pharmacia LKB Biotechnology Inc.). Briefly, a 26x1.5 cm column of blue Sepharose was equilibrated at room temperature with a 20 mmol/L sodiumphosphate buffer, pH 7.1. Culture medium with recombinant prorenin (9.6 mL) was applied to the column. Elution was performed in two steps i.e. with equilibration buffer and with the same buffer containing 1.4 mol/L NaCl. The flow rate was 1 mL/min and 2.0-mL fractions were collected. Renin content of the fractions was measured by immunoradiometric assay (see below). Prorenin content was determined with the same assay after activation with remikiren. Prorenin eluted in the step with NaCl. Prorenin-containing fractions were pooled and dialysed against a 100 mmol/L sodiumphosphate buffer, pH 7.4, and stored at -80 °C. This preparation contained approximately 8.5 x 10^6 μU/mL renin after proteolytic activation with plasmin (0.5 casein units/mL, 48 h at 4 °C).[2] The intrinsic AngI-generating activity of the non-activated prorenin preparation was less than 0.4 % of the fully activated prorenin. The intrinsic activity rose upon storage to maximally 2 %. The prorenin preparation did not contain prorenin-activating proteases; no activation was observed after 3 hours of incubation at 37 °C.

Chemicals and 'Dilution' Buffer. Remikiren, an active site-directed renin inhibitor, was provided by Dr. Fischli (Novartis, Basle, Switzerland). It is a non-peptide transition-state-analogue with a M_r of 726. The IC_{50} for purified human renin is $0.7x10^{-9}$ M.[10] The K_i for the reaction with human renin is $3x\ 10^{-10}$ mol/L.[11] Only freshly prepared solutions in dilution buffer (see below) were used. Plasmin was from Chromogenix, Mölndal, Sweden. Aprotinin was obtained from Bayer, Germany. 'Dilution buffer' is a 100 mmol/L sodium phosphate buffer, pH 7.4, containing 75 mmol /L NaCl and 0.1 % human serum albumin.

Assays

Table 2 shows some characteristics of the immunoradiometric assays (IRMAs) we used. IRMA-R was carried out as described earlier.[11] In this assay biotinylated mAb R3-36-16 was the primary antibody, which was bound to an avidin-coated bead. The secondary (developing) antibody was ^{125}I-labeled mAb R1-20-5 ($2.5x10^5$ cpm/assay tube, specific activity 740 kBq/mg). The assay was carried out with 200 μl sample or standard and 100 μl labeled antibody. Incubations were performed at 4 °C and incubation time was 6 hours.

The other assays were developed in our laboratory for the purpose of this study. mAbs R3-36-16, F257-16-2C3 and F258-37-B1 served as primary antibodies and were immobilized in Maxisorp Startubes (Nunc, Denmark). To this end 1.5 mg of R 3-36-16 or F 258-37-B1 or 3 mg of F257-16-2C3 were added per Startube and then incubated

Table 2. Characteristics of renin and prorenin immunoradiometric assays

Type of IRMA	Immobilized mAb	Method of immobilization	Developing mAb[1)]	Specificity of assay[2)]
R	R3-36-16	binding of biotinylated mAb to avidin-coated bead	R1-20-5	R : yes PR_n : no PR_a : yes
PR	R3-36-16	coating to Startube	R3-27-5	R : yes PR_n: yes PR_a : yes
PR-N	F257-16-2C3	coating to Startube	R3-27-5	R : no PR_n : no PR_a : yes
PR-C	F258-37-B1	coating to Startube	R3-27-5	R : no PR_n : no PR_a : yes

1) The developing monoclonal antibodies were labeled with ^{125}I.
2) This column shows whether or not renin or prorenin are recognized by the assay and which form of prorenin is recognized; R, renin;PR_n, native inactive prorenin; PR_a, reversibly activated prorenin

for 2 hours at 37 °C in 150 µl of a coating buffer (50 mmol/L sodiumcarbonate, 150 mmol/L NaCl and 0.02 % sodium azide, pH 8.6).[12] Tubes were then washed five times with phosphate-buffered saline (PBS, 150 mmol/L NaCl, 10 mmol/L sodium phosphate, pH 7.4). Any remaining adsorption sites in the tubes were blocked by incubation for 1 hour at 37 °C in PBS/1 % bovine serum albumin. Tubes were stored at 4 °C in this blocking buffer until use. Blocking buffer was then removed by five times washing with PBS. The secondary, developing antibody was labeled with ^{125}I by the Iodogen method according to the instruction of the manufacturer (Pierce, Rockford, IL, USA). Specific radio-activity was 360 kBq/µg.

IRMA-PR, IRMA-PR-N and IRMA-PR-C were carried out in the antibody-coated Startubes with 100 µl sample and 50 µl labeled antibody ($2.5x10^5$ cpm/tube). The samples were not pre-incubated with the immobilized antibody before the radiolabeled antibody was added. Incubation was at 4 °C for 6 hours. Then, the Startubes were washed three times with 2 mL dilution buffer. The remaining radioactivity in the Startubes was measured in a gamma counter.

Prorenin that is 'activated' by the renin inhibitor remikiren expresses all five epitopes against which the monoclonals were directed. We therefore used the recombinant prorenin preparation after it had been activated by remikiren as a standard in each IRMA. For this purpose the prorenin preparation was incubated at 4 °C for 48 hours with remikiren in a

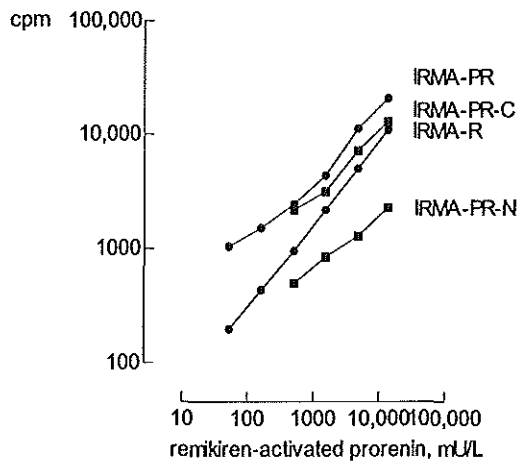


Figure 1. Calibration curves for the immunoradiometric assays used in this study. For characteristics and abbreviations, see Table 2.

final concentration of 100 nmol/mL. This causes complete activation of prorenin.[5] The remikiren-activated prorenin was calibrated against the WHO human kidney standard with the use of IRMA-R. This means that all IRMA results could be expressed as microunit equivalents of the WHO renin standard. Typical examples of standard curves are shown in Fig. 1. The interassay variability was 8 % for IRMA-R and 20 % for IRMA-PR-N and IRMA-PR-C (standard deviation of 8 assays of a standard sample of remikiren-activated prorenin at a concentration of 1500 μU/mL).

Renin and acid-activated prorenin were also measured by the enzyme-kinetic assay.[13] In this assay, renin or activated prorenin were incubated with saturating concentrations of sheep angiotensinogen at pH 7.4 and 37 °C in the presence of inhibitors to block AngI-converting enzyme, AngI-degrading enzymes and serine proteases. The incubation time was reduced to 5 minutes, in order to minimize the inactivation of acid-activated prorenin during incubation at pH 7.4. The AngI that was generated was quantitated by radioimmunoassay. Again, calibration was against the WHO human kidney renin standard, so that results of the enzyme-kinetic assay could be directly compared with the IRMA results.

Experimental Protocols

The following procedures of reversible non-proteolytic activation of prorenin were studied: cryoactivation, acid-activation and activation by remikiren. Prorenin in the activation experiments had a concentration between $8x10^3$ and $14x10^3$ μU/mL. Cryoactivation was performed by incubation of the prorenin-containing samples at 4 °C. Acid-activation was performed by incubation with one volume 0.05 mol/L glycin buffer, pH 3.3, containing 0.1 mol/L NaCl and 5 mmol/L EDTA, at 4 °C.[2] The activated prorenin samples were immediately assayed with the IRMAs. One-mL aliquots were snap-frozen and stored at -70 °C for later enzyme-kinetic assay.

Activation of prorenin was also studied by incubating one volume prorenin with 0.1 volume remikiren in dilution buffer at 4 °C (final concentration of remikiren 100 nmol/mL). Irreversible proteolytic activation of prorenin was studied by incubating one volume prorenin with 0.1 volume plasmin in dilution buffer at 4 °C (final concentration of plasmin 0.5 casein units/mL). At the end of the incubation with plasmin ice-cold aprotinin (final concentration 1000 kallikrein-inibiting units/mL) was added. This was sufficient to stop the proteolytic activation of prorenin.

In the activation experiments with remikiren, prorenin is exposed to the inhibitor not only during the incubation period prior to the IRMAs (pre-incubation) but also during the IRMAs themselves. In order to correct for the assay-related activation, we used the following calculations. The amount of activated prorenin that is measured ($PR_{measured}$) is given by the equation:

$$PR_{measured} = PR_{pre\text{-}incubation} + f \times (PR_{max} - PR_{pre\text{-}incubation}) \quad (1)$$

In this equation $PR_{pre\text{-}incubation}$ is the amount of prorenin that has been activated by remikiren in the pre-incubation period, PR_{max} is the amount of activated prorenin that is measured after all prorenin has been activated by remikiren. The amount of non-activated prorenin that has remained after the pre-incubation with remikiren, is given by the term $PR_{max} - PR_{pre\text{-}incubation}$. A proportion f of this remaining non-activated prorenin is activated during the assay, and is given by the term $f \times (PR_{max} - PR_{pre\text{-}incubation})$. The factor f can be measured by omitting the pre-incubation step and performing the IRMAs in the presence of remikiren; f is then the ratio between the measured amount of activated prorenin and PR_{max}.

Results

Table 3 shows the effects of non-proteolytic and proteolytic activation of prorenin on enzyme activity and the expression of the various epitopes. Incubation of prorenin with 100 nmol/mL remikiren at 4 °C for 48 hours causes full expression of the renin-specific epitope recognized with IRMA-R. Here, this result is taken as 100 percent activation. The term 'activation' is used to indicate that this epitope, which is not manifest on native enzymatically inactive prorenin, is also expressed on renin as well as acid-activated or cryoactivated prorenin, which possess enzymatic activity. Uniform expression of the results of the renin assays as microunits of the WHO renin standard enabled us to make quantitative comparisons between these assays in terms of degrees of activation.

IRMA-R indicated that both acid and plasmin caused complete activation of prorenin and this was confirmed by the enzyme-kinetic assay. IRMA-PR, which recognizes two epitopes that are both present on native prorenin as well as renin, showed that the activation procedures we used did not affect the assay results. Thus, it appears that these activation procedures did not lead to destruction of prorenin. Remikiren as well as acid caused full expression of the N-terminal and C-terminal propeptide epitopes that were recognized by IRMA-PR-N and IRMA-PR-C respectively. These epitopes were not manifest on plasmin-activated prorenin, which lacks the propeptide part of the molecule.

The results of IRMA-R and the enzyme-kinetic assay obtained after incubation of prorenin for 48 hours at 4 °C, were compatible with the well known fact that exposure of

Table 3. Effects of different activation procedures on the prorenin assay results

Pretreatment of prorenin	Type of IRMA				Enzyme-kinetic assay
	PR-N	PR-C	R	PR	
None	10±5	2±3	12±3	74±7	2±1
Remikiren 100 µmol/l 48 h, 4 °C	127±29	98±31	100	101±7	1±1
Plasmin 0.5 CU/ml 48 h, 4 °C	7±8	0±0	131±8	111±2	120±17
pH 3.3 48 h, 4 °C	88±22	92±22	128±11	118±17	87±6
pH 7.4 48 h, 4 °C	12±4	10±1	20±2	80±3	7±2

Data (mean ± SD, n=3) are expressed as percentage of the results obtained in the IRMA-R after maximal activation with remikiren. CU, casein-units.

prorenin to cold causes a small proportion of the prorenin molecules to attain an active conformation that is similar to the conformation of acid-activated prorenin.[3] Surprisingly the results obtained with IRMA-R without any pretreatment of prorenin also indicated some degree of activation, which was not seen with the enzyme-kinetic assay. The activation may have occurred during the incubation step of the assay. This is supported by the results of IRMA-PR-N. This assay, which recognizes the epitope on the N-terminal part of the propeptide, showed some degree of expression of this epitope on prorenin that had not been subjected to any form of pretreatment. An alternative explanation might be that antibody R1-20-5 is not completely renin-specific but crossreacts, to a small degree, with native, enzymatically inactive prorenin.

Results of kinetic studies are shown in Figs. 2-5. We detected no marked differences in activation kinetics between the results of IRMA-R, IRMA-N and IRMA-C, both during treatment of prorenin with remikiren (Fig. 2) or with acid (Fig. 3). There was some indication that, with acid-activation, the C-terminal propeptide epitope might be

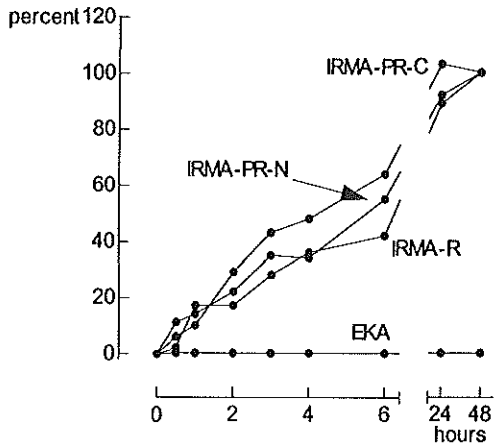


Figure 2. Progression curves for the expression of epitopes on recombinant human prorenin during incubation with the renin inhibitor remikiren (final concentration 100 nmol/ml) at 4°C. Results are means of 3 experiments. No enzymatic activity could be detected. For abbreviations, see Table 2.

expressed at a higher rate than the N-terminal propeptide epitope, but the reproducibility of our assay is not high enough to establish whether this difference is real. Results of the enzyme-kinetic assay showed kinetics of acid-activation that were very similar to those seen with the IRMAs. Kinetics of inactivation (pH 7.4, 37°C) of acid-activated prorenin are shown in Fig. 4. Epitope expression as determined by the IRMAs and enzymatic activity of acid-activated prorenin decreased at similar rates.

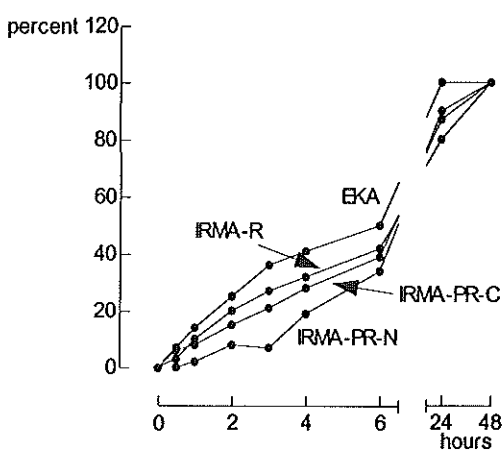


Figure 3. Progression curves for the expression of epitopes on recombinant human prorenin and the development of enzymatic activity during acid-activation (incubation at pH 3.3 and 4°C). Results are means of 3 experiments. For abbreviations, see Table 2.

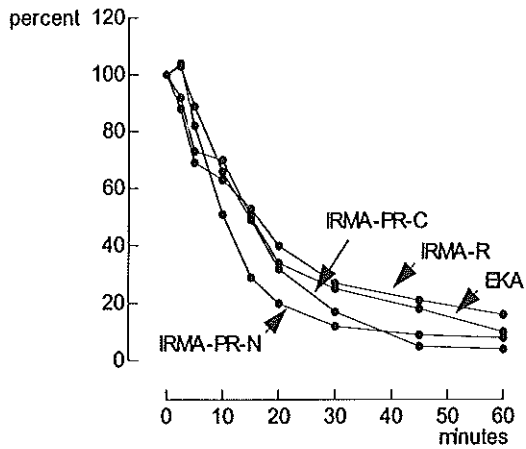


Figure 4. Disappearance of the expression of epitopes and enzymatic activity of acid-activated recombinant human prorenin at pH 7.4 and 37°C. Results are means of 3 experiments. For abbreviations, see Table 2.

During proteolytic activation by plasmin, the appearance of the renin-specific epitope coincided with the appearance of enzymatic activity (half-time approximately 60 minutes) (Fig. 5, left). Our experiments, in which we used the propeptide antibodies and in which the propeptide epitopes were unmasked by remikiren after the incubation with plasmin

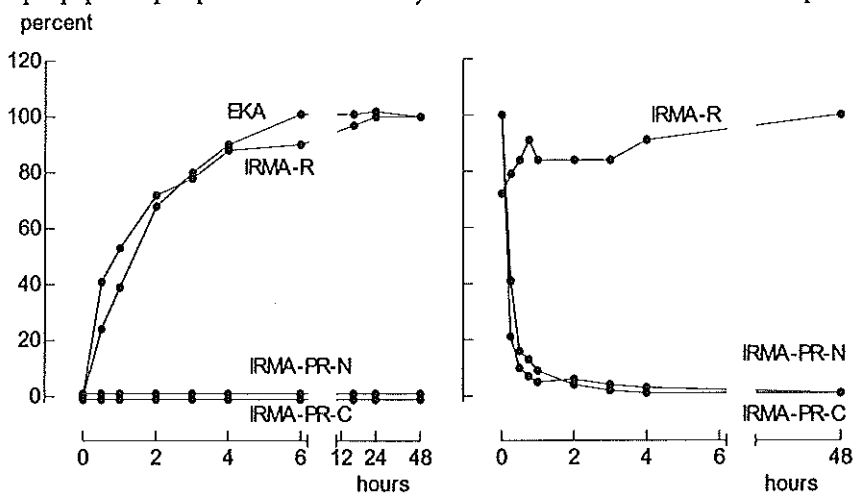


Figure 5. Activation of human recombinant prorenin by plasmin (final concentration 0.5 casein units/ml) at 4 °C. Left: progression curves for the expression of renin-specific and propeptide epitopes and the development of enzymatic activity. Right: same as left, however, the propeptide epitopes that were present in the remaining uncleaved prorenin, were unmasked with remikiren (final concentration 100 nmol/ml, 24 hours at 4 °C). For abbreviations, see Table 2.

(Fig. 5, right), indicated that the N- and C-terminal propeptide epitopes are cleaved off by plasmin at similar rates (half-time approximately 10 minutes). Apparently the cleavage of the propeptide by plasmin proceeds at a higher rate than the appearance of the renin-specific epitope and the appearance of enzymatic activity. We also used the propeptide antibodies in experiments in which prorenin was first treated with remikiren and then with plasmin. Both the N-and C-terminal propeptide epitopes disappeared rapidly (half-time approximately 10 minutes, results not shown), as they did when prorenin was first treated with plasmin and then with remikiren.

Discussion

Prorenin antibodies against peptide sequences of the N-terminal part of the propeptide fail to bind native prorenin,[7,14] but recognize acid-treated prorenin.[7] Antibodies against the C-terminal sequences of the propeptide may[8,15-19] or may not[7,16] recognize native prorenin. Prorenin recognition by these antibodies is enhanced by acid treatment[7,18] or renin inhibitor.[16] The prorenin antibodies we used had been elicited with the N-terminal (-p43-Leu to -p21-Leu) and C-terminal (-p24-Arg to -p1-Arg) halves of the propeptide. Our experiments show that these antibodies bound very little, if any, native prorenin but reacted well with acid-activated prorenin and with prorenin that had been treated with the renin inhibitor remikiren (remikiren-activated prorenin). This is fully compatible with the view that in native prorenin the propeptide is located in the active-site cleft and that for activation to occur, the propeptide has to move out of the cleft.

The propeptide antibodies we used proved most helpful to our study, because they enabled us to monitor the kinetics of the conformational changes of the propeptide during the non-proteolytic activation of prorenin and to do this independently of measurements of enzymatic activity. Such kinetic studies with the use of immunological methods are often not possible, because the conformational changes proceed rapidly relative to the binding rates of the monoclonal antibodies. The propeptide antibodies were also helpful to monitor the cleavage of the propeptide during the proteolytic activation of prorenin.

Our observations suggest a common rate-determining process for both the expression of the propeptide epitopes and the renin-specific epitope and the appearance of enzymatic activity, during activation with acid as well as during activation with remikiren. The activation with remikiren and acid-activation are known to be slow processes.[1,5] The remikiren concentration and the pH in our experiments were chosen in such a way that the rate of activation was close to the maximum rate, which is 0,14-0,20/hour for both procedures.[1,5] We had expected that the binding of the active-site ligand remikiren to prorenin, which displaces the propeptide from its original position in the cleft, would lead to the immediate unmasking of the propeptide epitopes. This apparently is not the case. We therefore assume that, after displacement, the propeptide is still held, for some time, in a 'stressed' conformation that somehow prevents its epitopes to be expressed on the surface of the prorenin molecule.

It has been reported that plasmin cleaves prorenin between -p2-Lys and -p1-Arg.[20] This, however, may not be the only site of cleavage by plasmin. The propeptide contains

a number of peptide bonds adjacent to lysyl and arginine residues that could be susceptible to serine protease attack (-p12, 18, 20, 24, 29, 30, 34, 35). The possibility that N-terminally truncated prorenin forms are generated by plasmin needs therefore to be addressed. Full-length prorenin is inactive but truncation may result in partial or full expression of enzymatic activity. We cannot exclude that our prorenin preparation contained some truncated prorenin, but, if it did, the truncation was not extensive, since our results showed that the whole C-terminal half of the propeptide and at least part of the N-terminal half were still present in prorenin. The intrinsic angotensin I-generating activity of our prorenin preparation was less than 2 % of fully activated prorenin. This may indicate that the amount of truncated prorenin, if present, is very small but it is also

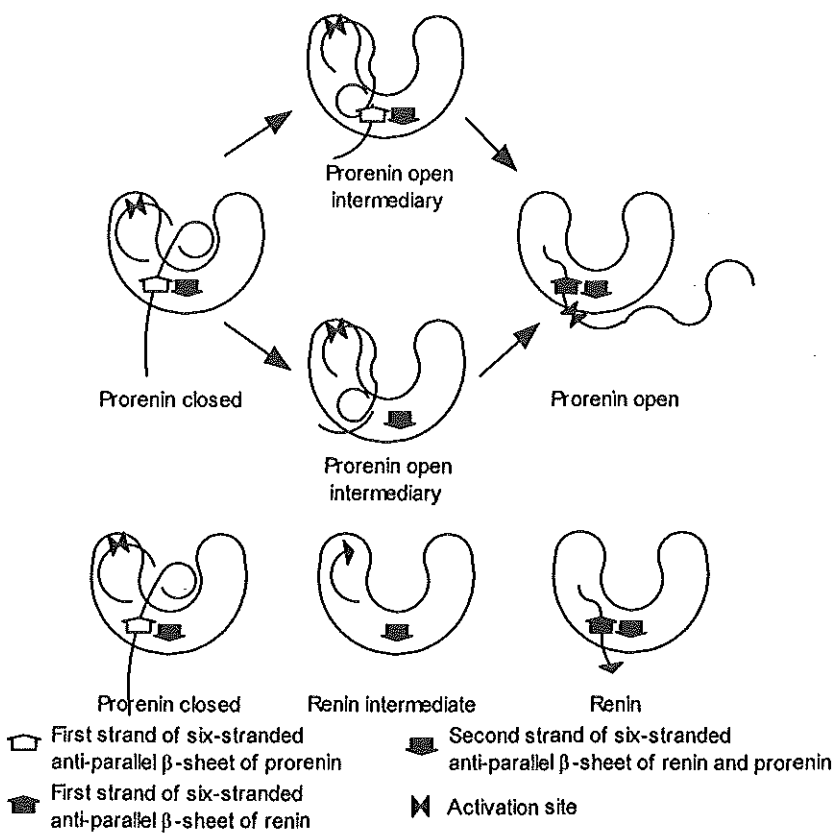


Figure 6. Cartoon depicting various steps in the activation of prorenin. (Upper panel) Proposed model for non-proteolytic activation of prorenin. Two alternative intermediary open prorenin forms are shown, the upper one with the first β-strand of the propeptide *in situ* in the six-stranded β-sheet, the lower one with an incomplete β-sheet. In both forms the propeptide is not accessible to propeptide-specific monoclonal antibodies. (Lower panel) Proposed model for proteolytic activation of prorenin. In both panels β-strands are only shown when they are embedded in the six-stranded antiparallel β-sheet.

Table 4. Some characteristics of various conformational species of renin and prorenin

	Prorenin closed (PR_c)	Prorenin open intermediate (PR_{oi})	Prorenin open (PR_o)	Renin intermediate (R_i)	Renin (R)
Conformation of propeptide	folded in enzyme cleft	out of cleft 'stressed'	out of cleft 'relaxed'	cleaved from enzyme	cleaved from enzyme
Enzymatic activity	absent	absent	present	absent	present
Binding of renin inhibitor	no	yes	yes	yes	yes
Expression of renin-specific epitope on surface of enzyme	no	no	yes	no	yes
Expression of propeptide-specific epitopes on surface of enzyme	no	no	yes	no	no

possible that the truncated form of prorenin has very little enzyme activity. Plasmin at the concentration we used, caused rapid disappearance of the N- and C-terminal propeptide epitopes. The disappearance rates were not different between the two epitopes and there was no evidence for the formation of a stable truncated form of prorenin lacking the N-terminal epitope.

The disappearance rate of the propeptide epitopes following treatment with plasmin, was similar for native prorenin and remikiren-activated prorenin, which indicates that in native prorenin the activation site is not hidden in the cleft in the same way as the two propeptide epitopes are. X-ray-diffraction analysis of the three-dimensional structure of pepsinogen supports that the cleavage site for the non-intramolecular proteolytic activation of pepsinogen is also located on the surface of the molecule.[6] Our results further suggest that proteolytic cleavage at the activation site of prorenin is not immediately followed by the appearance of enzymatic activity. A rate-determining conformational change seems to be required after the propeptide has been cleaved from the renin part of the molecule.

On the basis of our results we propose the sequence of events as presented in Fig. 6 and Table 4. This scheme also incorporates the knowledge about the structure and

activation of other aspartic proteases. From studies of pepsinogens and pepsin-like enzymes it is inferred that in these enzymes, which have a bilobal three-dimensional structure, a six-stranded antiparallel β-sheet is situated at the centre where the two lobes are connected; three strands belong to the N-terminal lobe and three to the C-terminal lobe.[21] The proper three-dimensional conformation of this β-sheet is thought to be essential for enzymatic activity. In the zymogen conformation, the first strand of this sheet belongs to the N-terminal part of the propeptide. In the active enzyme conformation, the first strand of this sheet is formed by amino acid residues in the N-terminal part of the enzyme part of the molecule. In the scheme of Fig. 6, it is assumed that the proper positioning of the N-terminal part of the propeptide in the six-stranded β-sheet, is important for keeping prorenin inactive, and that the proper positioning of the N-terminal part of the renin polypeptide chain to form the six-stranded β-sheet, is important for prorenin to attain its active conformation. The difference betweeen the two alternative pathways of non-proteolytic activation shown in Fig. 6, depends on whether or not the intact propeptide can move out of the active-site cleft while the N-terminal part of the propeptide remains in its original position in the six-stranded beta-sheet and is still in the 'stressed' conformation. These alternative pathways correspond with the pathways proposed for the early stages of the non-proteolytic activation of pepsinogen at low pH.[6] In pepsinogen, the eight N-terminal residues (-p44-Leu to -p37 -Arg) form the first strand. Sequence alignment indicates that in prorenin the residues -p36-Phe to -p29-Arg form the first strand.[22] The assumption that residues belonging to this strand are important to keep prorenin inactive, is supported by studies of truncated forms of prorenin. In these studies it was found that prorenin missing the first 9 N-terminal amino acids had little enzyme activity, whereas prorenin missing the first 14 N-terminal amino acids was fully active.[20] Further support is provided by studies on inhibition of active aspartic proteases by synthetic peptides corrresponding to the first β-strand of the propeptide. For instance, porcine pepsin is strongly inhibited by a peptide consisting of the first 11 amino acids of the propeptide (-p44-Leu to -p34-Ser).[22,23] Similarly, human renin is inhibited by the peptide -p38-Thr to -p27-Pro, which includes the first beta-strand of the propeptide.[22,24]

Our results provide more insight into the processes involved in the reversible activation of prorenin. This may help to answer the question of whether the open, enzymatically fully active, form of prorenin exists in vivo and whether the closed, enzymatically inactive, form of prorenin is reversibly activated in vivo. More detailed knowledge of the molecular basis of the reversible activation of prorenin might eventually lead to the development of agents that prevent the activation. Based on Figure 6 for instance, one can envisage that peptides or non-peptide analogs corresponding with amino acid sequences of the N-terminal part of the propeptide can act as activation inhibitors. Finally, a better insight into the reaction mechanisms that lead to the non-proteolytic activation of prorenin might be applicable to other asparticproteases including human immunodeficiency virus protease.

References

1 Lumbers ER. Activation of renin in amniotic fluid by low pH. Enzymologia 1971;40:329-36.
2 Derkx FHM, Schalekamp MPA, Schalekamp MADH. Two-step prorenin-renin conversion, J Biol Chem 1987;262:2472-77.
3 Osmond DH, Cooper RM. Cryoactivation and tryptic activation of blood prorenin in normal man and animals. Can J Physiol Pharmacol 1978;56:792-805.
4 Pitarresi TM, Rubattu S, Heinrikson R, Sealey JE. Reversible cryoactivation of recombinant human prorenin. J Biol Chem 1992;267:11753-59.
5 Derkx FHM, Deinum J, Lipovski M, Verhaar M, Fischli W, Schalekamp MADH. Non-proteolytic "activation" of prorenin by active site-directed renin inhibitors as demonstrated by renin-specific monoclonal antibody. J Biol Chem 1992;267:22837-42.
6 James MNG, Sielecki AR. Molecular structure of an aspartic proteinase zymogen, porcine pepsinogen, at 1.8 Å resolution. Nature 1986;319:33-8.
7 Price-Jones MJ, Charlton PA, Bessant CM, Harrison TM, Darke BM, Lees WE, Kay J. Analysis of latent forms of renin using antibodies raised against the propart segment of human prorenin: validation with representative samples of ovarian cyst and follicular fluids. Clin. Exp. Hypertension 1993;15:619-40.
8 Gaillard I, Fehrentz JD, Simon D, Badouaille G, Seyer R, Castro B, Pau B, Corvol P. Analysis of inactive renin by renin profragment monoclonal antibodies. FEBS Lett 1986;207:100-4.
9 Heusser CH, Bews JPA, Alkan SS, Dietrich FM, Wood JM, De Gasparo M, Hofbauer KG. Monoclonal antibodies to human renin: properties and applications. Clin Exp Hypertension 1987;A:1259-75.
10 Fischli W, Clozel JP, El Amrani K, Wostl W, Neidhart W, Stadler H, Branca Q. Ro 42-5892 is a potent orally active renin inhibitor in primates. Hypertension 1991;18:22-31.
11 Derkx FHM, de Bruin RA, van Gool JMG, van den Hoek MJ, Beerendonk CCM, Rosmalen F, Haima P, Schalekamp MADH. Clinical validation of renin monoclonal antibody-based sandwich assays of renin and prorenin, and use of renin inhibitor to enhance prorenin immunoreactivity. Clin Chemistry 1996;42::1051-63.
12 Nielsen MD, Rasmussen PH, Giese . A highly sensitive and reproducible immunoradiometric assay for total human renin using monoclonal antibodies, Iodogen labeling, and polystyrene star tubes. Clin Exp Hypertension 1987;A9:1391-1414.
13 Derkx FHM, Tan-Tjiong HL, Wenting GJ, Boomsma F, Man in't Veld AJ, Schalekamp MADH. Asynchronous changes in prorenin and renin secretion after captopril in patients with renal artery stenosis. Hypertension 1983;5:244-56.
14 Schumacher M, Nanninga A, Delfs A, Mukhopadhyay AK, Leidenberger FA. A direct immunoradiometric assay for human plasma prorenin: concentrations in cycling women and in women taking oral contraceptives, J Clin Endocrinol Metab 1992;75:617-623.
15 Ishizuka Y, Iizumi S, Akiyama F, Hori H, Poorman RA, Lobl TA, Murakami K. Characterization of monoclonal antibodies against human prorenin profragment and identification of active prorenins in plasma. J Biochem 1989;106:430-5.
16 Cohen P, Simon D, Badouaille G, Mani JC, Portefaix JM, Pau B. New monoclonal antibodies directed against the propart segment of human prorenin as a tool for the exploration of prorenin conformation. J Immunol Methods 1995;184:91-100.
17 Kim SJ, Hirose S, Miyazaki H, Ueno N, Higashimori K, Morinaga S,. Kimura T, Sakakibara S, Murakami.K Identification of plasma inactive renin as prorenin with a site-directed antibody. Biochem Biophys Res Commun 1985;126:641-5.
18 Atlas SE, Christofalo PE, Hesson T, Sealey JE, Fritz LC. Immunological evidence that inactive renin is prorenin Biochem Biophys Res Commun 1985;132:1038-45.
19 Naruse K, Naruse M, Hirose S, Nagano M, Kurimoto F, Sakurai H, Zeng ZP, Demura H, Inagami T, Shizume K. Immunoreactive prorenin and its profragment peptide are present in human juxtaglomerular cells. J Hypertension 1988;6(suppl. 4):S426-S428.
20 Heinrikson RL, Hui J, Zürcher-Neely H, Poorman RA. A structural model to explain the partial catalytic activity of human prorenin. Am J Hypertens 1989;2:367-80.

21 Cooper JB, Khan G, Taylor G, Tickle IJ, Blundell TL. X-ray analyses of aspartic proteases. II. Three-dimensional structure of the hexagonal crystal form of porcine pepsin at 2.3 Å resolution. J Mol Biol 1990;214:199-222.
22 Shiratori Y, Nakagawa S, Hori H, Murakami K, Umeyama H.Protein modeling of human prorenin using the molecular dynamics method. J Mol Graphics 1990;8::163-8.
23 Dunn BM, Deyrup C, Moesching WG, Gilbert WA, Nolan RJ, Trach ML. Inhibition of pepsin by zymogen activation fragments. J Biol Chem 1978;253:7269-7275.
24 Cumin F, Evin G, Fehrentz JA, Seyer R, Castro B, Menard J, Corvol P. Inhibition of human renin by synthetic peptides derived from the prosegment. J Biol Chem 1985;260:154-7.

2.3 Molecular Mechanism of Prorenin Activation. Studies with A Site-Directed Mutants at the Proline-4 Position

Summary

We hypothesized that the proline residue at the -4 position in the propeptide of human prorenin is involved in a conformational change of the proptide, probably a *cis-trans* isomerization, which is a prerequisite for both non-proteolytic and proteolytic activation of prorenin. A prorenin mutant in which the proline residue at the -4 position in the propeptide had been replaced by glycin, was compared with wild type prorenin as to its susceptibility to (non-)proteolytic activation. This mutation is expected to preserve the tertiary structure of the propeptide, whilst making any movement at the preceding peptide bond impossible. There was, however, no important difference in activation characteristics between wild-type and mutated prorenin, irrespective of activation mode. The hypothesis that *cis-trans* isomerization at the -4 position is important in prorenin activation has thus been refuted.

Introduction

The aspartic protease renin is produced in juxtaglomerular cells of the kidney. In the dense core granules in these cells, renin is generated from its precursor prorenin by proteolytic removal of a 43 amino acid N-terminal propeptide. The activating protease has not been identified with certainty although various candidates have been proposed.[1-3] By site-directed mutagenesis of the region around the processing site in prorenin Chu et al. have shown that the pair of basic amino acids at position -1 and -2 (*i.e.* in the propeptide) is important for correct processing as well as the proline residue at position -4.[4] Substitution of the proline residue at position -4 by alanine or phenylalanine caused a substantially reduced proteolytic activation of prorenin in mouse pituitary AtT-20 cells that had been transfected with mutated prorenin cDNA. By contrast, AtT20 cells transfected with wild-type prorenin cDNA correctly process prorenin to renin. Alanine or phenylalanine at the -4 position may destroy a reverse turn, which, according to computer calculations, is present at this site. The absence of this reverse turn may cause a conformation of the polypeptide chain that is unfavorable to either binding of a processing enzyme or proteolysis by this enzyme. Substitution of proline at -4 by glycine is expected to conserve this reverse turn, but nevertheless also resulted in reduced prorenin processing in transfected AtT20 cells. This appears to refute the hypothesis that the reverse turn at position -4 *per se* is essential for the processing enzyme.

Prorenin may also be activated non-proteolytically by acid, cold or incubation with a renin inhibitor (see chapter 2.1 and 2.2.). Kinetic experiments reported in these chapters suggest that activation by renin inhibitor occurs by interaction of the renin inhibitor with an intermediary, open form of prorenin that has no enzymatic activity, does not express renin or propeptide-epitopes, but allows access of the renin inhibitor to the active site in the enzymatic cleft of renin. This intermediary form is in equilibrium with the native form. It is not known if this intermediary form is also the physiological substrate for

prorenin processing enzymes, rather than the closed, native, conformation of prorenin. It could be that the importance of the proline-4 residue for prorenin processing is determined by the *cis-trans* isomerization it may allow in the peptide bond.[5] The postulated intermediary, open prorenin conformation may be a form in which the chain direction has changed at the –4 position. If this is true then this intermediary form is expected not to occur if another residue than proline is present at this site. Non-proteolytic as well as proteolytic activation of prorenin would then be seriously hampered.

In this study we assessed kinetics of non-proteolytic and proteolytic activation of wild type prorenin and a prorenin mutant with the proline residue at –4 replaced by glycin.

Materials and Methods

Materials

Prorenin Variants. Recombinant human prorenins were a gift by Dr. T. Reudelhuber (University of Montreal, Montreal, Canada). They were wild-type human prorenin (WT) and prorenin with glycine instead of proline at the –4 position (P/G-4), *i.e.* the fourth position preceding the cleavage site between the propeptide and the renin-body of the molecule. In AtT-20 cells this mutated prorenin is not processed to renin as is WT human prorenin.[4] Construction of vectors containing prorenin cDNAs has been described previously.[4] Rat pituitary GH4 CL cells had been transfected with these vectors and culture supernatants were used for the experiments. WT and P/G-4 prorenin were partially purified from these culture supernatants by Blue-Sepharose chromatography as described in chapter 2.2. Stock solutions of both prorenins contained about 150,000 and 80,000 μU/mL respectively and were kept in aliquots at – 80 °C. Spontaneous renin activity of the stock was 2.4% and 0.6% respectively.

Chemicals and 'Dilution' Buffer. Remikiren, an active site-directed renin inhibitor, was provided by Dr. Fischli (Hoffmann- LaRoche, Basle, Switzerland). It is a non-peptide transition-state-analogue with a M_r of 726. The IC_{50} for purified human renin is 0.7×10^{-9} M.[6] The K_i for the reaction with human renin is 3×10^{-10} mol/L.[6] Only freshly prepared solutions in dilution buffer (see below) were used. Plasmin was from Chromogenix, Mölndal, Sweden. Aprotinin was obtained from Bayer, Germany. 'Dilution buffer' is a 100 mmol /L sodium phosphate buffer, pH 7.4, containing 75 mmol /L NaCl and 0.1 % human serum albumin.

Assays

Active prorenin and renin were measured by IRMA as described earlier.[7] In this assay biotinylated mAb R3-36-16 is the primary antibody, which was bound to an avidin-coated bead. The secondary (developing) antibody is ^{125}I-labeled mAb R1-20-5 (2.5×10^5 cpm/assay tube, specific activity 740 kBq/μg). The assay was carried out with 200 μl sample or standard and 100 μl labeled antibody. Incubations were performed at 4 °C and incubation time was 3 hours.

Activation Protocols

WT and P/G-4 prorenin were activated by four activation methods. Concentration of prorenin in the experiments was about 10,000 μU/mL. Proteolytic activation was performed by incubation with plasmin at a final concentration of 0.5 CU/mL. Activation was at pH 7.4 and incubation temperature was 37 °C. At 0, 2, 5, 7, 10, 15, 20, 30, 45, 60, 90 and 120 minutes an aliquot was taken and proteolysis was stopped by addition of aprotinin (final concentration 1000 kallikrein inhibiting units/mL). Activation at low pH was performed by addition of 9 volumes 0.05 mol/L glycin buffer, pH 3.3, containing 0.1 mol/L NaCl and 5 mmol/L EDTA, and subsequent incubation at 4 °C. At regular time intervals an aliquot was taken, neutralized in dilution buffer and immediately assayed. Remikiren activation was at 100 μmol/L final concentration at 4 °C. In the activation experiments with remikiren, prorenin is exposed to the inhibitor not only during the incubation period prior to the IRMAs (pre-incubation) but also during the IRMAs themselves. In order to correct for the assay-related activation, we used the following calculations. The amount of activated prorenin that is measured ($PR_{measured}$) is given by the equation:

$$PR_{measured} = PR_{pre\text{-}incubation} + f \times (PR_{max} - PR_{pre\text{-}incubation})$$

In this equation $PR_{pre\text{-}incubation}$ is the amount of prorenin that has been activated by remikiren in the pre-incubation period, PR_{max} is the amount of activated prorenin that is measured after all prorenin has been activated by remikiren. The amount of non-activated prorenin that has remained after the pre-incubation with remikiren, is given by the term $PR_{max} - PR_{pre\text{-}incubation}$. A proportion f of this remaining non-activated prorenin is activated during the assay, and is given by the term $f \times (PR_{max} - PR_{pre\text{-}incubation})$. The factor f can be measured by omitting the pre-incubation step and performing the IRMAs in the presence of remikiren; f is then the ratio between the measured amount of activated prorenin and PR_{max}.

Cryoactivation was performed for 24 h at 4 °C and pH 7.4.

Results

Both WT and P/G-4 prorenin could be activated by remikiren, acidification, incubation at 4°C and plasmin. **Fig.1** shows typical progression curves for activation of prorenin WT and mutants by plasmin, remikiren and acidification. Activation appeared first-order. From these curves activation rate constants were calculated. Results are shown in **Table 1**. For cryoactivation the percentage active prorenin after 24 h is given. The rate constants for the WT and P/G-4 did not differ importantly for plasmin activation. Activation by acid or remikiren was slightly slower. Inactivation of prorenins at 37 °C and pH 7.4 after acid activation was similar for both prorenins.

Table 1. Activation rate of human prorenin, wild-type (WT) and the Pro-Gly mutant (P/G-4) by various methods

Method of activation	Degree or rate of activation	WT	P/G-4
Cryoactivation	% in 24 h	8.4	6.6
acid-activation	k (h^{-1})	0.219	0.167
Remikiren	k (h^{-1})	0.194	0.099
Plasmin	k (min^{-1})	0.019	0.023

Data are means of three experiments

Discussion

Although non-proteolytic activation (by acid, cold or remikiren) was slightly slower in the P/G-4 mutant compared with WT PR, these findings do not support the hypothesis that the proline residue at the –4 position in prorenin is involved in a *cis-trans* isomerization that induces a conformational change, preceding the transition to an active form of prorenin. In other words, the intermediary, open form of prorenin, postulated in chapter 2.1 is not likely to be caused by an isomerization of the propeptide at the proline-4 position.

The proteolytic processing of prorenin is a puzzling subject. According to computer calculation of prorenin structure,[8] the activation site of prorenin is located deep in the active site cleft. It is not sure how proteases reach this site, but a simple explanation may be that the activating site is brought to the surface before cleavage. Hints at this mechanism have been found in experiments which show that acid-treated prorenin is converted to renin by serine proteases at a faster rate than native prorenin.[9] Although the P/G-4 mutant is not processed to renin in the AtT20 expression system, plasmin was able to activate this mutated prorenin fully, indicating that plasmin's mode of activating prorenin is probably different from that of the activating enzyme in AtT20 cells.

Study of prorenins, mutated in their propeptide, have yielded some insight into the problem why prorenin is inactive. Change of arginine residues at the positions –33, -27 and –22 to glycin but not to lysin yielded fully active prorenins, indicating that positive charges of these Arg residues are important to maintain prorenin in an inactive state.[10]

Mutations in the propeptide of prorenin that are closer to the processing site, like the mutants we used in this study, may be useful to identify the processing enzyme that converts prorenin into renin in juxtaglomerular cells. This is illustrated by a recent study[11] that demonstrated that it is not likely that cathepsin B is the processing enzyme, as had been suggested earlier by Wang et al.[2] Identification of the processing enzyme is important, because in the age of transgenesis, it may be possible to knock-out the gene coding for this protease in a kidney-specific manner. This would permit to construct an animal

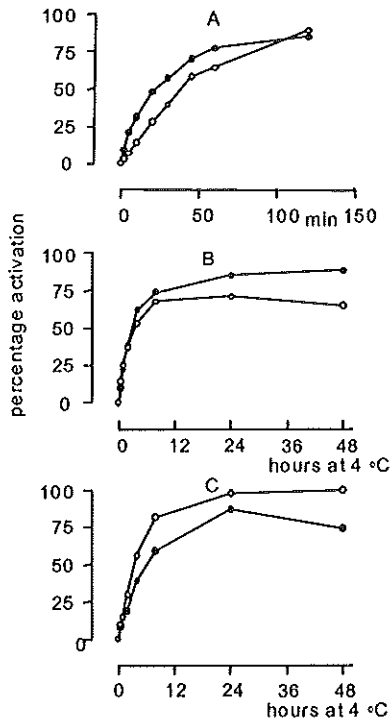


Figure 1. Progression curves for the expression of the renin-specific epitope on wild-type (wt) and mutated prorenin (P/G-4) during incubation with plasmin (A), incubation at pH 3.3 with subsequent neutralization (B) or during incubation with remikiren (100 µM) (C). Open symbols: WT prorenin, closed symbols: P/G-4 prorenin.

model that is essentially a renin-knock out, in which prorenin synthesis is normal. Such an animal would be an excellent tool to study prorenin physiology.

References

1 Hsueh WA, Baxter JD. Human prorenin Hypertension 1991;17:469-79.

2 Wang PH, Do YS, Macaulay L, Shinagawa T, Anderson PW, Baxter JD, Hsueh WA. Identification of renal cathepsin B as a human prorenin-processing enzyme. J Biol Chem 1991,266:12633-8.

3 Heinrikson RL, Hui J, Zurcher-Neely H, Poorman RA. A structural model to explain the partial catalytic activity of human prorenin. Am J Hypertension 1989: 2:367-80.

4 Chu WN, Mercure C, Baxter JD, Reudelhuber TL. Molecular determinants of human processing, Hypertension 1992; 20:782-7.

5 Creighton TE. *In*: Proteins, structures and molecular properties. Freeman and Co., New York 1993, p.312.

6 W. Fischli, J.-P. Clozel, K. El Amrani, W. Wostl, W. Neidhart, H. Stadler, Q Branca. Ro 42-5892 is a potent orally active renin inhibitor in primates. Hypertension 1991;18;22-31.

7 F.H.M. Derkx, R.A. de Bruin, J.M.G. van Gool, M.-J. van den Hoek, C.C.M. Beerendonk, F. Rosmalen, P. Haima, M.A.D.H. Schalekamp. Clinical validation of renin monoclonal antibody-based sandwich assays of renin and prorenin, and use of renin inhibitor to enhance prorenin immunoreactivity Clin. Chemistry 1996;42:1051-63.

8 Shiratori Y, Nakagawa S, Hori H, Murakami K, Umeyama H. Protein modeling of human prorenin using the molecular dynamics method. J Mol Graph 1990;8:163-8.

9 Derkx FHM, Schalekamp MPA, Schalekamp MADH. Two-step prorenin-renin conversion:isolation of an intermediary form of activated prorenin. J Biol Chem 1987;262:2472-7.

10 Yamauchi T, Nagahama M, Watanabe T, Ishizuka Y, Hori H, Murakami K. Site-directed mutagenesis of human prorenin. Substitution of three arginine residues in the propeptide with glutamine residues yields active prorenin. L Biochem 1990;107:27-31.
11 Jutras I, Reudelhuber TL. Prorenin processing by cathepsin B in vitro and in transfected cells. FEBS Lett 1999;443:48-52.

2.4 Cryoactivation of Prorenin is due to Inhibition of Inactivation

Summary

We studied the so-called cryoactivation of prorenin, a process in which prorenin slowly acquires up to 20% of maximal enzymatic activity at low temperature and pH 7.4. Subsequent incubation at 37°C causes a rapid return of prorenin to the inactive state. We incubated recombinant human prorenin at pH 3.3, which causes reversible activation of prorenin (acid-activation), which is similar to cryoactivation. In contrast with cryoactivation, however, acid-activated prorenin is fully activated. We then neutralized acid-activated prorenin at various temperatures and followed inactivation by either an enzyme-kinetic assay for AngI generation or an immunoradiometric assay specific for an epitope of renin and activated prorenin. We also studied activation kinetics of prorenin using a renin inhibitor, remikiren, which induces a conformation of prorenin that is indistinguishable from reversibly activated prorenin.

From our experiments we conclude that the inactivation rate is highly temperature dependent. At 37°C the inactivation rate constant amounts to 5.6 h^{-1}, compared to 0.032 h^{-1} at 4 °C. The maximal activation rate is much less dependent on temperature with values of 0.2 h^{-1} at 4 °C and 0.15 h^{-1} at 37°C. Thus, cryoactivation is due to slow inactivation. Inactivation of reversibly activated prorenin occurs 5 times slower in the presence of angiotensinogen. Reversible activation of prorenin at physiological conditions may well be possible, provided that inactivation can be inhibited. A likely candidate for inactivation inhibition is angiotensinogen. Tissue binding may also keep prorenin in an activated state, although this remains to be proven.

Introduction

So-called cryoactivation of prorenin occurs when prorenin is incubated at neutral pH and low temperature (-4 to +4 °C).[1] It is a slow process, taking seven days to activate up to 26% of prorenin.[2] In plasma cryoactivation is thought to be proteolytic and therefore irreversible. This proteolysis comprises cleaving of the propeptide of prorenin that covers the enzymatic cleft of the renin body of the molecule, thus rendering prorenin enzymatically inactive. Proteolysis depends on the serine proteases kallikrein and factor XII that are activated in the contact activation pathway of the coagulation system.[3,4] At low temperature endogenous plasma protease inhibitors lose their inhibitory activity on these proteases.[5] In the absence of these proteases, *e.g.* in solutions of purified or recombinant prorenin, activation after exposure to cold also occurs, but this activation is reversible.[6] It is thought that this non-proteolytic activation consists of a conformational change, in which the propeptide moves out of the enzymatic cleft, which is located between the two lobes of the renin molecule. Reversibly cryoactivated prorenin is rapidly inactivated again by incubation at neutral pH and 37 °C.[6]

The mechanism of reversible cryoactivation is not known and has not been studied extensively. Pitarresi et al. suggest that hydrophobic interactions between the propeptide

and the renin part, which keep the molecule inactive, are disrupted at low temperature.[6] Another model to understand non-proteolytic cryoactivation, suggested by the experiments in chapter 2.1, is that inactive prorenin is in equilibrium with a small amount of active prorenin, and that this equilibrium shifts to active prorenin because the rate constants of activation and inactivation have different sensitivities to temperature.

The purpose of this study is to investigate the kinetics of non-proteolytic cryoactivation of prorenin. A simple model is assumed in which porenin is at an equilibrium between an open, active form (PR_o) and a closed, inactive, form (PR_c), in which the propeptide blocks the enzymatic cleft.

Materials and Methods

Prorenin and Renin. Recombinant prorenin was a kind gift by Dr. W. Fischli (Hoffmann-La Roche, Basle Switzerland). It had been synthetized in CHO cells, which were stably expressing human prorenin after transfection with a vector containing human prorenin cDNA. Partial purification of the prorenin from culture medium was performed by chromatography on blue Sepharose (Pharmacia LKB Biotechnology Inc) as described in chapter 2.2. The aliquotted stock of partially purified prorenin had a concentration of $1{,}6x10^5$ mU/L and was kept at -80°C. Its AngI generating activity was less than 0.4 % of maximal activity after complete proteolytic conversion of prorenin to renin by plasmin.

Renin is the WHO human kidney renin standard, lot 68/356 (National Institute for Biologic Standards and Control, Potters Bar, Hertfordshire, UK). Angiotensinogen was from plasma from nephrectomized sheep.[7]

Chemicals. Remikiren, an active site-directed renin inhibitor, was provided by Dr. Fischli (Novartis, Basle, Switzerland). It is a non-peptide transition-state-analogue with a M_r of 726. The IC_{50} for purified human renin is $0.7x10^{-9}$ M.[8] The K_i for the reaction with human renin is $3x\ 10^{-10}$ mol/L.[9] Only freshly prepared solutions in dilution buffer were used. Dilution buffer is a phosphate-buffered saline solution at pH 7.4.

Methods

Assays. Active prorenin was measured directly in an immunoradiometric assay (IRMA)[9] and indirectly in an enzyme-kinetic assay (EKA). In the IRMA the biotinylated monoclonal antibody (mAb) R3-36-16 against renin and prorenin is immobilized on an 8mm polystyrene bead coated with avidin. MAb R1-20-5, which is specific for renin and active prorenin but does not recognize inactive prorenin, is the free radio-iodinated antibody. Incubation volume was 400 μL consisting of 100 μL sample or standards, 200 μL incubation buffer containing 1.5 μg of mAb R3-36-16 and 100 μL of ^{125}I-R1-20-5 (approximately $2{,}5 \times 10^5$ cpm/tube). The pH was 7.4 and incubation time was 4 hours at 4 °C. Standards for a calibration line were from a WHO human kidney renin standard in concentrations ranging from 0 to 16000 μU/mL. The indirect, enzyme-kinetic assay uses partially purified sheep angiotensinogen. In brief, samples were incubated with near-saturating concentrations (0.6 μM of sheep angiotensinogen, K_m 0.2 μM[7]) at 37 °C, pH 7.4 for 2-5 min in the presence of AngI-degrading enzyme inhibitors (8-hydroxy quinoline,

phenyl methonsulphonyl-fluoride, disodium EDTA and aprotinin[7]). AngI was quantitated in a radioimmunoassay.[10]

Generation of Active Prorenin. Non-proteolytic, reversible activation of recombinant human prorenin was brought about by dialysisis against a 0.05 M glycin buffer pH 3.3 for 24 h at 4 °C. This causes a reversible activation of all prorenin, since enzymatic activity was identical to that of a sample with the same prorenin concentration after exhaustive proteolytic activation by plasmin (not shown). Final concentrations of prorenin ranged from 8000 to 14000 μU/mL.

Experimental Protocols. For experiments of inactivation, acid-activated prorenin was neutralized in a 0.15 M sodium phosphate buffer, pH 7.4 at the temperature of the experiment. At various time points during each experiment samples were drawn and active prorenin was measured in the EKA and the IRMA. For experiments of activation prorenin was first incubated at 37 °C for 1 h to inactivate any active prorenin. Incubations were then started at the appropriate temperatures. For experiments in the presence of remikiren, prorenin-containing samples were diluted 1:1 in an appropriate remikiren solution. Inactivation in the presence of angiotensinogen was performed by adding reversibly activated prorenin at $t=0$ to a mixture, containing sheep angiotensinogen (0.6 μM final concentration) and protease inhibitors like described above for the EKA. The mixture was incubated at 37 °C. At regular time interval a sample was taken and assayed for AngI content. The disappearance rate of AngI generation was compared with the disappearance rate of AngI generating activity of reversibly activated prorenin, incubated at 37 °C in dilution buffer, from which at regular time intervals samples were taken that were assayed in the EKA.

Analysis of the Results

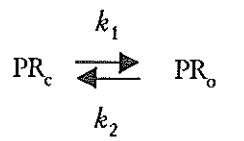
During incubation at 4 °C in the IRMA ongoing inactivation or activation may occur, but this is small compared to the total prorenin concentration (maximally 5% of fully activated or fully inactived prorenin respectively), so we ignored this. Likewise, in the EKA there will be inactivation at 37 °C but with the very short incubation times we also ignored this. When prorenin is activated with the renin inhibitor remikiren there is substantial ongoing activation during the IRMA step, but this was corrected for by the following formula:

$$PR_{measured} = PR_{pre\text{-}incubation} + f(PR_{max} - PR_{pre\text{-}incubation})$$

in which $PR_{measured}$ is the amount of reversibly activated prorenin after pre-incubation and incubation in the IRMA (4 hours at 4 °C). $PR_{pre\text{-}incubation}$ is the amount of prorenin that is activated during the incubation step preceding the IRMA-incubation. PR_{max} is the total amount of prorenin that can be activated. This is found by incubating prorenin for 48 hours with remikiren (100 nmol/mL final concentration). The factor f determines the fraction of inactive prorenin that is activated during the IRMA step. It is found by immediate IRMA incubation of inactive prorenin in the presence of remikiren for 4 h at 4 °C and dividing this value by PR_{max}.

Kinetic Analysis

Results were analysed according to a simple model:



(Scheme A)

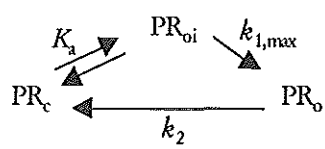
An equilibrium is assumed between an open and a closed form of prorenin (resp. PR_o and PR_c). Open and closed refer to the enzymatic cleft being accessible (open) or not (closed) to substrate and hence, prorenin being active or not. We presume that opening of prorenin is caused by the prosegment moving out of the cleft. The equilibrium between the two states is determined by an activation rate constant k_1 and an inactivation rate constant k_2. Progression curves for activation and inactivation of renin are described by the following equation

$$PR_{o,t} = \frac{k_1 PR}{(k_1 + k_2)} - \left(p - \frac{k_2}{(k_1 + k_2)}\right) PR \times e^{-(k_1 + k_2)t} \qquad (1)$$

PR is the total amount of prorenin (i.e. PR_o plus PR_c). $PR_{o,t}$ is the amount of PR_o at time t and p is the fraction of prorenin that is in the closed form (PR_c) at time 0 (*i.e.* PR_c/PR).

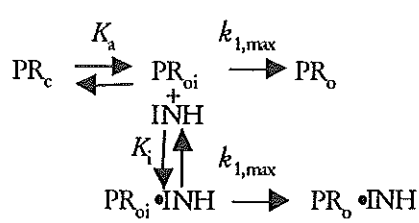
The rate constants were estimated by fitting the equation to the progression curves for either activation or inactivation of prorenin using a PowerMac computer and Graphpad Prism software (Graphpad Software, Inc., San Diego, CA).

Scheme A is a simplified scheme. From the results in chapter 2.1 it can be refined to:



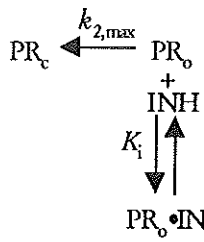
(Scheme B)

K_a is an equilibrium constant. The activating reaction and inactivating reaction in the presence of remikiren (INH) may be dissected as follows:
Forward (activating) reaction



(Scheme C)

Reverse (inactivating) reaction



(Scheme D)

The rate constants for the activating ($k_{1,obs}$, based on scheme C) and inactivating ($k_{2,obs}$, based on scheme D) reaction are given by the following equations:

$$k_{1,obs} = \frac{1+[INH]/K_i}{1+1/K_a+[INH]/K_i} \times k_{1,max} \qquad (2)$$

$$k_{2,obs} = \frac{1}{1+[INH]/K_i} \times k_{2,max} \qquad (3)$$

Even without knowing the exact values of K_i or K_a (estimated to be ~8 and ~40 nM respectively, see chapter 2.1) it can be seen easily that at high inhibitor concentration equation (2) reduces to $k_{1,obs} = k_{1,max}$. Likewise, at INH=0, equation (3) reduces to $k_{2,obs} = k_{2,max}$. If at different temperatures the proportional change in k_2 is similar at different remikiren concentration, then the term $1/(1+[INH]/K_i)$ remains constant. This means that K_i remains constant at different temperatures. The values of $k_{1,obs}$ and $k_{2,obs}$ were estimated by fitting the progression curves at a remikiren concentration of 10^{-4} M and zero respectively, to equation (1).

Results

Activation of Native Recombinant Prorenin and Inactivation of Reversibly Activated Prorenin. Active and inactive prorenin were incubated at pH 7.4 at three temperatures, 37 °C, 22 °C and 4 °C. Progression curves for the fraction of PR_o are shown in **Fig. 1**. From these curves k_1 and k_2 were calculated. This proved to be impossible for the curves at 22 °C and 37 °C, because hardly any activation was measurable (resp. 3 % and 1% of total PR after 24 h). **Table 1** shows the values of k_1 and k_2 when

Table 1. k_1 of the forward reaction and k_2 of the reverse reaction at various temperatures as calculated from data shown in Fig.1

	k_1		k_2	
Temperature	PR_o=0 at t=0	PR_c=0 at t=0	PR_o=0 at t=0	PR_c=0 at t=0
4 °C	0.014/h	0.012/h	0.034/h	0.032/h
22 °C	Not measurable	not measurable	not measurable	0.78/h
37 °C	Not measurable	not measurable	not measurable	5.7/h

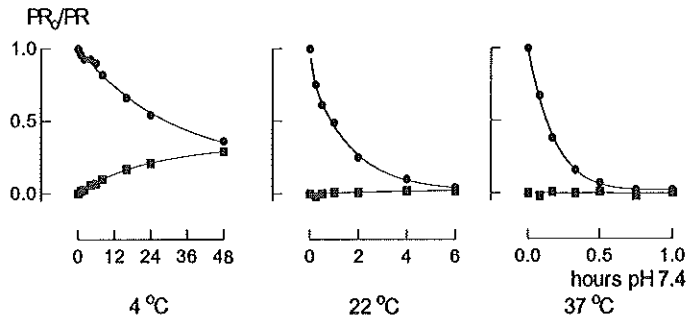


Fig.1. Progression curves for activation (PR_0/PR =0 at t=o, square symbols) and inactivation (PR_0/PR=1 at t=0, circles) of recombinant human prorenin at 4, 22 and 37 °C. Results were obtained by enzyme kinetic assay.

derived from either an activation or an inactivation progression curve. The inactivation constants are resp. 0.032, 0.78 and 5.7 h^{-1} at 4, 22 and 37 °C respectively. Therefore, inactivation appears to be highly dependent on temperature. The results shown in Fig. 1 were obtained using an EKA for active prorenin. Results with the IRMA were not different.

Influence of Remikiren on Inactivation Rate Constant k_2. When remikiren at various concentrations is incubated with active prorenin at pH 7.4 inactivation is slower depending on remikiren concentration (**Fig. 2** and **Table 2**). This effect is not caused by alterations of K_i at different temperatures, since the fractional increase of observed k_2 with decreasing

Table 2. k_2 of the reverse reaction at various remikiren concentrations and temperatures

	k_2			
temperature	remikiren 0 M	remikiren $5x10^{-10}$ M	remikiren 10^{-9} M	Remikiren 10^{-8} M
4 °C	0.032/h	0.015/h	0.011/h	0.002/h
22 °C	0.79/h	0.39/h	0.24/h	0.038/h
37 °C	5.6/h	2.8/h	1.8/h	0.26/h

Data are means of 3 experiments

Table 3. Fractional increase of k_2 with increasing temperature at various remikiren concentrations

Remikiren concentration	$k_{2,\ 37°C}$		$k_{2,\ 22°C}$		$k_{2,\ 4°C}$
0 M	176	:	25	:	1
$5x10^{-10}$ M	188	:	26	:	1
10^{-9} M	171	:	22	:	1
10^{-8} M	135	:	19	:	1

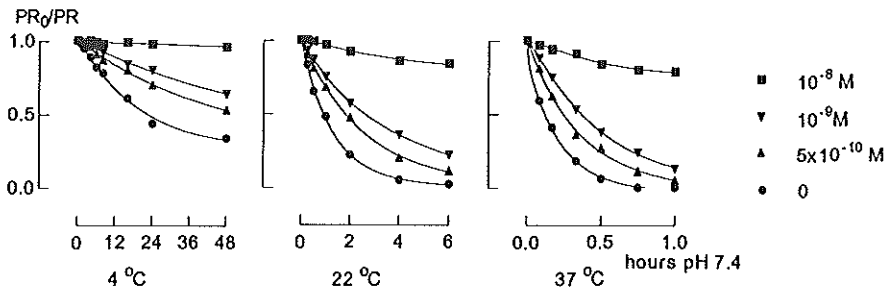


Fig.2. Progression curves of inactivation of recombinant human prorenin at various remikiren concentrations and at 4, 22 and 37 °C. Results were obtained by IRMA.

remikiren concentration was similar at each temperature (Table 3). The results depicted in figure 2 were obtained by IRMA.

Activation Rate Constant k_1 Increases with Increasing Remikiren Concentration but not with Increasing Temperature. At high concentrations of remikiren prorenin inactivation is blocked. This may be used to study the activation rate constant. From Fig. 3 it can be seen that with increasing remikiren concentration faster activation occurs. Influence of temperature on progression curves and, hence, on activation rate constant

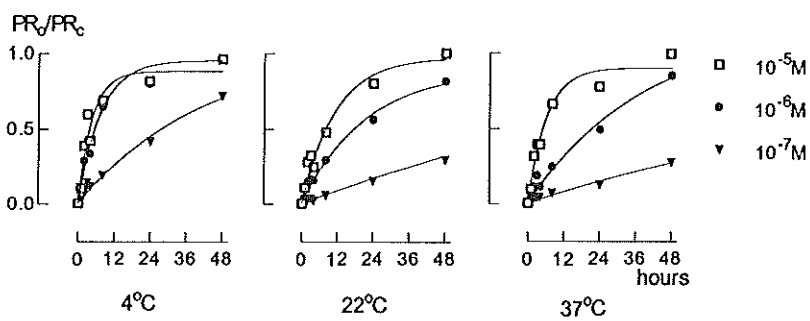


Fig. 3. Progression curves of activation of recombinant human prorenin at various (high) remikiren concentrations at 4, 22 and 37 °C. Results were obtained by IRMA.

Table 4. k_1 of the forward reaction at various remikiren concentrations and temperatures

Temperature	k_1		
	remikiren 10^{-7} M	remikiren 10^{-6} M	remikiren 10^{-4} M
4 °C	0.027/h	0.127/h	0.226/h
22 °C	0.007/h	0.05/h	0.107/h
37 °C	0.007/h	0.034/h	0.154/h

Data are means of 3 experiments. The k_1 values in the rightmost column are $\approx k_{1,max}$ values at the three temperatures

k_i is present but to a much smaller extent than influence on prorenin inactivation (Table 4).
Active prorenin (PR_a) was determined by IRMA in the experiments with remikiren, because the EKA can not be used in the presence of a renin inhibitor.

Inactivation of Reversibly Activated Prorenin in the Presence of Angiotensinogen.
Inactivation of prorenin, as evidenced by a decrease in AngI generating activity, proceeded with a rate constant of 0.03 min^{-1} when angiotensinogen was present. In a purified system, inactivation rate was 0.15 min^{-1} (results not shown).

Discussion

Our study confirms the finding of Pitarresi et al.[6] that recombinant human prorenin can be activated at low temperature. This cryoactivation is a slow process and is rapidly reversed at 37 °C. We studied the temperature dependency of this prorenin activation and inactivation separately. Time course of inactivation was studied by incubation of fully activated prorenin at different temperatures at neutral pH. Inactivation rate constants were highly dependent on temperature, with a 170-fold difference between inactivation rates at 4 °C and 37 °C. Inactivation in the presence of low concentrations of remikiren showed that the interaction between prorenin and renin inhibitor was not temperature-dependent. Activation was studied at high concentrations of remikiren, and the results suggest that temperature has little impact on activation rate constants, in contrast with the effect of temperature on inactivation rate constants.

We assumed that the form of prorenin induced by incubation at pH 3.3 is the same at the moment of neutralization as the form of active prorenin that is generated during cryoactivation at neutral pH. This is likely from studies in which we incubated cryoactivated prorenin at 37 °C. Inactivation rate was identical to inactivation rate of acid-activated prorenin (not shown). Also, epitope expression on cryoactivated prorenin is proportionally identical to acid-activated recombinant prorenin (see chapter 1.1). Nevertheless, we do not have definite proof that our assumption is correct.

From our experiments we conclude that the phenomenon of cryoactivation can be understood as caused by a decrease in the inactivation rate and not by an increase in activation rate. In fact, the activation rate constant is relatively temperature-independent. This is an important insight, because this means that *in vivo* activation will occur at 37 °C at neutral pH provided inactivation of active prorenin can be blocked. One obvious candidate for blocking inactivation of prorenin would be angiotensinogen. Indeed, inactivation of acid-activated prorenin proceeded at a 5 times lower rate in the presence of angiotensinogen. Activation of inactive prorenin by angiotensinogen, however, does not occur at physiological pH, temperature and concentrations of angiotensinogen (not shown), refuting the proposition at the end of chapter 2.1 that angiotensinogen may induce an active from of prorenin. The probable reason for this is that the intermediary form of prorenin, postulated to explain the effect of remikiren (see chapter 2.1), is not accessible to angiotensinogen or that affinity or angiotensinogen concentration are too low. Activation could also occur if some other factor would bind to the prosegment and in this way inhibit prorenin to assume its native conformation. If this inactivation-inhibiting

factor exists, it is probably present at the level of the tissues, because no evidence has been found for such a factor in plasma. It cannot be excluded that the recently described cell-binding of prorenin[11, 12] induces and/or fixes such a conformationally changed prorenin. The concept of a tissue factor causing nonenzymatic activation of prorenin is also supported by a recent article that reports *in-vivo* enzymatic activity of prorenin.[13] Transgenic mice expressing human prorenin and angiotensinogen exclusively in the somatotrophic cells of the pituitary gland, demonstrate angiotensin I production in the pituitary gland. This phenomenon appears to be specific for the extracellular space in tissues, since prorenin catalytic activity is not detectable in the circulation or in supernatants of cells cotransfected with prorenin and angiotensinogen.[13]

The maximal activation rate constant is about 0.2/h (Table 4). This is a low value for a conformational change. If activation of prorenin indeed plays a role *in vivo*, then this relatively slow activation would be a safeguard against very rapidly increasing angiotensin production. On the other hand, it can also be proposed that prorenin activation is not very important *in vivo*, because it is too slow a process.

The phenomenon of cryoactivation, however intriguing, has met relatively little interest, probably because of its slowness and because *in vivo* low temperatures do not occur, making its significance for physiology questionable. Cryoactivation would indeed deserve oblivion if activation rate constants would depend on temperature. The results we now present, however, suggest that prorenin activation is not limited by temperature, provided inactivation is precluded. Further understanding of these processes may therefore yield information on a role of prorenin *in vivo* other than the intrarenal percursor of renin.

References

1 Osmond DH, Cooper RM. Cryoactivation and tryptic activation of blood prorenin in normal man and animals. Canadian J Physiol Pharmacol 1978;56:792-805.

2 Derkx FHM, Bouma BN, Schalekamp MADH. Prorenin-conversion by the contact activation system in plasma: role of plasma protease inhibitors. J Lab Clin Med 1984;103:560-7.

3 Sealey JE, Atlas SA, Laragh JH, Silverberg M, Kaplan AP. Proc Natl Acad Sci USA 1979;76:5914-8.

4 Derkx FHM, Bouma BN, Schalekamp MPA, Schalekamp MADH. An intrinsic factor XII-prekallikrein-dependent pathway activates the human renin-angiotensin system. Nature 1979;280:315-6.

5 Derkx FHM, Schalekamp MPA, Schalekamp MADH. Two-step prorenin-renin conversion: isolation of an intermediary form of activated prorenin. J Biol Chem 1987 262:2472-7.

6 Pitarresi, TM, Rubattu Speranza, Heinrikson R, Sealey JE. Reversible cryoactivation of recombinant human Prorenin. J Biol Chem 1992;267:11753-9.

7 Derkx FHM, Tan-Tjiong HL, Wenting GJ, Boomsma F, Man in't Veld AJ, Schalekamp MADH. Asynchronous changes in prorenin and renin secretion after captopril in patients with renal artery stenosis. Hypertension 1983;5:244-56.

8 Fischli W, Clozel JP, El Amrani K, Wostl W, Neidhart W, Stadler H, Branca N. Ro 42-5892 is a potent orally active renin inhibitor in primates, Hypertension 1991;18:22-31.

9 Derkx FHM, de Bruin RA, van Gool JMG, van den Hoek M-J, Beerendonk CCM, Rosmalen F, Haima P, Schalekamp MADH. Clinical validation of renin monoclonal antibody-based sandwich assays of renin and prorenin, and use of renin inhibitor to enhance prorenin immunoreactivity. Clin. Chem 1996;42:1051-63.

10 Admiraal PJJ, Derkx FHM, Danser AHJ, Pieterman H, Schalekamp MADH. Metabolism and production of angiotensin I in different vascular beds in subjects with hypertension. Hypertension 1990;15:44-55.

11 van Kesteren CAM, Danser AHJ, Derkx FHM, Dekkers DHW, Lamers JMJ, Saxena PR, Schalekamp MADH. Mannose 6-phosphate receptor-mediated internalization and activation of prorenin by cultured neonatal rat cardiac myocytes and fibroblasts. Hypertension 1997;30:1389-96.
12 Sealey JE, Catanzaro DF, Lavin TN, Gahnem F, Pitarresi T, Hu LF, Laragh JH. Specific prorenin/renin binding (ProBP). Identification and characterization of a novel membrane site. Am J Hypertens 1996;9:491-502.
13 Methot D, Silversides DW, Reudelhuber TL. In vivo enzymatic assay reveals catalytic activity of the human renin precursor in tissues. Circ Res 1999;84:1067-72.

Chapter 3 Renin and Prorenin Measurements

3.1 Improved Immunoradiometric Assay for Plasma Renin

Summary

In a previous report we described new IRMAs to measure the plasma concentration of the enzyme renin and its inactive precursor prorenin. Compared to the enzyme-kinetic plasma renin concentration (PRC) assay, the level of renin was overestimated by IRMA, particularly in plasmas with a high prorenin-to-renin ratio. Here we demonstrate that the overestimation of renin was caused not so much by cross-reactivity of the renin-specific antibody with prorenin, but rather by an inadvertent conformational change of prorenin into an enzymatically active form during the assay. The inactive form of prorenin is known to pass slowly into an active form at low temperature. We therefore raised the assay-temperature from 22 °C to 37 °C, and shortened the incubation time from 24 to 6 h. With these modifications, the co-measurement of prorenin as renin was eliminated. Reagents were stable at 37 °C, and IRMA 37 °C, 6h and IRMA 22°C, 24h were comparable in terms of precision and accuracy. The functional lower limit of the assay (4 mU/L) was well below the lower reference limit (9 mU/L). Results of the modified IRMA in clinical samples with low, medium and high levels of renin showed excellent agreement with the levels measured by enzyme-kinetic assay. The IRMA results were not influenced by the plasma concentration of angiotensinogen and were, in plasmas with a normal angiotensinogen level, closely correlated with the results of the classical enzyme-kinetic assay of plasma renin activity (PRA). The modified IRMA takes less than one working day and is simple to perform. Its results are expressed in terms of the internationally recognized human renin standard and, therefore, permit ready comparison between different laboratories.

Introduction

The aspartic protease renin is rate-determining in the proteolytic cascade that leads to generation of Ang II, a key factor in the regulation of blood pressure and the maintenance of water and salt homeostasis.[1] Renin is produced exclusively in the kidney from its enzymatically inactive precursor prorenin. Prorenin is not only produced in the kidney, but in other organs, like adrenal gland,[2] ovary,[3] testis,[4] and the uteroplacental unit,[5] as well. Its secretion into the blood is continuous, in contrast to the tightly controlled release of renin. The level of prorenin in normal plasma is about 9 times that of renin and is much less subject to short-term fluctuations than renin.[6]

Enzyme-kinetic measurements of the so-called plasma renin activity (PRA) are often used to assess the *in-vivo* activity of the renin-angiotensin system, instead of the difficult and labor-intensive AngII measurement. In the PRA assay plasma is incubated at 37 °C. During the incubation renin acts on endogenous renin substrate, angiotensinogen, to generate Ang I. Conversion to Ang II and degradation of Ang I are blocked by protease inhibitors that have been added to the plasma. The *in-vitro* generated Ang I is quantitated by radioimmunoassay. Because angiotensinogen circulates at a concentration that is about

equal to K_m, the rate of Ang I generation in the PRA assay is as much dependent on the concentration of renin as on that of angiotensinogen. Plasma angiotensinogen levels may vary, and PRA is therefore not always a good measure of renin release by the kidney. To overcome this problem, exogenous angiotensinogen can be added to the incubation mixture at a saturating concentration. This is the principle of the enzyme-kinetic plasma renin concentration (PRC) assay.

Recently our group described the clinical validation of an immunoradiometric assay (IRMA) of plasma renin.[7] This assay measures renin directly, is better to standardize and is less laborious than the classical enzyme-kinetic PRA and PRC assays. Our paper, however, sparked a polemic on the specificity of the new assay,[8-10] because in the IRMA some prorenin is measured as renin so that the reliability of the assay at low renin levels might be insufficient. We therefore set out to improve our method, in order to minimize the co-measurement of prorenin.

We assumed that the problem was caused not so much by cross-reactivity of the renin-specific antibody with native, enzymatically inactive, prorenin but rather by inadvertent activation of prorenin during the assay procedure. It is known that prorenin spontaneously undergoes a conformational change by which it becomes enzymatically active (active prorenin). This change does not require the proteolytic cleavage of the prosegment and does not necessarily lead to generation of renin. The non-proteolytic activation of prorenin is time-and temperature-dependent. It occurs mainly at low temperature 4 °C.[11] Our original IRMA was carried out at room temperature. Spontaneous activation of prorenin over time has been detected at room temperature, and is virtually absent at 37 °C.[12] We therefore attempted to prevent the inadvertent activation of prorenin by shortening the incubation time of the assay, and by raising the incubation temperature to 37 °C.

Materials and Methods

Antibodies. The monoclonal antibodies (mAbs) R3-36-16 and R 1-20-5 against renin and prorenin were purchased in a kit by Nichols Diagnostics (San Juan Capistrano, CA). Briefly, the primary mAb, R3-36-16, is a biotinylated monoclonal mouse anti-human antibody, which binds renin as well as active and inactive prorenin. The secondary mAb, R1-20-5, is radio-iodinated (^{125}I) and only binds renin and active prorenin. Detailed information on the antibodies is given in ref. 13. mAb F258-37-B1 was provided by Dr. S. Mathews (Hoffmann-LaRoche, Basle, Switzerland). It was elicited against the C-terminal part of the propeptide of prorenin. The epitope of this part of the propeptide is hidden in native, enzymatically inactive prorenin, but is expressed on the active form of prorenin.[14] Like R-3-36-16 it was biotinylated.[15]

Avidin-coated Plastic Beads, Washing Buffer and Sheep Serum. Biotinylated bovine serum albumin was coupled to 8-mm-diameter polystyrene beads (Precision Plastic Balls Co., Chicago, IL), after which the beads were coated with avidin 15 and stored at 4°C. Washing buffer, phosphate buffered saline with 0.01% Triton X-100, was provided by Nichols Diagnostics. Serum from normal sheep was heat-inactivated for 1 h at 56 °C.

Renin Standards. Recombinant human renin was provided by Nichols Diagnostics. We calibrated this renin preparation against the WHO human kidney renin standard, the International Reference Preparation (IRP), lot 68/356 (National Institute for Biologic Standards and Control, Potters Bar, Hertfordshire, UK)[16] in the enzyme-kinetic PRC assay (see below). Serial dilutions of human recombinant renin to be used for constructing the calibration line were made up in undiluted sheep serum. Sheep serum content of the renin standards was therefore near 100 %, contrary to what was stated in our previous paper[7] on a renin IRMA. Renin standard-dilutions were then lyophilized and stored at 4 °C. Prior to assay, the lyophilisates were reconstituted in distilled water.

Renin Inhibitor. Remikiren, an active site-directed renin inhibitor, was obtained from Hoffmann-LaRoche (Basle, Switzerland).[17] It is a non-peptide transition-state-analogue with a M_r of 726. The IC_{50} for purified human renin is 0.7×10^{-9} M, and the K_i for the reaction with human renin is 3×10^{-10} mol/l.[17] Only freshly prepared solutions of remikiren were used.

Angiotensinogen. Angiotensinogen was prepared from plasma of nephrectomized sheep.[18] The partially purified preparation was dialyzed against 0.15 mol/L PBS, pH 7.4, and neomycin sulphate was added (final concentration 2 g/L). The final preparation had an angiotensinogen concentration of 1.2×10^{-6} mol/L, determined as described below.

Methods

Non-proteolytic Activation of Prorenin. Prorenin was treated with the renin inhibitor remikiren to change the molecular conformation from an inactive form into a form that is recognized by the mAb specific for renin.[19] We refer to this remikiren-induced conformational change of prorenin as 'activation', despite the fact that the prorenin-remikiren complex lacks enzymatic activity. To activate all the prorenin in plasma, we added 1 vol of remikiren (10^{-3} mol/L) to 10 vol of plasma, and incubated the mixture for 24 h at 4 °C.[19]

Assays

Enzyme-kinetic Assays of Renin. To measure PRC, we used our in-house assay, as described peviously.[20, 21] In short, we added 250 μL aliquots of sheep angiotensinogen solution to 25- 250 μL aliquots of plasma, and adjusted the volume to 500 μL with 0.15 mol/L PBS, pH 7.4, and added 17.5 μL of a protease inhibitor solution consisting of 2 vol 0.34 mol/L 8-hydroxyquinoline sulphate, 1 vol of 0.287 mol/L phenylmethylsulfonyl fluoride in ethanol, 2 vol of 0.5 mol/L disodium EDTA, and 2 vol of aprotinin (10,000 kallikrein-inhibiting units/mL). We incubated the mixture at 37 °C for at least two time periods between 0.25 and 3 h in order to verify linear Ang I generation. The final concentration of sheep angiotensinogen in the incubation mixture was 6×10^{-7} mol/L (K_m 2×10^{-7} mol/L). Only incubations in which less than 5% of angiotensinogen was hydrolyzed were accepted to calculate the concentration of naturally occurring renin. Parallel incubations at 0 °C served as blanks. The generated Ang I was measured by RIA.[22] Results of the PRC measurements are expressed as mU/

L, as calibrated with the international renin standard. Under the conditions of the assay, 1 mU of renin generates 163 pmol (212 ng) of AngI per hour. The lower limit of detection is 1 mU/L.

For the PRA assay we used a modification of the method proposed by Sealey et al.[23] The method was modified to include blank subtraction and the 18 hour incubation for low renin samples was eliminated. In this assay 50 μL of maleic acid, pH 5.7, and 12.5 μL of a protease inhibitor solution consisting of 1 vol 0.287 mol/L phenylmethylsulfonyl fluoride in ethanol, 2 vol. 0.5 mol/L disodium EDTA, and 2 vol. 10% (w/v) neomycin sulphate, were added to 0.5 mL plasma. The mixture was then incubated at 37 °C for at least two time periods between 0.5 and 3 h to check for linear Ang I generation. The generated Ang I was quantitated by RIA.[22] Results are expressed as nmol Ang I/L/h. The lower limit of detection was 0.08 nmol Ang I/L/h.

Assay of Angiotensinogen. The concentration of angiotensinogen in plasma was determined as the maximal quantity of Ang I that was generated during incubation at pH 7.4 and 37 °C of plasma to which recombinant human renin had been added at a final concentration of 20,000 mU/L. Results are expressed as nmol/L.[21]

Immunoradiometric Assays. To 200 µL aliquots of untreated plasma, remikiren-treated plasma or renin standards, we added 100 µL of an equivolume mixture of biotinylated mAb R3-36-16 (0.5 µg/L) and radiolabeled (approximately 250 000 cpm) mAb R1-20-5. We incubated this mixture with a polystyrene, avidin-coated bead in duplicate for either 24 h at room temperature (IRMA22 °C, 24h) or for 6 h at 37 °C (IRMA37 °C, 6h). After incubation we washed the beads three times and transferred the washed beads to a clean tube. Radioactivity of bound antibody was counted for 5 min in a gamma-counter. The assay measures renin in the untreated plasmas and renin + prorenin in the remikiren-treated plasmas. Prorenin is the difference between the results of the two measurements.

In order to test our hypothesis that co-measurement of prorenin as renin was caused by a conformational change of prorenin we also performed another, two-step sandwich assay. This assay is a modification of an assay we described earlier[14] and measures prorenin levels directly after remikiren activation. In this assay an avidin-bead carrying 1 μg of biotinylated F258-37-B1, was incubated for 6h at 37°C or for 24h at 22 °C with plasma from a patient with high plasma prorenin (prorenin 2757 mU/L, renin 53.1 mU/L. This F258-37-B1-bead can only bind the active form of prorenin by its exposed propeptide,[14] but will not bind renin and native, inactive prorenin. Half-time of binding of active prorenin to the bead at 37 °C is about 12 minutes. After incubation we rinsed the bead three times with 2 mL of phosphate buffered saline, containing 0.1% bovine serum albumin, to remove all renin and inactive prorenin. Then we incubated the bead with sheep serum containing remikiren (10^{-4} M) and radiolabeled R 1-20-5 for 24 h at 22 °C. The tracer antibody binds the active prorenin trapped on the bead. We added remikiren in this second step to ensure that any active prorenin captured on the bead in the first step, is kept in its active conformation. After incubation we washed the beads with 3x2 mL washing buffer and we counted bound radioactivity after transferral to a clean tube.

A standard line of remikiren-treated recombinant human prorenin in sheep serum was used.[14] The lower limit of detection of this assay is about 10 mU/L active prorenin. The interassay coefficient of variation at levels below 100 mU/L is about 10%.

Collection of plasma samples

Plasma samples were from normal subjects, patients with essential hypertension without therapy or on enalapril, patients with renovascular hypertension without therapy or on enalapril, patients with hepatic cirrhosis and patients with primary aldosteronism (Conn's syndrome). Individuals with conditions where a high prorenin-to-renin ratio is not unusual, i.e. pregnant women, women with ovarian hyperstimulation, women with preeclampsia, women taking oral contraceptives and patients with diabetes mellitus, were also studied. Blood sampling was performed according to our in-house protocol; after cannulation of an antecubital vein and 30-45 minutes supine rest, blood was collected into tubes containing 0.2 mL of 0.646 mol/L citrate per 10 mL blood. The blood was immediately centrifuged at room temperature, and plasma was stored at -20 °C. Shortly before each assay plasma samples were rapidly thawed and kept at room temperature.

Results

Performance characteristics of the renin IRMA at 37°C

Calibration line. Fig. 1 depicts a comparison of the calibration line of IRMA 37°C, 6h with the calibration lines of IRMA 37°C, 24 h, and of IRMA 22°C, 24h. Results, expressed

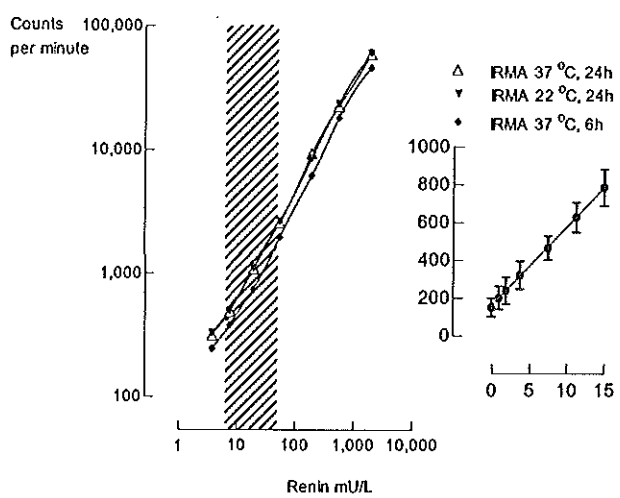


Figure 1. Calibration lines of IRMAs. Ordinate: total binding of labeled antibody. Abscissa: renin concentration. The shaded area represents the normal range. *Inset*: low range of the calibration line of IRMA 37 °C, 6h (mean ± 3 SD).

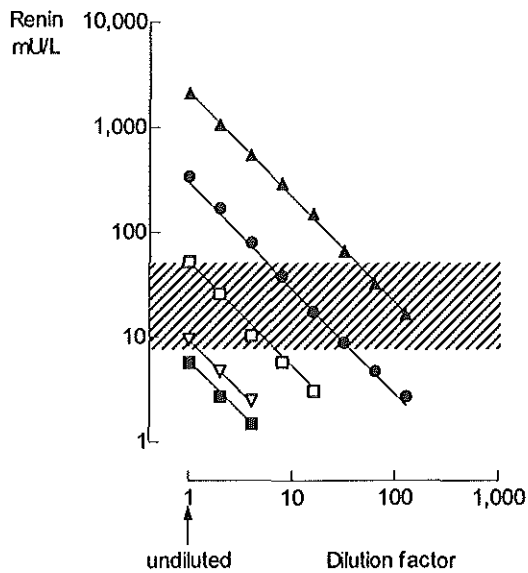


Figure 2. Plasma dilution tests for plasmas from a patient with renal artery stenosis, treated by enalapril (▲), a patient with renal artery stenosis, untreated (•), a pregnant woman (□), a patient with low-renin hypertension (▽) and a patient with Conn's syndrome (■). Ordinate: results of IRMA 37 °C, 6h. The shaded area represents the normal range.

Table 1. Recovery of human kidney renin added to plasma

Plasma	Renin added mU/L	Renin concentration measured before addition of renin mU/L	Renin concentration measured after addition of renin mU/L	Recovery of added renin %
A	10	7.4	17.1	97
B	20	10.1	30.2	102
C	50	24.5	75.6	102
D	50	25.1	75.6	101
E	50	28.1	80.1	107
F	10	37.0	46.5	95
G	20	42.3	61.9	98
H	70	53.1	115.4	89
H	130	53.1	203.9	116
I	130	155	265.5	85
I	260	155	446	110

as counts per minute, were linear over a wide range of renin concentrations. IRMA 37°C, 24h and IRMA 22°C, 24h produced identical results. It appears therefore that the reagents were stable at 37°C. The calibration line of IRMA 37°C, 6 h was parallel to those of the 24h-IRMAs but results were 25% lower. This difference was fully explained by the first-order rate constant for the binding of the radiolabeled mAb to renin at 37°C (0.23 h^{-1}, data not shown). For practical purposes we chose IRMA 37°C, 6h instead of IRMA 37°C, 24 h as our routine assay, although the quantity of radiolabeled mAb trapped by renin was somewhat lower after 6 h than after 24 h.

Linearity was tested by preparing dilutions of plasma samples with renin ranging from 5.7 to 2106 mU/L. Dilutions were made up in the same matrix, *i.e.* sheep serum, as used in the calibration line. Plots of the results, expressed as mU/L, against the dilution factor gave straight lines with the correct slope (**Fig. 2**).

Analytical recovery. The human kidney renin IRP was added to plasma samples with renin concentrations ranging from 7.4 to 155 mU/L. As shown in **Table 1** results of IRMA 37°C, 6h demonstrate excellent analytical recovery of both low and high quantities of added renin. The mAbs we used are capable of inhibiting the AngI-generating activity of renin.[13] To investigate whether angiotensinogen might interfere with the binding of renin to these antibodies, we added the human kidney renin IRP to a plasma, obtained from a pregnant woman. This plasma (plasma D in Table 1) had an angiotensinogen concentration of 3208 nmol/L, which is about 3 times normal. Angiotensinogen, at this high concentration, did not influence the results of IRMA 37 °C, 6h (see Table 1).

Assay variation and sensitivity. As shown in Fig.1, the detection limit of IRMA 37°C, 6h, defined as 3 SD above the zero-standard was 1.3 mU/L. The zero-standard of this standard line gave about 150 counts per minute, and the 4 mU/L-standard about 300 counts per minute. Thus, at this low renin concentration, the result is close to the blank. This raises concern about the reliability of renin measurements by IRMA in the low

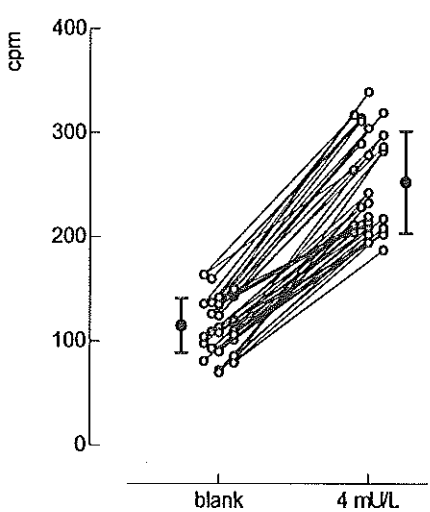


Figure 3. Binding of labeled antibody to zero-standard-beads (blank) and to the lowest, 4 mU/L, renin standard-incubated beads, for all 32 IRMAs, 37C, 6h performed from January 1st, 1998 through September 1st 1998. Indicated are the mean ± SD on the left and right for the blank and the 4 mU/L standard respectively.

Table 2. Intraassay and interassay CVs determined in plasma samples

Renin concentration (Enzyme-kinetic PRC assay)	Intraassay CV (%) n=6			Interassay CV(%) n=10		
mU/L	IRMA 22 °C, 24h	IRMA 37 °C, 6h	Enzyme-kinetic PRC assay	IRMA 22 °C, 24h	IRMA 37 °C, 6h	Enzyme-kinetic PRC assay
3.6	11.3	9.5	8.2	25.5	21.5	14.5
7.3	5.9	7.6	6.2	13.5	16.4	9.6
34.3	4.9	3.9	3.6	6.6	5.2	5.1
154	3.9	3.8	3.7	5.7	5.3	4.6

range of renin levels. This issue is addressed in Fig. 3 which gives the results, expressed as counts per minute, for human recombinant renin at a concentration of 4 mU/L (specific binding of radiolabeled mAb = total binding by 4 mU/L renin-standard minus the binding by zero-standard (blank), both as the mean value of duplicate measurements). Fig. 3 shows adequate separation between the 4 mU/L-standard and the blank in each of the 32 assays. Variability of the duplicate blanks was low compared to specific binding by the 4 mU/L renin-standard as depicted in Fig. 3. The difference between the duplicate blanks was 13.5 (1-38) cpm or 11 (1-35)% of the mean blank (median(range), n=32). The difference of the duplicate 4 mU/L-standard was 14(1-32) cpm or 6(0-15)% of specific binding.

In Table 2 intra-assay and inter-assay CVs in low-, medium- and high-renin concentration renin plasmas are given for IRMA 37°C, 6 h and are compared with the CVs for IRMA 22°C, 24 h and the enzyme-kinetic PRC assay. Intra-assay CVs were similar for the three types of assay in plasmas with high renin concentration as well in plasmas with medium or normal renin. Interassay CVs in the plasmas with low renin

Table 3. Renin concentration determined in a pool of human plasma and in a dilution of human kidney renin in sheep serum

	IRMA 22 °C, 24h	IRMA 37 °C, 6h	Enzyme-kinetic PRC assay
	mU/L	mU/L	mU/L
Normal plasma pool	30.6±3.6	24.5±1.5	25.1±1.6
Kidney renin	50.2±2.9	51.1±2.8	50.0±1.5

Values are mean±SD, n=3. Plasma samples from 8 normal subjects were pooled; the prorenin concentration of this pool was 238±13 mU/L

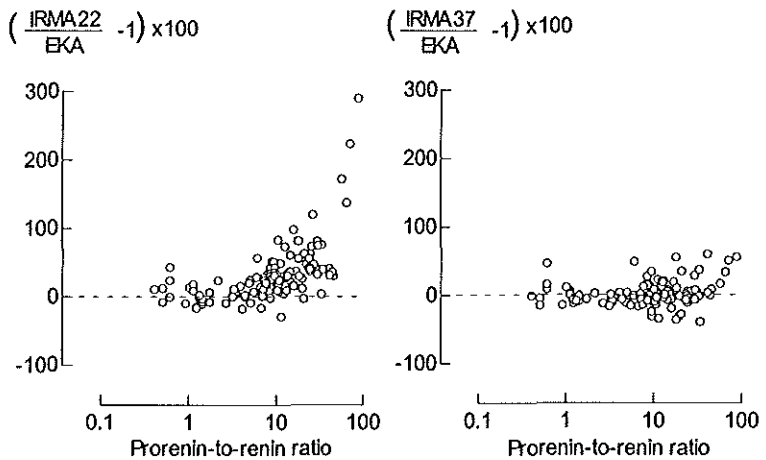


Figure 4. Comparison of IRMA 22 °C, 24 h (IRMA 22) and IRMA 37 °C, 6h (IRMA 37) with the enzyme-kinetic PRC assay (EKA). Ordinate: difference between IRMA and the enzyme-kinetic PRC assay, expressed as a percentage of the enzyme-kinetic PRC assay. Abscissa: prorenin –to-renin ratio as measured by IRMA 37 °C, 6h. Slopes of regression lines for IRMA 22 and IRMA 37 (not depicted) are 41 and 5.9 ($p<0.001$ and NS for comparison with slope=0). Y-intercept is –7.2 and 3.7 % resp. The x-variable is log[prorenin-to-renin ratio]. The outlier most on the right was omitted from analysis

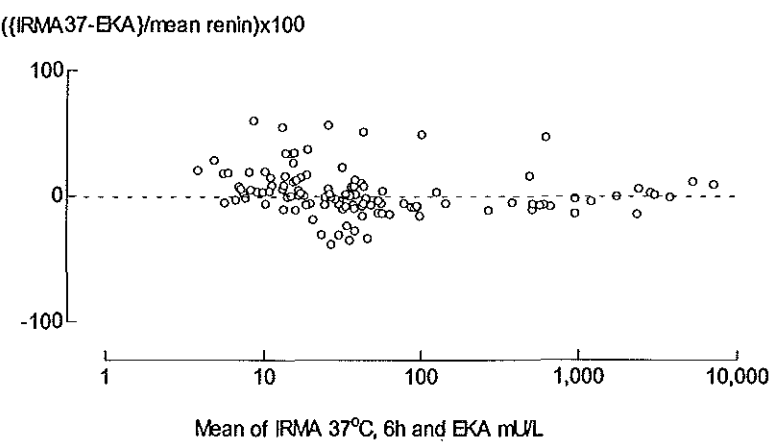


Figure 5. Bland-Altman plot comparing IRMA 37 °C, 6h (IRMA 37) with the enzyme-kinetic PRC assay. Ordinate: difference between IRMA and the enzyme-kinetic PRC assay (EKA), expressed as a percentage of the renin level (mean renin=mean of IRMA and EKA). Abscissa: mean of IRMA and enzyme-kinetic PRC assay. The mean of the difference between IRMA 37 °C, 6h and enzyme-kinetic PRC assay is 0.4%, SD =17% (n=121)

Table 4. Plasma concentrations of renin, renin plus prorenin and angiotensinogen, and the level of plasma renin activity in normal subjects and in various groups of patients

Subject groups	n	Renin			Renin plus prorenin		PRA	Angio-tensinogen	PRA-to-renin ratio
		IRMA 22 °C, 24 h	IRMA 37 °C, 6 h	Enzyme-kinetic PRC assay	IRMA 22 °C, 24 h	IRMA 37 °C, 6 h			
		mU/L	mU/L	mU/L	mU/L	mU/L	nmol Angl/L/h	nmol/L	
Controls	10	29.7a,b 15.9-62.2	24.9 14.6-38.9	23.3 10.2-36.2	198 131-347	202 123-344	2.01 1.15-6.09	918 823-1003	0.079 0.064-0.118
Essential hypertensives									
No treatment	20	11.0a,b 6.7-21.9	9.1 5.4-16.1	8.5 5.0-16.9	139 49-527	138 48-486	0.58 0.19-1.58	1159 729-1628	0.073 0.029-0.114
Enalapril	9	38a,b 20.7-73.3	30.9 17.8-58.0	32.7 13.2-68.2	346 213-559	330 219-470	2.42 1.06-5.97	876 627-1189	0.077 0.029-0.114
Renovascular hypertension									
No treatment	8	100.2a,b 73.3-158	86.3 58-140	94.7 68.1-149	426 346-475	411 385-445	7.43 5.97-9.00	1334 1189-1628	0.079 0.061-0.093
Enalapril	9	527 153-1392	496 128-1188	533 124-1238	1233 405-2034	1171 383-1923	31.9 11.8-78.1	804 526-1139	0.060c 0.038-0.095
Hyperaldosteronism (Conn syndrome)	2	4.4,9.4	4.1,6.4	3.4, 5.4	44.0, 64.1	43.7, 68.2	0.33,0.57	1105,1244	0.081, 0.080
Liver cirrhosis	12	1892 525-6821	1847 528-7436	1777 456-6830	ND	3569 807-4605	27.7 13.5-70.6	252c 115-626	0.015d 0.007-0.040
Diabetes mellitus (insulin-dependent)	8	61.6a,b 45.4-82.4	36.8 24.4-54.4	38.3 25.0-57.5	691 381-904	678 381-859	3.28 1.79-7.08	903 784-1025	0.076 0.061-0.117
Pregnancy	10	67.7a,b 33.7-127	52.5 29.6-121	56.2 30.7-147	648 367-1609	658 426-1348	9.62 5.35-20.8	3535c 2631-4675	0.157d 0.065-0.210
Preeclampsia	9	27.3a,b 7.6-56.3	18.5 5.4-40.4	19.8 4.2-44.0	484 139-1137	485 129-1137	2.83 1.09-4.93	2501c 1723-3711	0.113d 0.064-0.189
Gonadotropin-treated women	12	47.2a,b 19.9-110	27.9 14.0-57.8	27.2 12.4-56.7	ND	914 355-2345	3.31 1.54-7.64	2707c 1563-3981	0.122d 0.076-0.161
Women on oral contraceptives	10	18.7a,b 12.1-36.8	16.9 11.0-34	15.9 9.2-33.0	122 95-152	130 101-135	2.01 0.85-4.68	2884c 1390-6776	0.133d 0.092-0.187

Values are mean and 95% confidence interval. a: Significantly different from IRMA 37°C, 6h, P<0.01; b: Significantly different from enzyme-kinetic PRC assay, P<0.01; c and d: Significantly different from control subjects, P<0.05 and 0.01 resp. ND: not determined

concentration were, however, higher for the IRMAs than for the enzyme-kinetic PRC assay. The functional sensitivity, i.e. the minimum renin concentration that can be measured from assay to assay with < 20% CV, is about 4 mU/L (see Table 2).

Co-measurement of prorenin

Table 3 compares the results of IRMA 37°C, 6 h, IRMA 22°C, 24 h and the enzyme-kinetic PRC assay in a normal plasma pool. The renin concentration in the plasma pool measured by IRMA 37°C, 6 h was equal to that measured by the enzyme-kinetic PRC assay and was about 25% lower than the concentration measured by IRMA22°C, 24h. In contrast, results obtained by IRMA 37°C, 6h and by the enzyme-kinetic PRC assay in a dilution of the human kidney renin IRP in sheep serum were not different from the concentration measured by IRMA 22°C, 24 h. This is an indication that prorenin interferes with the measurement of renin in IRMA 22°C, 24 h. To further prove that a conformational change of prorenin at 22 °C is the culprit of renin overestimation, we also performed at 22 and 37 °C a two-step IRMA that measures the active conformation of prorenin. When in the first step of the assay incubation is performed at 37°C for 6h, very little active prorenin is measured in the second step (18 mU/L, less than 1% of total prorenin and close to the detection limit of the assay). In contrast incubation of the same plasma with the same beads at 22 °C for 24 h yielded a much higher level of active prorenin measured in the second step (149 mU/L, i.e. 5.4% of total prorenin). This indicates that in the course of incubation prorenin is activated and then captured by the F258-37-B1-coated bead at 22 °C, but not at 37°C. This observation could be repeated and was also made in 3 other plasmas with a high prorenin level. Activation of prorenin at 22 °C, but not at 37 °C, also explains the results presented in Fig. 4. In this figure we compare the results of IRMA 22 °C, 24 h and IRMA 37°C, 6h with the results of the enzyme-kinetic PRC assay, in plasmas having different prorenin-to-renin concentration ratios. Overestimation of renin was frequent in IRMA 22°C, 24 h, and was most pronounced at high prorenin-to-renin ratios (Fig.4, left-hand plot). Overestimation of renin was not seen in the IRMA 37 °C, 6h. Fig. 5 depicts a Bland-Altman plot, comparing the IRMA 37 °C, 6h with the enzyme-kinetic PRC assay. Since renin levels ranged over three orders of magnitude and differences were proportional to the renin level, the mean of the two assays was log-transformed and the difference corrected for by the renin level. The plot demonstrates good agreement between IRMA 37°C, 6 h and the enzyme-kinetic PRC assay, in plasmas with a renin level ranging from very low to very high.

In Table 4, the results presented in Figs. 4 and 5 are grouped according to diagnostic categories. Table 4 also compares the three types of renin concentration measurement (IRMA 22°C, 24h; IRMA 37°C, 6h; enzyme-kinetic PRC assay) with the measurement of plasma renin activity (PRA) in different patient groups. The percent overestimation that was seen in IRMA 22°C, 24h was most pronounced in patients with diabetes mellitus, in pregnant women with or without preeclampsia, and in gonadotropin-treated women. These are the patient groups with the highest prorenin-to-renin ratio. IRMA 22°C, 24 h did not overestimate renin in patients with renovascular hypertension treated with enalapril

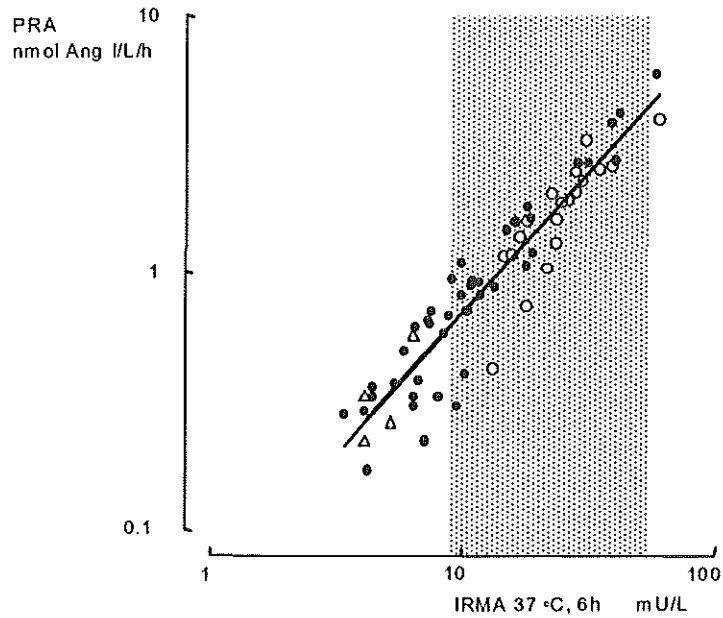


Figure 6. Comparison of IRMA 37 °C, 6h with the enzyme-kinetic PRA assay in patients with Conn's syndrome (triangles), patients with essential hypertension (closed circles) and from control subjects (open circles). The regression line was obtained by a least-square regression analysis after logarithmic transformation of the results. The *y*-intercept (given as the antilog) is 0.056, slope is 1.097, n=65.
The shaded area represents the normal range.

and patients with liver cirrhosis. In these patient groups the prorenin-to-renin ratio is abnormally low.

Many laboratories measure PRA instead of renin concentration. The results of the PRA assay are determined not only by the concentration of renin but also by the concentration of angiotensinogen in plasma. We compared the results of IRMA 37°C, 6 h with those of the PRA assay. Fig. 6 shows the results in normal subjects, in patients with untreated essential hypertension and in patients with Conn's syndrome. The angiotensinogen concentration in these plasma is normal and renin is normal or low. Results of the PRA assay (nmol angiotensin I/L/h)were 0.07 times those of IRMA 37°C, 6 h (mU/L). A similar correlation was seen in other patient groups having normal renin and angiotensinogen concentrations in their plasma (see Table 4). As expected, the PRA-to-IRMA ratio was higher in patients with abnormally high angiotensinogen, and the ratio was lower in patients with abnormally low angiotensinogen.

Discussion

The original IRMA of renin we described previously, was not 100% specific for renin.[7] It also measured a small fraction (0.5-2%) of prorenin. Measurements in plasma therefore

led to an overestimation of renin. In normal subjects and in patients with essential or renovascular hypertension, renin is overestimated by 20% or less but the deviation can be larger in situations in which plasma prorenin comprises more than 90% of total renin such as low-renin essential hypertension, pregnant women, in patients with Conn syndrome and in patients with diabetes mellitus.

The present study demonstrates that this problem is solved in the improved assay. The tertiary structure of renin and prorenin has the form of two lobes separated by a cleft that contains the enzyme's active site.[24,25] In enzymatically inactive, intact, prorenin the propeptide is folded in this cleft so that angiotensinogen cannot reach the active site. This so-called closed form of prorenin is in dynamic equilibrium with an open form where the propeptide, while still covalently linked with the renin part of the molecule, has been displaced from the cleft so that angiotensinogen can now reach the active site.[14] The transition of the closed form into the open, active form takes hours and the equilibrium is shifted towards the open form by lowering the temperature (cryoactivation)[12] or by the addition of active site-directed renin inhibitors.[19] Our results, especially those of the two-step IRMA that captures active prorenin, demonstrate that the co-measurement of prorenin we observed in the original IRMA 22°C, 24h, is caused by the formation of open prorenin. This is almost completely prevented in the IRMA 37°C, 6h.

IRMAs are usually performed at room temperature. One may therefore wonder whether the reagents we used are stable at 37°C. Our observation that the calibration lines of IRMA 37°C, 24h and IRMA 22°C, 24 h were identical strongly suggests that the reagents are indeed stable at 37°C.

The renin standard was diluted in sheep serum in order to minimize unspecific binding of the mAbs. Theoretically, the binding characteristics in the sheep serum matrix might be different from those in human plasma. Additions of the renin standard to human plasma, however, resulted in complete recovery, and serial dilutions of human plasma resulted in decrements of binding that were linearly correlated with the dilution factor. The close agreement between the results of IRMA 37°C, 6h with those of the enzyme-kinetic PRC assay also indicates that the difference in the reaction milieu between plasma samples and standards did not affect the results.

The IRMA 37°C, 6h in plasma samples with a normal or elevated renin concentration was as precise as the enzyme-kinetic PRC assay (interassay CV $< 10\%$). In plasma samples with a low renin concentration (< 10 mU/L), the precision of IRMA 37°C, 6h, was less than that of the enzyme-kinetic PRC assay. The functional sensitivity, defined as the minimum concentration that can be measured from assay to assay with $< 20\%$ CV, was approximately 4.0 mU/L. Our results show this to be sufficient for measuring the concentration of renin in plasmas from patients with suppressed renin, like patients with Conn's syndrome or low-renin essential hypertension.

Many laboratories still use the enzyme-kinetic PRA assay. From our results it appears that the patients who were diagnosed by IRMA 37°C, 6h as having a low concentration of renin in this plasma, also had abnormally low PRA.

The improved IRMA is specific for renin and appears to be a simple, precise and accurate method for measurements in clinical plasma samples. This assay is easier to standardize than the enzyme-kinetic assays that rely on endogenous angiotensinogen like the PRA-assay. Its results are expressed in terms of the internationally recognized human renin standard and, therefore, permit ready comparison between different laboratories.

References

1 Sealey JE, Laragh JH. The renin-angiotensin-aldosterone system for normal regulation of blood pressure and sodium and potassium homeostasis. In: Laragh JH, Brenner BM, eds. Hypertension: pathophysiology, diagnosis and management. New York: Raven Press, 1995:1763-96.

2 Racz K, Pinet F, Gasc JM, Guyenne TT, Corvol P. Coexpression of renin, angiotensinogen, and their messenger ribonucleic acids in adrenal gland. J Clin Endocrinol Metab 1992;75:730-7.

3 Itskovitz J, Sealey JE, Glorioso N, Rosenwaks Z. Plasma prorenin response to hCG in ovarian hyperstimulated women: correlation with the number of ovarian follicles and steroid hormone concentrations. Proc Natl Acad Sci USA 1987;84:7285-9.

4 Sealey JE, Goldstein M, Pitarresi T, Kudlak TT, Glorioso N, Flamengo SA, et al. Prorenin secretion from human testis: no evid ence for secretion of active renin or angiotensinogen. J Clin Endocrinol Metab 1992;75:730-7.

5 Poisner AM, Dowing GJ, Poisner R. Prorenin secretion from villous placenta: regulation by cyclic AMP and angiotensin. Placenta 1994;15:487-99.

6 Toffelmire EB, Slater K, Corvol P, Menard J, Schambelan M. Response of plasma prorenin and active renin to chronic and acute alterations of renin secretion in normal humans. Studies using a direct immunoradiometric assay. J Clin Invest 1989;83:679-87.

7 Derkx FHM, de Bruin RJA, van Gool JMG, van den Hoek M-J, Beerendonk CCM, Rosmalen F, et al. Clinical validation of renin monoclonal antibody-based sandwich assays of renin and prorenin, and use of renin inhibitor to enhance prorenin immunoreactivity. Clin Chem 1996;42:1051-63.

8 Sealey JE, Laragh JH. Renin and prorenin: advances and declines in methodology [Editorial], Clin Chem 1996;42:993-4.

9 Derkx FHM, Schalekamp MADH. More on renin [Letter]. Clin Chem 1997;43:694-5.

10 Sealey JE, Catanzaro D, Laragh JH. [Letter]. Clin Chem 1997;43:695-7.

11 Osmond DH, Cooper RM. Cryoactivation and tryptic activation of blood prorenin in normal man and animals. Can J Physiol Pharmacol 1978;56:792-805.

12 Pitarresi TM, Rubattu S, Heinrikson R, Sealey JE. Reversible cryoactivation of recombinant human prorenin. J Biol Chem 1992;267:11753-9.

13 Heusser CH, Bews JPA, Alkan SS, Dietrich FM, Wood JM, de Gasparo M, et al. Monoclonal antibodies to human renin; properties and applications. Clin. Exp. Hypertens 1987;A9:1259-75.

14 Deinum J, Derkx FHM, Schalekamp MADH. Probing epitopes on human prorenin during its proteolytic and non-proteolytic activation. Biochim Biophys Acta 1998;1388:386-96.

15 Odell WD, Griffin J, Zahradnik R. Two-monoclonal-antibody sandwich-type assay for thyrotropin, with use of an avidin-biotin separation technique. Clin Chem 1986;32:1873-8.

16 Bangham R, Robertson I, Robertson JIS, Robinson CJ, Tree M. An international collaborative study of renin assay: establishment of the International Reference Preparation of human renin. Clin Sci Mol Med 1975;48:135S-59S.

17 W. Fischli, J.-P. Clozel, K. El Amrani, W. Wostl, W. Neidhart, H. Stadler, et al. Ro 4205892 is a potent orally active renin inhibitor in primates. Hypertension 1991;18:22-31

18 Skinner SL, Dunn JR, Mazzetti J, Campbell DJ, Fidge NH. Purification, properties and kinetics of sheep and human renin substrates. Aust J Exp Biol Med Sci 1975;53:77-88.

19 Derkx FHM, Deinum J, Lipovski M, Verhaar M, Fischli W, Schalekamp MADH. Nonproteolytic "activation" of prorenin by active site-directed renin inhibitors as demonstrated by renin-specific monoclonal antibody. J Biol Chem 1992;267:22837-42.
20 Derkx FHM, Tan-Tjiong HL, Wenting GJ, Boomsma F, Man in 't Veld AJ, Schalekamp MADH. Asynchronous changes in prorenin and renin secretion after captopril in patients with renal artery stenosis. Hypertension 1983;5:244-56
21 Derkx FHM, Stuenkel C, Schalekamp MPA, Visser W, Huisveld IH, Schalekamp MADH. Immunoreactive renin, prorenin, and enzymatically active renin in plama during pregnancy and in women taking oral contraceptives. J Clin Endocrinol Metab 1986; 63:1008-15.
22 Admiraal PJJ, Derkx FHM, Danser AHJ, Pieterman H, Schalekamp MADH. Metabolism and production of angiotensin I in different vascular beds in subjects with hypertension. Hypertension 1990;15:44-55.
23 Sealey JE. Plasma renin acitvity and plasma prorenin assay. Clin Chem 1991;37:1811-9.
24 Hsueh WA, Baxter JD. Human prorenin. Hypertension 1991;17:469-79.
25 Szeczi PB. The aspartyl proteases. Scand J Clin Lab Invest 1992;52 (suppl 210):5-22.

3.2 Renin and Prorenin Assays: The Next Generation

Summary

In search of renin and prorenin assays that (1) do not co-measure prorenin as renin, (2) do not use a proteolytic step, which may destroy renin or prorenin, (3) can be easily standardized, and (4) are easy to perform, we compared a traditional enzyme kinetic assay (EKA) for renin, a direct immunoradiometric assay (IRMA) for renin (R-IRMA) and a new, direct IRMA for prorenin (PR-IRMA).

The EKA was performed by measurement of angiotensin I generation from sheep angiotensinogen. R-IRMA was performed by immobilization of renin and prorenin with a renin- and prorenin-binding monoclonal antibody (mAb) R3-36-16. Renin and prorenin treated with the active site-directed renin inhibitor remikiren, were detected by a radiolabeled mAb R1-20-5 that is specific for an epitope expressed solely on renin and remikiren-treated prorenin. PR-IRMA was performed by selective immobilization of remikiren-treated prorenin by a mAb F258-37-B1 against the propeptide, followed by binding of the radiolabeled antibody R1-20-5. Standards in the IRMAs were the WHO kidney renin standard or remikiren-treated recombinant human prorenin, that expresses the renin-specific epitope and the propeptide-epitope, both hidden in native prorenin. Plasma from 6 healthy subjects and 50 with various causes of hypertension were studied as well as 7 patients treated by remikiren.

EKA and R-IRMA gave identical results for renin. EKA and R-IRMA were also used to assess total renin after proteolytic treatment and remikiren treatment respectively. This was used to determine prorenin level indirectly. Comparison with direct prorenin quantification in the PR-IRMA gave identical results in 56 clinical samples. Patients on renin inhibitor therapy showed disproportionally high renin levels, probably caused by inhibitor-induced activation of prorenin.

We conclude that for routine purposes the R-IRMA is reliable for direct renin and indirect prorenin measurement and that a new standard for both renin and prorenin assays could be remikiren treated recombinant prorenin. Measurement of renin and prorenin by both R-IRMA and PR-IRMA may become important with further clinical development of renin inhibitors.

Introduction

Renin is the regulated compound of the renin-angiotensin system, and determines the rate of generation of angiotensin II, the effector of the system. Therefore, plasma renin measurement has been important to study the physiology of the renin-angiotensin system since the early days of renin-angiotensin research.[1] In the clinical setting plasma renin measurement gained a place in the diagnostic work-up of renovascular hypertension and hypokalemic hypertension. Initially renin levels were estimated by the hypertensive response after injection of a renin-containing sample into an animal. Later on, enzyme kinetic assays (EKAs) were developed that measured angiotensin I production by renin from angiotensinogen (for an overview of the various methods see ref. 2). Angiotensin I was quantitated by either bio-assay or by radioimmunoassay. A third phase in renin

measurement methodology was entered some fifteen years ago when antisera and monoclonal antibodies against renin became available, allowing development of direct immunoassays for renin.[3] Despite the long history of renin research and the availability of an international reference preparation[4] little consensus exists regarding which of the aforementioned assays is to be preferred. Because of this, methods abound and differ considerably. Results of renin assays are hardly ever comparable between laboratories and hence, reference values have never been established.

Another problem in renin measurement is the presence in plasma of prorenin, the enzymatically inactive precursor of renin. Its concentration in plasma is on average 9 times that of renin. Prorenin has a structure that is very similar to renin (see chapter 1). Prorenin may be co-measured as renin, either in enzyme-kinetic assays because it acquires enzymatic activity, e.g. after storage in the cold,[5] or in immunologic assays because of its considerable structural homology to renin,[6] or because of, again, a change into an active conformation (see previous chapter). Accounting for contribution of prorenin to measured renin levels is difficult, because few laboratories dispose of direct prorenin assays. The usual way of prorenin measurement is to convert prorenin to renin by proteolytic removal of the propeptide and to measure the the total, i.e. renin plus prorenin content. The difference in renin concentration before and after proteolytic activation is then a measure of the prorenin concentration.

We and others[7] feel that there is a need for specific, standardized assays for renin and prorenin, that preferably avoid a proteolytic step when total renin is to be measured. In the present paper we compared a second-generation, enzyme kinetic assay, the plasma renin concentration (PRC) and two third-generation assays, an immunoradiometric assay for renin (R-IRMA) and a direct prorenin IRMA (PR-IRMA), for their use in renin and prorenin quantification. The EKAs like the PRC assay rely on proteolytic activation of prorenin, but the two IRMAs use remikiren-'activation' as described in chapter 2.1. The PR-IRMA is a new development and in this chapter we describe the performance of this assay. Another new feature is that for both renin and prorenin measurement by IRMAs an identical standard was used. Finally we discuss the application of these assays.

Materials and Methods

Antibodies. Three mouse anti-human monoclonal antibodies (mAbs) were used. Purity was more than 98%, as verified by sodium dodecyl sulphate polyacrylamide electrophoresis. Biotinylated mAb R3-36-16 and ^{125}I-labeled mAb R1-20-5 were purchased as part of a kit from Nichols Institute Diagnostics, Wijchen, The Netherlands. mAb F258-37-B1 was from the laboratories of Hoffmann-La Roche, Basle, Switzerland. mAb R-3-36-16 binds to an epitope that is expressed on both renin and prorenin.[8] mAb R1-20-5 is able to bind to an epitope expressed on renin, but not on native, inactive prorenin.[8] It does, however, bind active prorenin (see chapter 2.2). mAb F258-37-B1 was raised against a synthetic peptide, consisting of the -p24-Leu to -p1-Arg sequence of the propeptide of prorenin, *i.e.* the C-terminal part of the propeptide. This propeptide is absent in renin.

Chemicals and Buffers. Remikiren, an active site-directed renin inhibitor, was provided by Dr. Fischli (Hoffmann-La Roche, Basle, Switzerland). It is a non-peptide transition-state-analogue with a M_r of 726. The IC_{50} for purified human renin is 0.7×10^{-9} M.[9] The K_i for the reaction with human renin is 3×10^{-10} mol/L. Only freshly prepared solutions were used. Sepharose-bound trypsin was prepared by coupling trypsin (Sigma, St. Louis, MO) to CBr-activated Separose 4B (Pharmacia, Uppsale, Sweden).[10] Sheep serum was used as a dilution buffer in the IRMAs. It was purchased at Biotrade, The Netherlands. In addition to irradiation it had been heated at 56 °C for 1 h. Washing buffer was a phosphate buffered saline, supplemented with 0.5% bovine serum albumin.

Renin and Prorenin Standards. Recombinant renin standards were included in the Nichols Institute Diagnostics kit. They were prepared as described previously.[11] Briefly, recombinant renin was calibrated in an enzyme kinetic assay against the WHO human kidney renin standard, lot 68/356 (National Institute for Biologic Standards and Control, Potters Bar, Hertfordshire, UK). Renin standards were made up in sheep serum. Recombinant human prorenin was a gift by Dr. Fischli (Hoffmann-La Roche, Basle, Switzerland). It was produced in CHO cells transfected with a vector containing human prorenin cDNA. Native prorenin is only recognized by mAb R3-36-16 (see chapter 2.2). For use of recombinant prorenin as a calibrator in the IRMAs the renin and propeptide epitopes of prorenin were unmasked by preincubation with remikiren at 10^{-4} M for 48 h at 4°C (see chapter 2.2). Remikiren-treated prorenin stock was diluted in sheep serum, containing 10^{-4} M of remikiren, to establish a range of calibration samples. The renin-specific epitope of these prorenin standards was calibrated with the reference human kidney standard, lot 68/356, in the renin IRMA

Avidin-coated Plastic Beads. Polystyrene beads with a diameter of 8 mm, coated with biotinylated bovine albumin to which avidin was coupled, were from Precision Plastic Balls Co., Chicago, IL.

Methods

Prorenin Activation

In order to measure plasma prorenin by IRMA, it was 'activated' prior to the assay by remikiren (10^{-4} M final concentration) for 48 h at 8°C. Although the complex of prorenin and remikiren is enzymatically inactive we use the term 'activation' to indicate that prorenin becomes recognizable by mAb R1-20-5, *i.e.* the renin-specific mAb, after remikiren incubation. Plasma prorenin was also activated proteolytically by mixing 200 μL of Sepharose-trypsin (0.5 mg of trypsin) with 1 mL of plasma and incubation of the mixture for 24 h at 4 °C.[10] The trypsin-Sepharose was then removed by centrifugation.

Assays

IRMAs. Fig. 1 depicts the principles of the two immunoradiometric assays we employed. R-IRMA measures renin and active prorenin. In this assay biotinylated mAb R3-36-16 is the primary antibody, which is bound to an avidin-coated bead. The secondary (developing) antibody is ^{125}I-labeled mAb R1-20-5 (2.5×10^5 cpm/assay tube, specific activity 740 kBq/mg). The assay was carried out with 200 μl sample or standard and 100 μl of an equivolume mixture of biotinylated mAb R3-36-16 (0.5 mg/L) and labeled

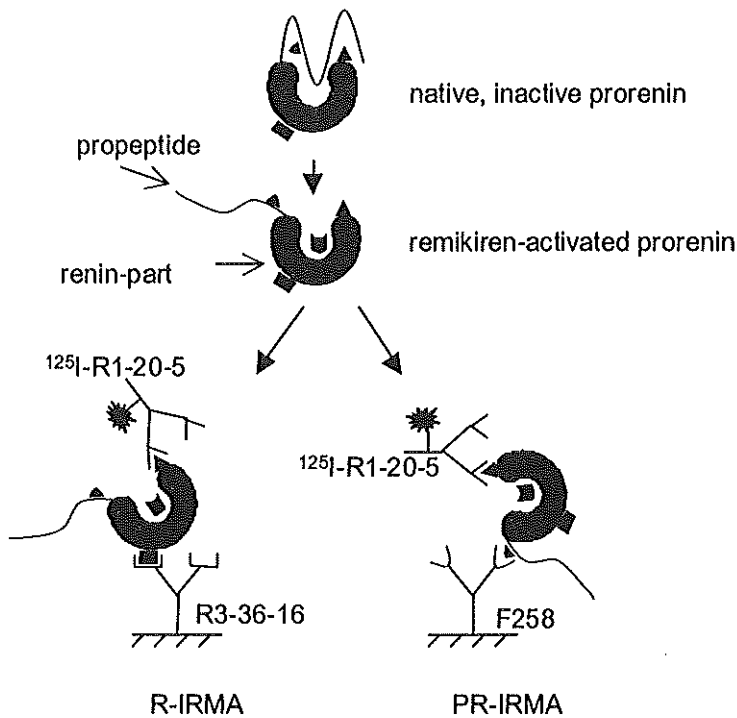


Figure 1. Schematic representation of the principle of prorenin measurement in the R-IRMA (left) and the PR-IRMA (right). The 'square' epitope is exposed on both renin and prorenin. The 'triangled' epitope is exposed in renin as well as in remikiren-treated prorenin, but is hidden in native prorenin. The 'dome' epitope on the propeptide is specific for prorenin and only exposed after remikiren incubation

mAb R1-20-5. Incubations were performed at 37°C and incubation time was 6 hours. After incubation beads were washed three times with 2 mL washing buffer. Beads were transferred to clean tubes and radioactivity was counted for 5 minutes in a gamma-counter. The characteristics of the R-IRMA are described in chapter 3.1.

Prorenin IRMA. For the direct prorenin IRMA (PR-IRMA) 1 μg of F-258-37-B1, biotinylated with 11% biotin (w/w),[12] in a volume of 250 μl of dilution buffer with 20%(vol/vol) assay buffer, was coupled per avidin-coated bead by incubation for 48 h at 4 °C. To remove unbound antibody, beads were washed three times with 2 mL of washing buffer. Two hundred μl of sample or standard and 50 μl of assay buffer were added per bead and left to incubate for 6 h at 37°C. In this step 'activated' prorenin is bound to the F-258-37-B1-coated beads through its exposed propeptide (see chapter 2.2). Then the bead was washed three times in 2 mL of washing buffer, followed by an incubation with 100 μl ^{125}I-labeled R1-20-5 (approximately $3x10^5$ cpm) and 200 μl assay buffer, containing 100 μM of remikiren, for 24 h at 22°C. Addition of remikiren in this second step was done to ensure that activated prorenin, if bound to the immobilized F-

258-37-B1 was kept in the 'activated' state and hence would not dissociate from the bead.

Enzyme-kinetic Assay. Plasma renin, and plasma renin plus trypsin-Sepharose-activated prorenin, were also measured by an EKA. In this assay, the sample was incubated with saturating concentrations of sheep angiotensinogen at pH 7.4 and 37 °C in the presence of inhibitors to block angiotensin I-converting enzyme, angiotensin I-degrading enzymes and serine proteases.[13] Two incubation times between 15 and 60 minutes were used in order to verify linear AngI generation. Generated AngI was quantitated by radioimmunoassay.[13] Calibration was against the WHO human kidney renin standard, so that results of the EKA could be directly compared with the IRMA results.

Patients. Blood was taken from an indwelling venous catheter after patients had rested for 45 minutes in the supine position. It was anticoagulated with trisodium citrate (0.2 mL of 0.646 mol/L per 10 mL of blood) and centrifuged within 30 minutes from collection for 10 minutes at 3000 g. Plasma was stored at -20 °C in 1-mL aliquots. Just before the assay, samples were rapidly thawed and kept at room temperature.

Two groups of patients were studied. The first group of 56 patients consisted of: healthy subjects (n = 6), patients with primary hyperaldosteronism (n = 2), renovascular hypertension (n = 6), essential hypertension with either low or high renin (n = 6 and 6 resp.), diabetes mellitus and microvascular complications (n = 6), heart failure (n = 6) and subjects that either used oral contraceptives (n = 6) or were pregnant (n = 6). To illustrate a potential use of the IRMAs we restudied the plasmas of seven patients that had been treated by oral remikiren. Time course of renin or propeptide epitope after oral ingestion of 600 mg of remikiren was followed at 0, 15, 30 minutes and 1,2,4,6 hours. This study was performed in 1990 and has been reported in reference.[14] One patient was not included in the series of the present report, because of lack of plasma. Plasmas had been thawed once in the meantime and had been kept stored at -80 °C.

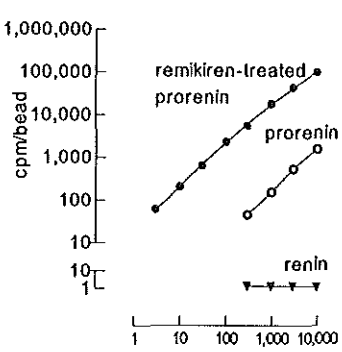


Figure 2. Calibration curves for remikiren treated human recombinant prorenin in sheep serum, native recombinant human prorenin in sheep serum, and a human kidney renin standard (lot 68/356).

Table 1. Effect of dilution of plasma by sheep serum on prorenin measurement in the PR-IRMA

		Prorenin (mU/L)		
sample	Dilution factor	Measured	Expected	Ratio (%)
1		165.1		
	2	81	82.6	98
	4	41	41.3	100
	8	16.7	20.6	81
	16	9.1	10.3	88
2		323.7		
	2	172.5	162	94
	4	83.1	81	97.5
	8	37.4	40.5	92
	16	18.7	20.3	92
3		968		
	2	475	484	98
	4	233	242	96
	8	115	121	95
	16	55	60.5	91

Results

Characteristics and Performance of the Direct Prorenin Assay. In preliminary experiments we found that plasma that had been incubated with trypsin-Sepharose did not elicit a detectable signal in the PR-IRMA. Pretreatment with remikiren gave values for prorenin that were similar to the prorenin levels estimated by the EKA after trypsin proteolysis (results not shown). Binding of remikiren treated prorenin to the F-258-37-B1-coated beads occurred with a half-time of about 20 minutes at 37 °C. **Fig. 2** shows the standard curve of the PR-IRMA. If standards were used consisting of native prorenin in the PR-IRMA, a parallel curve to the remikiren-treated, activated prorenin-standard curve was observed, indicating a cross-reactivity of native prorenin in the assay of approximately 0.8%. Renin standards did not produce an appreciable signal in the PR-IRMA. Serial dilution of plasma with sheep serum, with subsequent remikiren treatment gave parallel, proportional decreases in prorenin content (**Table 1**). Prorenin added to sheep serum or plasma was fully recovered (**Table 2**). **Table 3** gives coefficients of intra- and interassay variation. The analytical lower limit of detection, defined as the prorenin level at a binding level of 3SD of non-specific binding (n=6) above the mean non-specific binding was 4 mU/L. It proved to be impossible to assess the functional

Table 2 Recovery of added prorenin in sheep serum and three plasmas

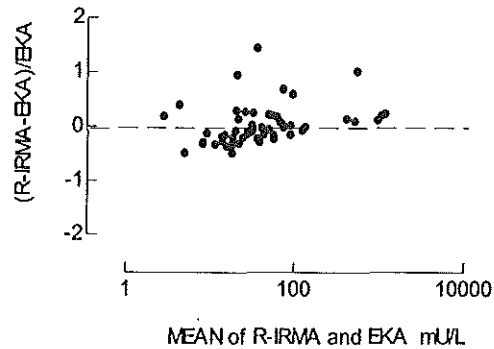
	Prorenin conc., mU/L				
	Initial	Added	Expected	Measured	recovery (%)
Sheep serum	0	157	157	182	116
		314	314	309	98
		628	628	577	92
		1256	1256	1177	94
Plasma 1	145	157	302	290	93
		314	459	446	96
		1256	1401	1391	99
Plasma 2	272	314	586	627	113
		628	900	1027	120
		1256	1528	1760	119
Plasma 3	587	314	901	871	91
		628	1215	1140	88
		1256	1843	1820	98
Plasma 4	897	395	1292	1339	112
Plasma 5	1348	395	1743	1752	102

lower limit of sensitivity, defined as the lowest prorenin level with an interassay coefficient of variation of less than 20%, because our lowest plasma pool (45 mU/L of prorenin) had an interassay CV of 10.5%.

Renin and Prorenin in Clinical Samples According to Different Assay Types. R-IRMA and EKA gave identical results in renin levels (Fig. 3). Prorenin results of the three assay types for 56 different plasmas were compared by a variant of the Bland-Altman plot (Fig. 4). The difference of the result in each assay with the mean of the three

Table 3. Coefficients of variation (CV)of the PR-IRMA

Prorenin level (mU/L)	Interassay CV (%) n=8	Intraassay CV (%) n=6
45	10.5	4.5
279	19.3	6.4
559	10.9	7.5
735	15.4	5.6



assays was plotted against this mean. No significant differences of the EKA, the R-IRMA and the PR-IRMA with this mean were observed.

Renin and Prorenin Measurements in Patients on Remikiren Treatment. The results of the R- and PR-IRMA of serially taken plasmas from patients that had taken the renin inhibitor remikiren are depicted in Figure 4. Plasma samples were studied in both assays before as well as after a 48 h preincubation with remikiren. There is a discrepancy between the renin values on one hand and the prorenin and total renin values on the other hand. Moreover, there appears to be expression of propeptide epitope in the first two time-points after administration of remikiren, even though the blood samples had not been preincubated with remikiren. We conclude from this observation that after ingestion remikiren reaches levels in circulating blood that are sufficient to 'activate' prorenin. It seems therefore that, contrary to earlier reports, the kidney does not respond to acute renin inhibition with an immediate –*i.e.* within minutes- release of renin, but instead, with a more gradual rise, and that the early peak in renin, observed with the R-IRMA, in a large part is remikiren-'activated' prorenin.

Discussion

In the preceding chapter we demonstrated the good performance of the R-IRMA for renin measurement. Now we demonstrate the adequacy of a direct PR-IRMA for prorenin measurement. Indirect *renin* measurement by subtracting the PR-IRMA result from total renin (defined by the mean of EKA and R-IRMA after trypsin and remikiren treatment respectively) gave identical results to those by the EKA or R-IRMA (results not shown), albeit with too large a variation to be applicable in all samples. In particular, samples with very low renin content often gave negative values or a relatively large overestimation of renin.

A problem we anticipated but that did not surface in the PR-IRMA, was the phenomenon of proteolytic cryoactivation. The unmasking of the propeptide epitope of prorenin by remikiren was performed at 8°C (the temperature of our cold room) for 48 h and since spontaneous proteolytic activation may occur in plasma at this temperature, a lower level of prorenin could be expected, compared to other methods. However, as demonstrated in Fig.4, there was no underestimation of prorenin. Results of

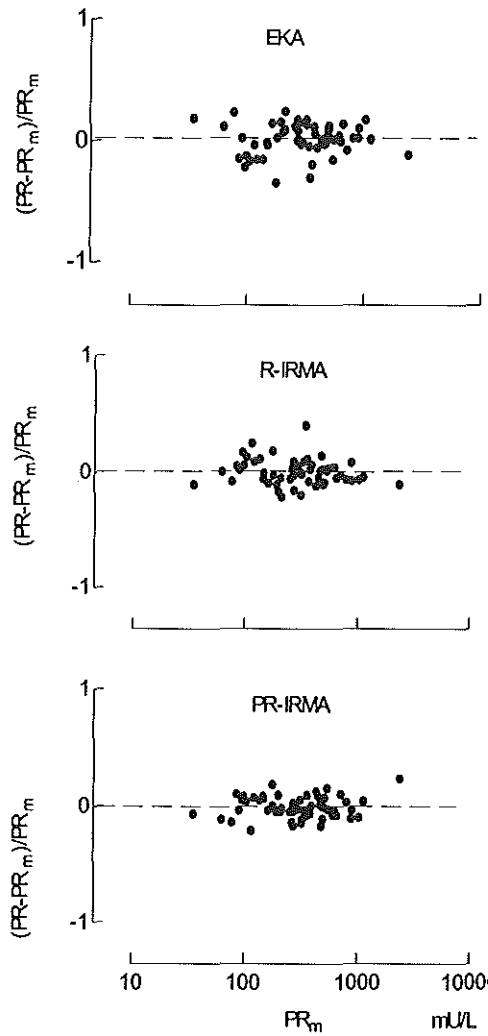


Figure 4. Comparison of prorenin measurement in 56 human plasmas, indirectly by the enzyme-kinetic assay after trypsin-activation (EKA), by R-IRMA after remikiren treatment (R-IRMA) and directly by PR-IRMA. PR=prorenin as measured by the assay. PR_m = mean of the three assay types for prorenin. Mean±SD of the differences with PR_m was 1±12, 0±11 and −1±9% for EKA, R-IRMA and PR-IRMA resp.

preincubation at higher temperatures gave identical results. The absence of proteolytic cryoactivation may be explained by the near absence of proteolytic prorenin-activation at this temperature or by only limited proteolysis that leaves the C-terminal epitope of the propeptide intact and attached to the renin body of prorenin.

A direct prorenin assay has been developed before by Schumacher et al.[15] Their IRMA employed a polyclonal antiserum against a propeptide sequence consisting of amino-acids −12p-+3p of the prosegment. In contrast with our assay their assay could be performed with native prorenin, although it is not sure whether sensitivity could have been improved by preincubation with remikiren. In contrast to their assay, which found prorenin levels 20% lower in the direct IRMA compared to an indirect prorenin assay,

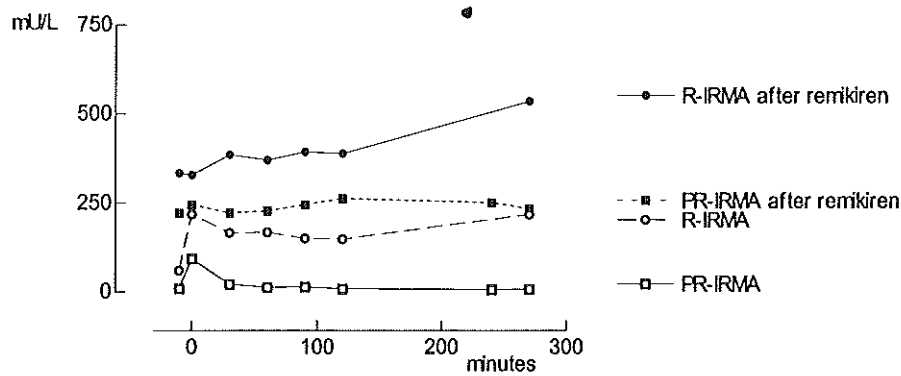


Figure 5. Time course of plasma levels total renin, prorenin, renin and 'active' prorenin after oral administration of 600 mg of remikiren at t=-15 min. Shown are geometric means for 7 subjects. Error bars have been omitted for clarity.

our PR-IRMA had identical results to indirectly determined prorenin. Furthermore, our PR-IRMA appears to be a little more sensitive and permits to measure prorenin over a wide range of concentrations that encompasses all concentrations that are encountered in clinical samples.

The three assays had an identical calibrator, recombinant human prorenin. The consistency in the results obtained by three assays that differ in principle, suggests that what is measured in either of them refers to the same molecules, with the restriction that PR-IRMA is not suitable for total renin measurement. It also shows the advantage of a universal standard. We would like to stress the importance and the potential of such a standard. The only official renin standard that is now available is the WHO standard that we used for calibration of our prorenin standard. This standard is defined by its blood pressure-increasing effect upon injection and is in fact a first-generation renin assay standard. Our remikiren-treated prorenin may be an attractive alternative to this renin standard. It can be used in both our R-IRMA and PR-IRMA and has the potential of being expressed in molar units. Prerequisite is that a prorenin standard has as much renin epitope as prorenin epitope. This can be verified in enzymatic assays, in which the native prorenin standard must not have enzymatic activity. Our preparation has an enzymatic activity of less than 2% which means that prorenin levels may be overestimated at most by 2%. We do not know, however, whether this spontaneous acitivity is due to the presence of renin or to the presence of active prorenin. In the latter case the propeptide is still present and prorenin will not be underestimated. Another check can be made if in control plasmas both renin and prorenin are measured directly by the R- and PR-IRMA respectively and total renin directly in a R-IRMA after full activation of prorenin. Directly measured renin and prorenin should add up to the same level as has been found in the total renin assays, which is the case in our assays.

What role could direct renin and prorenin IRMAs play in the clinical and research setting? It is still unknown whether prorenin has any role in the (patho)physiology of angiotensin generation, and, therefore, the relevance of prorenin measurement may be

questionable. Some conditions exist in which prorenin is elevated in excess of renin, for instance in diabetes mellitus with microvascular complications.[16, 17] It can not be excluded that prorenin measurement can become of interest in the management of diabetic patients (see chapter 4.1).

From the present results it seems that the R-IRMA may cover all needs of renin and prorenin measurements in plasma, because it reliably can measure renin as well as, indirectly after preincubation with remikiren, prorenin. The EKA also performs well, but it is much more labor-intensive. Furthermore, we have preliminary evidence that the R-IRMA for renin and prorenin and the PR-IRMA for prorenin quantification have good performance in non-plasma samples, like tissue homogenates, cell culture media and human follicular fluid. The EKA measures renin reasonably well in these samples but prorenin is usually highly underestimated. Presumably this is caused because proteolytic activation of prorenin by trypsin-Sepharose is not complete, since measurement of trypsin-Sepharose samples in the R-IRMA yields low concentrations unless an additional preincubation with remikiren is performed (unpublished observation).

There appears to be little need for the PR-IRMA, but one can imagine situations in which active prorenin may be present which would normally be interpreted as renin in an enzyme-kinetic assay, whereas our PR-IRMA would recognize this as active prorenin. Evidence for active prorenin has been found,[18] although in the present report we did not find discrepancies between direct and indirect prorenin assays in plasma. A further use for a direct renin and a direct prorenin assay may be in studies of renin inhibitors. As shown in the samples of a study performed some years ago even a direct renin assay may not yield reliable results, because inhibitor-activated prorenin may be measured as renin. Since the prorenin-'activating' character of renin inhibitors is probably a class effect (see chapter 2.1), using the R- and PR-IRMA allows to dissect meticulously what happens to renin and prorenin levels during renin inhibitor therapy.

Finally, we want to emphasize that this study is not concerned with superiority of the renin assay over the plasma renin activity assay. In our view and that of others,[19] the PRA can be considered as 'a poor man's' angiotensin II assay. It is much easier to perform than angiotensin II measurements and yet a good measure of the activity of the renin-angiotensin system. Nevertheless, the IRMAs described in this paper have the considerable advantage that they will enable interlaboratory comparison and may be used to establish reference values. It is our conviction that direct assays for the components of the renin-angiotensin system will be of great help in the elucidation of the physiology of angiotensin generation. What is needed now is to further develop a new renin/prorenin standard, for which we propose the remikiren-treated recombinant prorenin preparation, and a multi-center testing of these assays.

References

1 Pickering GW, Prinzmetal M. Some observations on renin, a pressor substance contained in normal kidney, together with a method for its biological assay. Clin Sci 1938;3:211-27.

2 Bangham DR, Robertson I, Robertson JIS, Robinson CJ, Tree M. An international collaborative study of renin assay: establishment of the international reference standard preparation of human renin. Clin Sci Mol Med 1975, 48:135s-159s.

3 Galen FX, Devaux C, Atlas S, Guyenne TT, Menard J, Corvol P, Simon D, Cazaubon C, Richer P, Badouaille G, Richaud JP, Gros P, Pau B. New monoclonal antibodies directed against human renin: powerful tools for the investigation of the renin system. J Clin Invest 1984;76:723-35.
4 Bangham DR, Robertson I, Robertson JIS, Robinson CJ, Tree M. An international collaborative study of renin assay: establishment of the international reference preparation of human renin. Clin Sci Mol Med 1975;48: 135s-159s
5 Osmond DH, Cooper RM, Cryoactivation and tryptic activation of blood prorenin in normal man and animals. Can J Physiol Pharmacol 1978;56:792-805.
6 Nielsen MD, Rasmussen PH, Giese J. A highly sensitive and reproducible immunoradiometric assay for total human renin using monoclonal antibodies, Iodogen labeling, and polystyrene Startubes. Clin Exp Hypertens 1987;A9:1391-1414.
7 Robertson JIS, Nicholls MG. Standardization and standards. In: Robertson JIS, Nicholls MG, eds. The renin-angiotensin system. London, Gower Medical Publishing, 1993:A7.
8 Heusser CH, Bews JPA, Alkan SS, Dietrich FM, Wood JM, De Gasparo M, Hofbauer KG. Monoclonal antibodies to human renin: properties and applications. Clin.. Exp. Hypertension 1987;A9:1259-75.
9 Fischli W, Clozel JP, El Amrani K, Wostl W, Neidhart W, Stadler H, Branca Q. Hypertension 1991;18: 22-31.
10 Derkx FHM, Tan-Tjiong HL, Wenting GJ, Boomsma F, Man in't Veld AJ, Schalekamp MADH. Asynchronous changes in prorenin and renin secretion after captopril in patients with renal artery stenosis. Hypertension 1983;5:244-56.
11 Derkx FHM, de Bruin RJA, van Gool JMG, van den Hoek M-J, Beerendonk CCM, Rosmalen F, Haima P, Schalekamp MADH. Clinical validation of renin monoclonal antibody-based sandwich assays of renin and prorenin, and use of renin inhibitor to enhance prorenin immunoreactivity. Clin Chem 1996; 42:1051-63.
12 Odell WD, Griffin J, Zahradnik R. Two-monoclonal-antibody sandwich-type assay for thyrotropin, with use of an avidin-biotin separation technique. Clin Chem 1986;32:1873-8.
13 Admiraal PJJ, Derkx FHM, Danser AHJ, Pieterman H, Schalekamp MADH. Metabolism and production of angiotensin I in different vascular beds in subjects with hypertension. Hypertension 1990;15:44-55.
14 van den Meiracker AH, Admiraal PJJ, Derkx FHM, Kleinbloesem C, Man in't Veld AJ, van Brummelen P, Mulder P, Schalekamp MADH. Comparison of blood pressure and angiotensin responses to the renin inhibitor Ro 42-5892 and the angiotensin converting enzyme inhibitor enalapril in essential hypertension. J Hypertens 1993;11:831-8.
15 Schumacher M, Nanninga A, Delfs T, Mukhopadhyay AK, Leidenberger FA. A direct immunoradiometric assay for human plasma prorenin: concentration in cycling women and in women taking oral contraceptives. Journal of Clinical Endocrinol Metab 1992;75: 617-23.
16 Luetscher JA, Kraemer FB, Wilson DM, Schwartz HC, Bryer-Ash M. Increased plasma inactive renin in diabetes mellitus: a marker of microvascular disease. N Engl J Med 1985 3121:1412-7.
17 Fanken AAM, Derkx FHM, Man in't Veld AJ, Hop WCJ, van Rens GH, Peperkamp E, de Jong PTVM, Schalekamp MADH. High plasma prorenin in diabetes mellitus and its correlation with some complications. J Clin Endocrinol Metab 1990;71 1008-15.
18 Ishizuka Y, Iizumi S, Akiyama F, Hori H, Poorman RA, Lobl TJ, Murakami K. Characterization of monoclonla antibodies against human prorenin profragment and identification of active prorenins in plasma. J Biochem (1989)106:430-435.
19 Sealey JE. Personal communication.

Chapter 4 Prorenin Regulation in Diabetes Mellitus

4.1 Increase in Serum Prorenin Precedes Onset of Microalbuminuria in Patients with Insulin-Dependent Diabetes Mellitus

Summary

The renin-angiotensin system is possibly involved in the pathogenesis of diabetic nephropathy. The most striking change in renin-angiotensin system components in blood of patients with diabetic nephropathy is an increased prorenin concentration. We investigated prospectively serum concentrations of renin-angiotensin system components and the time course of prorenin increase in normoalbuminuric diabetics developing microalbuminuria. Patients (n = 199) with Type I diabetes mellitus and normoalbuminuria at baseline were prospectively followed for 10 years. The prorenin concentration and other variables possibly associated with the occurrence of microalbuminuria, were investigated by Cox-regression analysis. Of the patients 29 developed microalbuminuria. Glycated hemoglobin concentrations were higher at baseline in these patients. Serum prorenin was similar at baseline, but rose in the 29 patients before the development of microalbuminuria and was stable in patients with stable albumin excretion. Renin, angiotensinogen and angiotensin converting enzyme serum concentrations were stable in both groups. Prorenin and glycated hemoglobin were independent prognostic factors for the development of microalbuminuria. A prognostic index, based on these variables, was constructed to estimate the relative risk of developing microalbuminuria.

Increase in serum prorenin precedes onset of microalbuminuria in normotensive patients with insulin-dependent diabetes mellitus. High concentrations of prorenin in combination with high values of glycated hemoglobin may be used as a predictor of development of microalbuminuria.

Introduction

Progression of microalbuminuria (defined as urinary albumin excretion rate (AER) of 30 -300 mg/24h[1]) to overt diabetic nephropathy in patients with type I (insulin-dependent) diabetes mellitus is retarded by treatment with inhibitors of angiotensin converting enzyme (ACE). Even at the microalbuminuric stage, pathologic changes are already present in the kidney[2] together with widespread endothelial dysfunction.[3,4] Microalbuminuria is associated with excess cardiac morbidity.[5] It may therefore be worthwile to identify diabetic patients at risk before the stage of microalbuminuria, to start preventive treatment. Several risk factors for progression to microalbuminuria have been proposed,[6,7] such as poor metabolic control, hypertension and high-normal AER.

The most attractive factors for identifying risk are those involved in the pathogenesis of the disease. The favorable response to ACE inhibitors[8,9] suggests that the renin angiotensin system is one which is supported by experimental evidence indicating that angiotensin II (AngII) is an important factor in the pathogenesis of diabetic nephropathy.[10] The major change detected in this system in diabetic patients with microalbuminuria or the ensuing stage of diabetic nephropathy is an increased plasma concentration of prorenin.[11,12] Prorenin is the inactive precursor of the aspartic protease renin, which is

produced by the kidney. Renin is essential in the proteolytic cascade leading to production of AngII, a key hormone in blood pressure regulation and fluid and electrolyte homeostasis. Data on the change in prorenin concentration over time, in relation to the onset of nephropathy are scarce. In a retrospective longitudinal survey[13] total renin (i.e. prorenin + renin) increases before the onset of microalbuminuria. Here we report on a longitudinal 10-year follow-up study in 199 patients with Type I diabetes mellitus who were normoalbuminuric at the outset. Microalbuminuria developed in 29 patients during follow-up. The aim of this study was to investigate whether the increase in prorenin that has been observed in type I diabetes mellitus, precedes the development of microalbuminuria.

Subjects and Methods

Patients

Patients (n = 209) with Type I diabetes mellitus from the outpatient clinic at the Steno Diabetes Centre (Copenhagen, Denmark) were included between October 1982 and January 1983. Characteristics of the cohort have been reported in detail.[6] All patients fulfilled the following criteria: age between 18 and 50 years, onset of diabetes before the age of 30 years, duration of diabetes 10 to 30 years, diastolic blood pressure < 100 mmHg and AER less than 30 mg per 24 hour in one urine sample collected at home, and

Table 1. Clinical data of patients at entry of study

	Group with persistent normoalbuminuria	Group developing microalbuminuria
	n=170	n=29
Sex (f/m)	74/96	13/16
Age (years)	34±8	34±8
Duration of diabetes(years)	17±5	17±5
AER (mg/24h)	12 (3-29)	17 (3-29)*
HbA_{1c} (%)	8.4±1.4	9.3±1.6*
Retinopathy		
None	85	10
Background	80	18
Proliferative	5	1
Systolic blood pressure (mm Hg)	125±16	128±13
Diastolic blood pressure (mm Hg)	78±11	81±7
Renin (mU/L)	26.0 (23.6-28.7)	29.1 (25.0-33.9)
Prorenin (mU/L)	270 (250-291)	313 (254-386)
Creatinine (μM)	82±11	80±10

AER; albumin excretion rate; values are means ± S.D. or geometric mean (95% C.I.);* $p<0.05$

remaining normal in at least 2 out of 3 samples during the first year of observation. None of the patients received medication, including oral contraceptives, other than insulin. Patients were followed for ten years. Every four months albumin excretion was determined in a 24 h urine collection. The patients were classified as having microalbuminuria when AER was 30-300 mg/24 h in two out of three urine samples in two consecutive years. Blood pressure was measured and ophthtalmoscopy was carried out annually. Blood samples for the measurement of glycated hemoglobin (HbA_{1c}) was also collected annually. Serum obtained annually was kept at -20 °C for later measurement of prorenin, renin, angiotensinogen and angiotensin converting enzyme. Four patients were lost during follow-up and five patients were excluded due to severe diseases (AIDS, cancer, connective tissue disease, hyperthyroidism and chronic pyelonephritis). One patient could not be evaluated because of missing serum samples. Finally 199 patients were evaluated.

During follow-up twelve patients became pregnant. Serum concentrations of prorenin and renin during pregnancy were excluded from analysis because prorenin is elevated during pregnancy.[15] Of the patients, 13 developing microalbuminuria, developed hypertension (defined as a blood pressure exceeding 140/95 mm Hg), 5 before and 8 after the onset of microalbuminuria. Antihypertensive treatment was initiated in only 15 patients of the control group. The antihypertensive drugs that were used were mainly diuretics and calcium channel blockers. Because antihypertensive drugs might influence the serum concentrations of renin and prorenin, only data obtained from patients while not on antihypertensive therapy were included in risk analysis.

Methods

Blood pressure was measured with a standard sphygmomanometer after a 10-min rest in the sitting position. The disappearance of Korotkoff sounds (phase V) was used to determine diastolic blood pressure. Blood for measurement of prorenin, renin, angiotensinogen and angiotensin converting enzyme, was taken after a short rest in the sitting position. Serum was stored at -20 °C. Retinopathy was assessed by direct ophthalmoscopy by an ophthalmologist. Retinal lesions were categorized as either absent or present, combining background or proliferative retinopathy.

AER was measured until May 1985 by a radial immunodiffusion technique.[17] Thereafter an enzyme-linked immunoassay was used.[18] HbA_{1c} (normal range 4.1-6.4%) was measured by HPLC (Biorad, Diamat, Richmond, CA, USA). Serum creatinine was measured by autoanalyser.

Assays of Renin and Prorenin. Serum renin and prorenin were measured with a commercial kit (Nichols Diagnostics, San Juan Capistrano, CA, USA), based on an immunoradiometric assay (IRMA). In this assay renin is measured directly. Total renin, i.e. renin plus prorenin, is measured after enhancement of immunoreactivity of prorenin by preincubation with the renin inhibitor remikiren as described in ref. 18 .The difference between total renin and renin is prorenin. The 95% reference limits are 7.7 - 54.8 mU/L for renin and 88.1 - 390 mU/L for prorenin.

Assays of Angiotensin Converting Enzyme and Angiotensinogen Assays. These assays were performed in the 29 patients developing microalbuminuria and in a group

of 29 patients remaining normoalbuminuric, matched for age, sex and duration of diabetes. Serum ACE activity was determined with a colorimetric kit (ACEcolor, Fujirebio Inc, Tokyo, Japan), using Gly-His-His as substrate. The concentration of angiotensinogen in serum was determined as the quantity of AngI that is generated after complete hydrolysis of angiotensinogen by recombinant human renin at pH 7.4 and 37 °C in the presence of inhibitors of AngI degrading enzymes.[14] AngI was measured by radioimmunoassay.[14] Results are expressed as nmol/L.

Statistical Analysis

Continuous data were compared using the t-test. Serum renin, serum prorenin and urinary albumin excretion were log-transformed before statistical analysis and presented as geometric mean. The relation of various time-dependent clinical characteristics with the occurrence of microalbuminuria was investigated using Cox-regression analysis.[18] Parameters with significant predictive value in univariate analysis were multivariately analysed. Backward elimination was used to determine which factors were independently associated with the occurrence of microalbuminuria. Statistical software used in the analyses was SPSS 7.0 (SPSS Inc. Chicago, IL).

Results

Clinical Data at Entry and during the Course of the Study

Twenty nine patients progressed to microalbuminuria during the ten years of observation and 170 continued with a normal AER. Eight of the 29 patients that developed microalbuminuria, progressed to overt diabetic nephropathy, i.e. an AER exceeding 300 mg/24 h. Clinical characteristics of the patients are given in **Table 1**. In the patients who later developed microalbuminuria, AER values and HbA_{1c} were higher at outset. Serum prorenin and renin were not significantly different at the time of inclusion. During the 10 year follow-up serum prorenin and renin did not correlate with serum creatinine, glycosylated HbA_{1c}, diastolic or systolic blood pressure. At baseline the concentrations of prorenin were not different in patients with background or proliferative retinopathy, compared to patients without retinopathy (not shown).

Fig. 1 shows the concentrations of prorenin, renin, angiotensinogen and ACE during follow-up. The year of first occurrence of microalbuminuria was designated as year zero. For patients who remained normoalbuminuric throughout follow-up year zero was chosen to be 1987, since this was the average year of onset of microalbuminuria in the other group. Data for angiotensinogen and ACE demonstrate stable concentrations during follow-up. Data in the upper two panels of Fig.1 suggest that serum prorenin and possibly renin increase before microalbuminuria sets in. Univariate Cox-regression analysis of occurrence of microalbuminuria showed significant relations with the concentrations of serum prorenin, HbA_{1c}, and systolic and diastolic blood pressure, and with retinal status. **Table 2** summarizes the size of the effect of these variables. No univariate significant relations were found for the duration of diabetes, and the serum concentrations of renin and creatinine. Subjecting the factors prorenin, HbA_{1c}, diastolic and systolic blood pressure, and the existence of retinopathy to multivariate analysis, we found that the

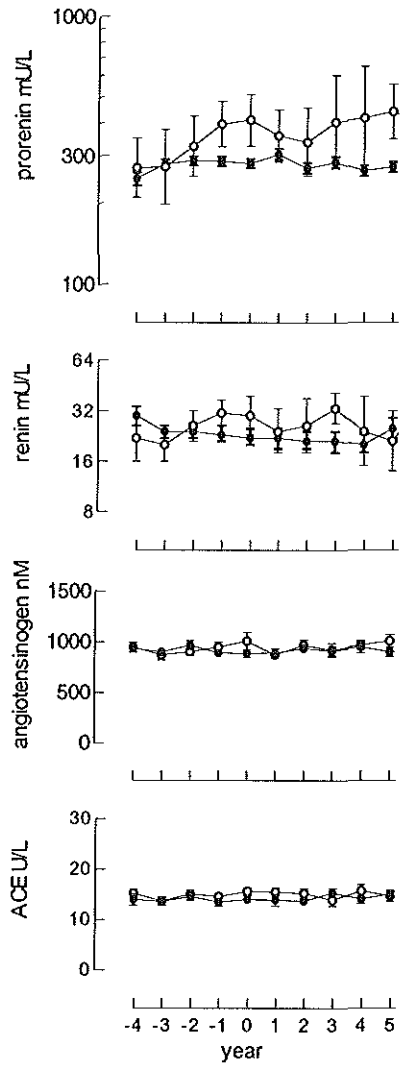


Figure 1. Serum prorenin, renin, angiotensinogen and ACE in patients remaining normoalbuminuric and (•)and in patients developing microalbuminuria (○). Year 0 is the first year of microalbuminuria or 1987 for patients remaining normoalbuminuric. Note the logarithmic scale in the upper two panels. Shown are geometric mean ± 95% confidence interval (upper two panels) and mean ± SE (lower two panels)

serum concentrations of prorenin and HbA_{1c} were the most important factors predictive of microalbuminuria. No additional prognostic value was found for diastolic and systolic blood pressure, and for retinal status. Furthermore, we observed that the effect of prorenin was significantly affected by the duration of diabetes. Prorenin, but not HbA_{1c}, lost its significance as a prognostic factor when the duration of diabetes exceeded 25 years. Table 3 gives the results of the multivariate analysis separately for the duration of diabetes up to 25 years and thereafter. With DM present for less than 25 years the risk of developing microalbuminuria increases 4.7-fold when prorenin concentrations double. The relative risk associated with a 1% increase in HbA1c is 2.0.

Table 2. Results of univariate Cox-regression analysis for the occurrence of microalbuminuria in 199 patients with type I diabetes mellitus.

Variable	Relative Risk[#]	p coefficient
Prorenin	2.6[a]	0.001
HbA_{1c}	2.3[b]	<0.001
Systolic BP (mmHg)	1.3[c]	0.019
Diastolic BP (mmHg)	1.6[c]	0.019
Retinopathy	2.7[d]	0.043

[#] As compared to: 2-fold lower value (a), 1% lower value (b), 10 mm Hg lower value (c), no abnormalities (i.e. no retinopathy)(d). BP=blood pressure

The results allow to construct a prognostic index (PI) for the development of microalbuminuria when diabetes has been present for less than 25 years. This is given by the following formula:

$$PI = 1.56 * \log_2 \text{prorenin} + 0.68\, HbA_{1c}$$

In Fig. 2, the relative risk of developing microalbuminuria is graphed, according to the PI-value. A PI-value of 18, corresponding to a prorenin concentration of 400 mU/L and a HbA_{1c} of 8.9%, was arbitrarily chosen as a reference. If a patient in the present study group had a PI that was continuously below 19, corresponding to, for instance, a prorenin concentration of 500 mU/L with a HbA_{1c} concentration of 9.7%, the chance of developing microalbuminuria was very small. However, patients scoring a PI exceeding 19, had about 30% chance to develop microalbuminuria in the next 5 years (data not shown).

Discussion

This longitudinal, 10-years follow-up study on 199 patients with type I diabetes mellitus confirms and extends earlier data[13] that indicate that an increase in serum prorenin

Table 3. Relative risk of development of persistent microalbuminuria, determined by prorenin and glycated hemoglobin levels

	Duration of diabetes mellitus							
	< 25 years				≥25 years			
Variable	Regression coefficient	RR	95% C.I.	p-value	Regression coefficient	RR	95% C.I.	p-value
Log_2(PR)	1.56	4.7	2.3-9.9	<0.001	-1.41	0.2	0.1-1.2	0.085
HbA_{1c} (%)	0.68	2.0	1.4-2.8	<0.001	1.05	2.9	1.1-7.7	0.036

PR=serum prorenin; RR=relative risk, per doubling of value for prorenin or per percent increase for HbA_{1c}, 95% CI=95% confidence interval

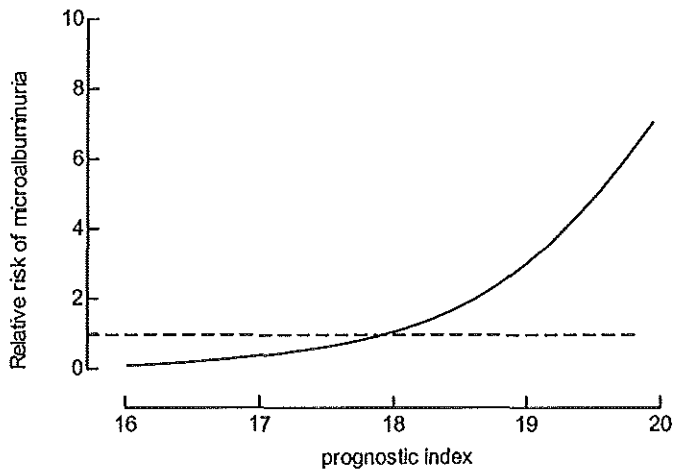


Figure 2. Relative risk of microalbuminuria in relation to the derived prognostic index PI. The risks are expressed relative to the risk at PI=18

precedes development of persistent microalbuminuria, the earliest phase in the development of diabetic nephropathy that can be recognized clinically.

The cause of the early rise of prorenin in patients with type I diabetes mellitus developing nephropathy is not known. Normally, and also under pathological conditions, an increase in prorenin is associated with an increase in renin, the latter being proportionally larger than the former.[19] In the patients with type I diabetes mellitus the increase in prorenin was not accompanied by an increase in renin, which suggests a derangement of the regulation of the renin-angiotensin system. Whether this derangement plays a causative role in the development of microalbuminuria and nephropathy or is merely a marker of early renal damage, more sensitive than others like albuminuria, is a matter of speculation.

To view our results in a clinical perspective we derived from them a prognostic index (PI), based on HbA_{1c} and serum prorenin. This PI indicates the risk of future microalbuminuria and hence, of diabetic nephropathy. It underlines once again the importance of good metabolic control. The PI fulfills the following criteria: it has strong predictive power, it is easily determined and it may have pathophysiological relevance in view of the involvement of hyperglycemia and, with regard to the favorable response to ACE inhibition, of the renin-angiotensin system in the development of diabetic nephropathy. Its use must be restricted, however, to normotensive diabetic patients. Moreover, use of drugs, in particular of contraceptive hormones, that considerably affect prorenin concentrations, precludes application of this PI.

A threshold value for PI could be agreed upon above which additional treatment is considered besides optimal therapy for restoring glucose to normal. An obvious choice would be ACE inhibition. The EUCLID-study showed that ACE inhibition in normotensive, normoalbuminuric patients had little effect on AER.[20] Use of the

proposed PI might have identified a subset of these patients that would have benefited from ACE-inhibition. Obviously, a PI like the one proposed, needs to be validated in other longitudinal studies and work remains to be done to establish international standardization of glycated hemoglobin and prorenin assays.[21] Moreover, whether treatment based on this index improves prognosis on a cost-effective basis needs to be determined. Finally, we would like to stress that a reassuring level of the PI value would not be an excuse for inadequate metabolic or blood pressure control.

The model that predicts microalbuminuria included an interaction term for prorenin and duration of diabetes. It is not sure if this statistically obtained variable has a biologic meaning. It could mean that microalbuminuria occurring before a duration of 25 years, has a different pathogenesis, with involvement of the renin-angiotensin system, from microalbuminuria that occurs very late in the course of type I diabetes mellitus. This is possibly compatible with reports that the incidence of diabetic nephropathy decreases after 16 years duration of diabetes but has a second smaller peak at about 32 years duration.[22]

In conclusion, an increased prorenin concentration in the blood is a herald of microalbuminuria and diabetic nephropathy. Serum prorenin together with glycated hemoglobin can be used to calculate a prognostic index that may be used as a predictor of clinically manifest renal disease in normotensive patients with type 1 diabetes mellitus.

References

1 Mogensen CE, Christensen CK, Vittinghus E (1983) The stages in diabetic renal disease with emphasis on the stage of incipient diabetic nephropathy. Diabetes 32(suppl 2): 64-78.

2 Østerby R, Anderson AR, Gunderson HJ, Jørgensen HE, Mogensen CE, Parving HH (1984) Quantitative studies of glomerular ultrastructure in juvenile diabetics with incipient nephropathy. Diabetic Nephropathy 3: 95-100.

3 Feldt-Rasmussen B (1986) Increased transcapillary escape rate in type 1 (insulin-dependent) diabetic patients with microalbuminuria. Diabetologia 29: 282-6.

4 Jensen T (1991) Albuminuria – a marker of renal and generalized vascular disease in insulin-dependent diabetes mellitus. Dan Med Bull 38: 134-44.

5 Deckert T, Yokoyama H, Mathiesen ER et al. (1996) Cohort study of predictive value of urinary albumin excretion for atherosclerotic vascular disease in patients with insulin-dependent diabetes mellitus. Brit Med J 312: 871-4.

6 Mathiesen ER, Rønn B, Storm B, Poght H, Deckert T (1995) The natural course of microalbuminuria in insulin-dependent diabetes: a 10-year prospective study. Diabetic Medicine 12: 482-7.

7 Poulsen PL, Ebbehøj E, Hansen KW, Mogensen CE (1997) High normo- or low microalbuminuria: Basis for intervention in insulin-dependent diabetes mellitus. Kidney International 52(suppl 63): S-15-S-18.

8 Marre M, Chantellier G, Leblanc H, Guyenne TT, Menard J, Passa P (1988) Prevention of diabetic nephropahty with enalapril in normotensive diabetics with microalbuminuria. Brit. Med J 297: 1092-5.

9 Mathiesen ER, Hommel E, Giese J, Parving H.-H (1991) Efficacy of captopril in postponing nephropathy in normotensive insulin-dependent diabetic patients with microalbuminuria. Brit Med J 303: 210-6.

10 Kennefick TM, Anderson S (1997) Role of angiotensin II in diabetic nephropathy. Seminars in Nephrology 17: 441-7.

11 Feldt Rasmussen B, Mathiesen ER, Deckert T et al. (1987) Central role for sodium in the pathogenesis of blood pressure changes independent of angiotensin, aldosterone and catecholamines in type I (insulin-dependent) diabetes mellitus. Diabetologia 30: 610-7.

12 Luetscher JA, Kraemer FB, Wilson DM, Schwartz HC, Bryer-Ash M (1985) Increased plasma inactive renin in diabetes mellitus. N Engl J Med 312: 1412-7.
13 Allen TJ, Cooper ME, Gilbert RE, Winikoff J, Skinner SJ, Jerums G (1996) Serum total renin is increased before microalbuminuria in diabetes. Kidney International 50: 902-7.
14 Derkx FHM, Stuenkel C, Schalekamp MPA, Visser W, Huisveld IH, Schalekamp MADH (1986) Immunoreactive renin, prorenin, and enzymatically active renin in plasma during pregnancy and in women taking oral contraceptives. J.Clin Endocrinol Metab 63: 1008-15.
15 Mancini G, Carbonara AO, Heremans JF (1965) Immunochemical quantitation of antigens by single radial immunodiffusion. Immunochemistry 2: 235-9.
16 Feldt-Rasmussen B, Dinesen B, Deckert M (1985) Enzyme-immunoassay: an improved determination of urinary albumin in diabetics with incipient nephropathy. Scand J Clin Lab Invest 45: 539-44.
17 Derkx FHM, de Bruin RJA, van Gool JMG (1996) Clinical validation of renin monoclonal antibody-based sandwich assays of renin and prorenin, and use of renin inhibitor to enhance prorenin immunoreactivity. Clin Chem 42:1051-63.
18 Cox DR (1972) Regression models and life tables. J. Roy Stat Soc 34: 187-220.
19 Deinum J, Schalekamp MADH. Renin and prorenin. In Oparil S, Weber M Eds. Hypertension, W.B. Saunders Company, Philadelphia, in press.
20 THE EUCLID STUDY GROUP (1997) Randomized placebo-controlled trial of lisinopril in normotensive patients with insulin-dependent diabetes and normoalbuminuria or microalbuminuria. Lancet 349: 1787-92.
21 Robertson JIS, Nicholls MG (1993) Appendix II.Standardization and Standards. In Robertson JIS, Nicholls MG (Eds) The renin-angiotensin system; vol.2 Gower Medical Publishing, London. pp P.A.7-A.9.
22 Andersen AR, Sandahl Christiansen J, Andersen JK, Kreiner S, Deckert T (1983) Diabetic nephropathy in type I (Insulin-Dependent) Diabetes: An epidemiological study. Diabetologia 25: 496-501.

4.2 Plasma Renin and Prorenin and Renin Gene Variation in Patients with Insulin-Dependent Diabetes Mellitus and Nephropathy

Summary

The most striking abnormality in the renin-angiotensin system in diabetic nephropathy (DN) is increased plasma prorenin. Renin is thought to be low or normal in DN. In spite of altered (pro)renin regulation the renin gene has not been studied for contribution to the development of DN.

We studied plasma renin, prorenin and four polymorphic markers of the renin gene in 199 patients with IDDM and DN, and in 192 normoalbuminuric IDDM controls matched for age, sex and duration of diabetes. Plasma renin and total renin were measured by immunoradiometric assays. Genotyping was PCR-based.

Plasma renin was increased in patients with nephropathy (median (range), 26.3 (5.2-243.3) vs. 18.3 (4.2-373.5)μU/ml in the normoalbuminuric group, $p < 0.0001$). Prorenin levels were elevated out of proportion to renin levels in nephropathic patients (789 (88-5481) vs. 302 (36-2226) μU/ml, $p < 0.0001$). Proliferative retinopathy had an additive effect on plasma prorenin, but not on renin. DN was associated with a Bgl I RFLP in the first intron of the renin gene (bb-genotype: n = 106 *vs.* 82 in DN and normoalbuminuric patients resp., $p = 0.037$), but not with three other polymorphisms in the renin gene. A trend for association of higher prorenin levels with the DN-associated allele of this renin polymorphism was observed in a subgroup of patients with DN (bb *vs.* Bb+BB $p = 0.07$).

The results indicate that in DN there is an increase in both renin and prorenin levels. A renin gene polymorphism may contribute weakly to DN. Although speculative, one of the renin gene alleles could lead to increased renin gene expression, leading to higher renin and prorenin levels. These may play a role in the pathogenesis of DN.

Introduction

A major cause of morbidity and death in insulin dependent diabetes mellitus (IDDM) is diabetic nephropathy (DN), characterized by proteinuria, steady decline of kidney function, hypertension and cardiovascular disease.[1] Although poor metabolic control plays a role in the development of DN, it appears that 60 to 70 percent of the diabetic population is protected from nephropathy despite inadequate metabolic control.[2] It has therefore been suggested that susceptibility to DN is innate and determined by genetic factors. This hypothesis is supported by reports on familial clustering of DN[3] and by studies demonstrating association with familial predisposition to hypertension.[4]

The favorable effect of ACE inhibition on the development[5] and progression[6] of DN is suggestive evidence that the renin-angiotensin system (RAS) is involved in the pathogenesis of DN and that variation at RAS gene loci might determine susceptibility to DN. In many studies, however, plasma renin and angiotensin II are normal or suppressed in diabetics (for review see 7 and 8) and reports on association between DN and

polymorphic markers have been equivocal for the angiotensinogen gene (ref. 9-11 positive and 12 negative) and negative for the AT1-receptor gene.[13, 14] Three meta-analyses of the numerous studies on association of the ACE I/D polymorphism with DN show that the D-allele may confer an increased risk of DN.[15-17] Interaction between the ACE I/D polymorphism and an angiotensinogen allele may also present elevated risk of DN.[18] Similarly, poor metabolic control may interact with the AT1-receptor polymorphism to augment risk of DN.[14] The renin gene that codes for prorenin and renin, has not yet been investigated in DN in large study groups. Prorenin is the enzymatically inactive biosynthetic precursor of renin, the rate-determining enzyme in the proteolytic cascade leading to formation of angiotensin II, the effector of the RAS. Several reports have described increased plasma prorenin levels when diabetes is complicated by microvascular disease like DN and retinopathy.[19-22] In the present case-control study we measured plasma levels of renin and prorenin and determined genotypes of the renin gene in patients with IDDM complicated by DN and in matched normoalbuminuric controls.

Subjects and Methods

Subjects

In 1993 242 IDDM patients with DN had their glomerular filtration rate measured at the Steno Diabetes Centre (Copenhagen, Denmark). Plasma and DNA samples were obtained from 199 patients of this cohort. Nephropathy in this study was defined as the presence of a urinary albumin excretion rate > 300 mg/24 h in at least two out of three consecutive urine samples, combined with the presence of retinopathy, while clinical or laboratory evidence of urinary tract disease other than diabetic glomerular sclerosis was absent. A group of 192 IDDM patients with persistent normal urinary albumin excretion rate (< 30 mg/24 h) and matched for sex, age and duration of diabetes, served as controls. A detailed description of the subjects with and without diabetic nephropathy can be found in references 23 and 24.

Methods

Blood pressure was measured with a Hawksley random zero standard sphygmomanometer after a ten minutes rest in the sitting position. The disappearance of Korotkoff sounds (phase V) was used to determine diastolic blood pressure. Retinopathy was assessed by fundus photography after pupillary dilatation. Retinal lesions were categorized as either no, background or proliferative retinopathy. EDTA-anticoagulated blood for measurement of plasma prorenin and renin was collected in the morning after an overnight fast from an indwelling catheter in a forearm vein after the patient had been in the supine position for at least fifteen minutes. The blood was centrifuged at 3000 g at room temperature and plasma was stored at -80 °C. Sixty nine patients with nephropathy and 9 with normoalbuminuria did not want to stop their antihypertensive drugs at least 8 days before the day investigations were performed.

Laboratory procedures. All measurements were done by technicians who were unaware of the status of the patients. Urinary albumin excretion rate (AER) was measured from 24 hour-collections by an enzyme-linked immunoassay.[25] Hemoglobin A_{1c} (HbA_{1c}) was

measured by high performance liquid chromatography (HPLC, Biorad, Diamat, Richmond, CA, USA). The normal range is 4.1-6.4 %. Serum creatinine was measured by autoanalyser.

Plasma Prorenin and Renin Determination. Renin and total renin (i.e. renin + prorenin) were measured by an immunoradiometric assay (IRMA), purchased as a kit from Nichols Institute, Wijchen, The Netherlands.[26] In this assay renin is sandwiched between a biotinylated monoclonal antibody (mAb) R3-36-16 against human renin, immobilized on an avidin-coated polystyrene bead, and a ^{125}I-labeled mAb R1-20-5 against a renin-specific epitope not expressed on prorenin. Therefore, the radiolabeled mAb does not recognize native prorenin. Yet, prorenin can be measured with this assay, after preincubation with the active-site directed renin inhibitor remikiren, which causes exposure of the renin-specific epitope recognized by mAb R 1-20-5.[26] The difference between the results with and without remikiren, i.e. the difference between total renin and renin, is a measure of the prorenin concentration. The assay was modified by shortening incubation to 6 hours and performing the assay at 37 °C. These modifications prevent inadvertent activation of prorenin and therefore exposure of the renin-specific epitope during the assay. Intra- and interassay variation for prorenin (320 mU/ml) is 4 and 13% respectively and for renin (22.2 mU/ml) 8 and 15%. Renin and prorenin of each patient were always measured in the same assay. Results of the renin and prorenin measurements are expressed as mU/ml. Standards in the assay were dilutions of the international reference preparation of human kidney renin 68/356 of the National Institute for Biological Standards and Control (Potters Bar, Hertfordshire, UK). The geometric mean and 95 % reference limits for 100 normal subjects (50 women) with an age range of 19-62 years was 22.0 (7.7-54.8) mU/ml for renin and 199 (88.1-390) mU/ml for prorenin.

DNA Extraction. Genomic DNA was extracted from peripheral leukocytes by a standard technique. It was aliquoted and stored at -80 °C until use.

Genotyping. Four polymorphisms were studied at the REN locus (**Fig. 1**). They consist of two restriction fragment length polymorphisms (RFLPs)[27, 28] and two microsatellites.[29, 30] The Taq I RFLP is located 4063 bases upstream of the start codon and consists of a C-T mutation. The Bgl I RFLP is a C-T mutation located at base 1161 of intron A. The $(ACAG)_n$ microsatellite is located in intron G. The CA-repeat is located downstream of the REN locus. Genotyping was PCR-based. Primers were obtained from Eurogentec (Seraing, Belgium), as were Goldstar Taq polymerase and the restriction

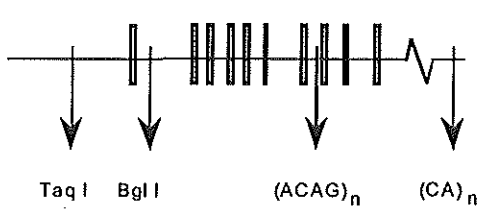


Figure 1. Schematic representation of the human renin gene. Shown are the exons (boxes) and the sites of the four polymorphic markers that were studied (see text). Length from Taq I marker to final exon is about 16 Kb.

Table 1. Primer sequences and PCR conditions for genotyping of renin gene polymorphisms

polymorphism	*Primers*	*Product size* (bp)	*PCR*
Bgl I-RFLP	5'-GGGGAAGCAGCTTGATATCGTGG 5'-CTAGGCTGGAGCTCAAGCGATC	772 (515/257)*	3' 95 °C, 30 x (30" 92 °C, 30" 60 °C, 1' 72 °C), 4' 72 °C
Taq I-RFLP	5'-GCTGTCTTCTGGTGGTACTGCC 5'-TGCTGGCCATGAACTGGTTCTAGC	964 (394/570*	5' 95 °C, 30 x (45" 95 °C, 30" 60 °C, 1'30" 72 °C), 6' 72 °C
Taq I/Bgl I haplotype	5'-GCTGTCTTCTGGTGGTACTGCC 5'-CTAGGCTGGAGCTCAAGCGATC	6079 (5324/5687/ 5717)§	2' 94 °C, 10 x (10" 94 °C, 30" 65 °C, 5' 68°C), 15 x (10" 94 °C, 30" 65 °C, 5' plus 20"/cycle 68 °C), 7' °C
tetranucleotide repeat	5'-AGAGTACCTTCCCTCCTCTACTCA[1] 5'-CTCTATGGAGCTGGTAGAACCTGA	255, 263, 267, 271	5' 95 °C, 15 x (1' 92 °C, 1' 59 °C, 2' 72 °C‡), 15x (1' 92 °C, 1' 59 °C, 1' 72 °C),
dinucleotide repeat	5'-GCGGGATATTTGAGTTGTGT 5'-GAACTGTTCAACTGGAGCCT[1]	126-144 (10 alleles)	5' 72 °C

* size of bands after digestion if restriction site is present

§ size of major bands if both Bgl I and Taq I, only Taq I or only Bgl I restriction sites are present respectively

enzymes. Except for haplotyping the Bgl I and Taq I loci (see below), PCR volume was 25 μL, consisting of 200 ng of DNA, 75 mM Tris-HCl, 20 mM $(NH_4)_2SO_4$, 0.01% Tween 20, 0.5 μM of each primer, dNTP concentrations of 10 μM each and a polymerase activity of 1U/25 μl PCR volume. Primer sequences and cycling conditions are summarized in Table 1.

Genotyping of the RFLPs was performed by PCR of the chromosomal region around the marker locus, followed by digestion by the restriction enzyme. Alleles were called B and T or b and t, indicating respectively digestion by Bgl I and Taq I or not. Controls with different genotypes, verified by direct sequencing, were run in each assay. Haplotyping of the Bgl I and Taq I loci in double heterozygotes was performed by a PCR on genomic DNA, using the Expand Long Template PCR System according to instructions by the manufacturer (Boehringer Mannheim, Germany). PCR volume was 50 μl and primer and DNA concentrations were the same as above. Primer sequences and cycling conditions are given in Table 1. PCR products were resolved on a 0.5 % agarose gel. Sequencing of the RFLP-loci was performed with PCR products as templates and a dye-Terminator technique (Perkin-Elmer, Applied Biosystems Division, Foster City, CA) using a nested primer. Electrophoresis was on the ABI-Prism 377 automatic sequencer. Results were analyzed with ABI Sequencing software.

Microsatellite loci were amplified in a duplex PCR, with one of each primer pair labeled with the fluorochrome FAM. Labeled PCR products were separated by electrophoresis on a denaturing 5% polyacrylamide gel, with fluorescence detection by an ABI Prism 377 automatic sequencer (Perkin Elmer, Applied Biosystems Division, Foster City, CA). In each lane a TAMRA-labeled calibration ladder was run. Allele lengths were calculated by Genescan software and checked by eye by two independent observers.

Statistical Analysis. Continuous normally distributed data were expressed as mean (standard deviation). AER, creatinine, plasma renin and prorenin were log transformed

before statistical analysis in order to obtain normal distribution. They are presented as median (range). Comparisons between groups were performed by a Student t-test or ANOVA. Chi square analysis was used to compare the distribution of genotypes and alleles and for comparison between groups for non-continuous variables. Two-sided P-values ≤ 0.05 were considered significant. All aforementioned analyses were performed using the statistics package Statgraphics (Manugistics, Princeton NJ). Multi-allelic polymorphisms were tested using the CLUMP program. CLUMP employs Monte Carlo simulation and has been developed for use in genetic case-control studies. A description of the program can be found in reference.[31]

Results

Patient Characteristics. Table 2 describes the features of the patients that were enrolled in the study. Patients with nephropathy and those with normoalbuminuria were well matched with regard to sex, age and duration of diabetes. In the nephropathic group systolic and diastolic blood pressure were higher and serum creatinine was increased compared to the normoalbuminuric group. Furthermore, HbA_{1c} levels were higher in nephropathic patients.

Renin and Prorenin are Increased in Diabetic Nephropathy. When plasma levels were evaluated according to the use of antihypertensive medication (aHT) at the time of blood sampling, both renin and prorenin levels in the 69 patients with nephropathy on aHT were higher than those in the patients not on aHT at the time of blood sampling. Normoalbuminuric patients showed no difference in renin or prorenin levels between patients with or without aHT at the time of blood sampling (Table 3). In the 130 patients with DN and the 183 normoalbuminuric patients that either used no drugs or had stopped aHT for at least 8 days, renin and prorenin levels were elevated in nephropathic patients (26.3 (5.2-243.3) and 789 (88-5481) mU/ml resp. *vs.* 18.3 (4.2-373.5) and 302 (36-2226) mU/ml in normoalbuminuric patients, $p < 0.0001$ both for renin and prorenin). If nephropathic patients that had never used antihypertensive drugs ($n = 48$) were compared to 48 normoalbuminuric patients, matched for age, sex and diabetes duration, prorenin levels were 527 (110-1653) vs. 296 (80-1812) mU/ml and renin levels were 23.3 (5.8-114.8) vs. 19.4 (4.2-81.6) mU/ml ($p < 0.001$ and $p = 0.059$ resp.). The increase in renin in nephropathic patients was not due to lower sodium intake as assessed by 24 hour urinary excretion of sodium chloride. Sodium excretion was the same in both groups (150 ± 84 *vs.* 151 ± 66 mmol/24 hour, NS). Sodium excretion was also identical in nephropathic patients using aHT at the time of blood sampling, nephropathic patients that had stopped aHT and patients not on aHT medication. Log[renin] and log[creatinine] as well as log[prorenin] and log[creatinine] were correlated in the nephropathic group (R resp. 0.31 and 0.41, $p = 0.0003$ and $p < 0.0001$ respectively).

Influence of Retinopathy on Renin and Prorenin Levels. Proliferative retinopathy appears to have an effect on prorenin that is independent of the presence of nephropathy (Table 4). The effect on renin levels is at most modest and equivocal, depending on the

Table 2. Clinical characteristics of the case-control study group, consisting of 199 patients with diabetic nephropathy and 192 diabetic patients with normoalbuminuria

	Nephropathy	Normoalbuminuria
n	199	192
sex (m/f)	122/77	118/74
age (years)	40.9±9.6	42.7±10.2
duration of diabetes (years)	27.7±7.9	26.8±8.5
BMI (kg/ m^2)	24.0±3.3	23.6±2.5
HbA_{1c}(%)	9.6±1.5	8.5±1.1*
UAER (mg/24 h)	796	8
	(16-14,545)	(1-30)
serum creatinine (μM)	103	76*
	(54-684)	(40-116)
systolic BP (mm Hg)	151±23	132±18*
Diastolic BP (mm Hg)	86±13	76±10*
Antihypertensive treatment (n)	150	23*
ACE-inhibitor	106	9
Beta-blocker	28	3
Calcium-entry blocker	44	6
Diuretic	129	15
Retinopathy (n)		
Nil	0	68
Simplex	62	105
Proliferative	137	19

Data are means ±SD, median (range), or n(%). * p<0.001

test used. The results of the statistical analysis by two-way ANOVA should be considered with caution, because groups are unbalanced.

Relationship between Diabetic Nephropathy and Renin Gene Polymorphisms

Table 5 gives the frequencies of genotypes or alleles of the renin gene polymorphisms in the two groups. Frequencies of Taq I and Bgl I polymorphisms and of the microsatellites are in accordance with frequencies found in other Caucasian populations.[27-30] Genotypes of the RFLPs were in Hardy-Weinberg equilibrium. The power of detecting a 20% increase in frequency of allele b of the Bgl I polymorphism is 98%, and 60 % for a 10%

Table 3. Plasma renin and prorenin levels in patients with diabetes mellitus with or without diabetic nephropathy

		nephropathy	normoalbuminuria
renin (μ/ml)	no anti-HT	26.3* (5.2-243.3) n=130	18.3 (4.2-373.5) n=183
	+anti-HT	55.2‡ (10.9-506.3) N=69	26.8 (6.6-78.5) n=9
prorenin (μU/ml)	no anti-HT	789* (88-5481) n=130	302 (36-2226) n=183
	+anti-HT	1085‡ (178-10740) n=69	288 (173-686) n=9

Data are median(range). *p<0.0001 compared to levels in normoalbuminuric patients ‡p<0.01 compared to levels in patients with same renal status but without antihypertensive medication. anti-HT= antihypertensive therapy

increase. Similarly, the power to detect a 20 % increase in frequency of the t-allele of the Taq I polymorphism is 99% and 72% for a 10 % increase. There were no significant differences in allele frequencies of the Bgl I and Taq I polymorphism in the two groups. However, genotype frequency distribution appeared to differ. Chi square analysis of BB *vs.* Bb *vs.* bb in the two groups yielded a p-value of 0.056. Grouping of Bgl I genotypes based on the presence of either at least one B-allele (BB+Bb *vs.* bb) or at least one b-allele (bb+Bb *vs.* BB) gave p-values of 0.037 and 0.87. This suggests an overrepresentation of bb-genotypes in the nephropathic group. The unadjusted odds ratio for nephropathy for subjects with a bb-genotype is 1.56 (95% C.I. 1.05-2.33). The odds ratio for nephropathy, as determined by multiple logistic regression analysis, and adjusted for sex, age, duration of diabetes, glycated hemoglobin level and systolic blood pressure, was 1.70 for the bb-genotype (p=0.03). Haplotyping of the Bgl I and Taq I loci revealed the absence of bt alleles. Therefore only 6 different genotypes were observed. We studied with the Clump-package the distribution of two microsatellites at the renin gene locus and the genotypes of the Bgl I/Taq I haplotypes, arranged in 2xN tables as shown in Table 5. Estimated 'p-values' for the least conservative of the four available tests was 0.188 for the Bgl I/Taq I haplotypes, 0.81 for the tetranucleotide repeat and 0.088 for the dinucleotide repeat (each value calculated after 1000 simulations), arguing against association between nephropathy and these polymorphic markers.

Although a trend towards higher prorenin levels exists in bb homozygotes (bb vs. BB+Bb, p=0.07) (Table 6), we did not find significant differences in plasma levels between the other renin genotypes, neither in the nephropathic nor in the normoalbuminuric group (results not shown).

Table 4. Renin and prorenin plasma levels in patients with or without diabetic nephropathy, according to the presence or absence of retinopathy

	nephropathy		normoalbuminuria	
retinopathy	renin (μU/ml)	prorenin* (μU/ml)	renin (μU/ml)	prorenin‡ (μU/ml)
no			17.5	267
	-	-	(4.2-66.8)	(36-711)
			n= 65	
background	26.5	553	18.1	321
	(5.2-82.4)	(88-2301)	(5.3-373.5)	(45-1124)
	n = 43		n = 100	
proliferative	26.1	869	19.4	414
	(7.7-243.3)	(167-5481)	(10.5-81.6)	(224-2226)
	n = 87		n = 18	

<0.001 proliferative vs. background. ‡ ANOVA: p=0.0004, proliferative vs.background: p=0.001, Proliferative + background Shown are median (range). Patients taking antihypertensive medication are not included.

* p vs. nul: p=0.04. Renin levels are not significantly different in different stages of retinopathy by this analysis. A two-way ANOVA of log-transformed renin and prorenin levels in a collapsed table (proliferative retinopathy YES/NO and nephropathy YES/NO) demonstrates a significant effect of the presence of nephropathy and proliferative retinopathy on prorenin levels (p<0.0001). In contrast with the results of ANOVA on the table above a two-way ANOVA on a collapsed table demonstrates an effect of retinopathy on renin levels (p 0.02). The effects of retinopathy and nephropathy on renin and prorenin levels are independent (p=0.30 and 0.91 resp, two-way ANOVA).

Finally, we analysed the data for association of renin gene polymorphisms with coronary heart disease, hypertension and retinal status. No statistically significant differences were revealed (data not shown).

In multiple logistic regression analysis, with presence or absence of nephropathy as dependent variable and the renin Bgl I and Taq I polymorphisms, ACE I/D polymorphism, angiotensinogen M235T and T174M polymorphisms and the angiotensin receptor type 1 A/C polymorphism at position 1166 as independent variables, only the renin bb-genotype, not surprisingly, remained in the final model (p = 0.037), but more importantly, no interactions were observed between the bb-genotype and the polymorphisms in the angiotensinogen, ACE and AT1R genes.

Discussion

In this report we confirm previous findings that in IDDM patients plasma prorenin levels are increased by 150% when DN is present. We also found plasma renin to be 50% higher in nephropathic than in normoalbuminuric patients with IDDM. Furthermore,

Table 5. Renin gene polymorphisms in patients with insulin-dependent diabetes mellitus

Genotype/ alleles	nephropathy n	normoalbuminuria n
TT	158	140
Tt	36	48
tt	5	4
BB	21	19
Bb	71	91
bb	107	82
haplotype genotypes		
BT/BT	7	7
Bt/Bt	5	4
bT/bT	107	82
BT/Bt	9	8
BT/bT	44	51
Bt/bT	27	40
tetranucleotide repeat (length in basepairs)		
255	314	309
259	0	0
263	38	32
267	43	41
271	3	2
dinucleotide repeat (length in basepairs)		
126	0	4
128	37	32
130	165	149
132	19	20
134	52	53
136	68	80
138	30	34
140	22	12
142	4	0
144	1	0

the presence of diabetic nephropathy is weakly associated with one of the genotypes of a Bgl I polymorphism in the first intron of the renin gene.

Although there are other reports of increased renin levels in DN,[8, 20, 32] general impression has always been that renin levels are low in DN (for a list of references see ref. 7). The apparent discrepancy with our finding of increased renin levels could be due to various factors. The first may be the poorly developed measurement methodology for renin.[33] Most studies date from the seventies and early eighties and employed variants of an enzyme-kinetic, plasma renin activity (PRA) assay, which measures angiotensin I production by renin from endogenous angiotensinogen. The PRA assay depends on both renin and angiotensinogen concentration. Kinetics of the enzymatic reaction of renin with angiotensinogen, often performed at non-physiologic pH in PRA assays, may differ in diabetic plasmas, including altered renin reactivity and non-linear angiotensin I generation, but no reports exist that address this problem. The immunologic, direct

assay we employed, measures molecules and is unlikely to be affected by the diabetic state. Another recent study that measured renin by IRMA, also found a trend to higher renin levels in diabetic subjects with microalbuminuria -the early phase of DN- compared to normoalbuminuric diabetics.[21] This suggests again that the cause of the discrepancy may be assay-related. Renin IRMAs may have a problem in co-measurement of prorenin as renin. Our IRMA employs a monoclonal antibody specific for renin. Prorenin may also assume a renin-like conformation by so-called cryoactivation, even at room temperature, which is the temperature of the assay incubation.[34] Although this activation amounts to not more than 1 to 2 %, renin levels may be overestimated, especially if prorenin levels are high. In our assay co-measurement of prorenin as renin is eliminated by performing the assay for 6 hours at 37 °C. We are therefore confident that our results on plasma renin in DN are valid. Another explanation for the discrepancy of previous reports with our observation of increased renin may be that study groups were often not homogeneous. Data for NIDDM and IDDM patients were often pooled in earlier studies and it is not known whether this is justified. Moreover, the various stages of DN were not always separated, partly because at the time these stages were not yet discerned. A final possible explanation for our high renin levels is that these are due to previous antihypertensive use. High renin levels may be observed with ACE-inhibitors and diuretic use (AT1-receptor antagonists were not yet being prescribed to these patients). Patients had stopped antihypertensive medication at least 8 days before blood-sampling. This is usually sufficient for any effect of ACE-inhibition to wear off.[35] After cessation of diuretic use secondary hyperaldosteronism may persist for longer periods than 8 days. However, this, together with a higher blood pressure, is expected to cause decreased renin levels, rather than the increased levels we found. Unfortunately, data on aldosterone that may clarify the issue somewhat, are not available. Finally, nephropathic patients virgin to antihypertensives have somewhat less increase in plasma renin and prorenin levels. This might argue in favor of a carry-over effect of antihypertensives on prorenin and renin levels in the hypertensive subgroup. On the other hand, this normotensive subgroup too does not show suppressed renin levels as is expected from older literature,[7] but rather increased levels.

Increased plasma renin and prorenin levels in DN support the hypothesis that the RAS is overactive and possibly even pathogenetic in the development or progression of DN, especially since increase in total renin occurs early.[22] A possible mechanism could be higher intra-glomerular pressure, through angiotensin II-mediated constriction of the efferent arteriole, with resulting proteinuria, but this remains speculative. The origin of elevated renin is most probably the kidney, since no other source of renin has been described.[36] Whether an excess prorenin also originates from the kidney is not known. Franken et al. did not observe an increase in renal vein-to-artery ratio for prorenin in patients with near end-stage diabetic nephropathy and high prorenin, suggesting that there is no markedly increased production of prorenin in the kidney.[20] Prorenin may also originate from extra-renal sites like ovaries, testes and adrenals.[36] Whether prorenin itself acquires enzymatic activity in DN is an unsolved question. Several mechanisms of prorenin activation have been described.[36 37]

Some controversy exists concerning the relation between retinopathy and plasma prorenin. We observed increased prorenin levels with proliferative retinopathy. This is in agreement with Franken et al., who found an increase of plasma prorenin with increasing severity of retinopathy.[20] However, contrary to their findings, we did not find a difference between patients without and those with background retinopathy in the normoalbuminuric group. This is similar to the result obtained by Allen et al., who did not observe -in a longitudinal survey- an increase in plasma prorenin when patients progressed from no to background retinopathy[22] Thus, apparently retinopathy and nephropathy have an additive effect on plasma prorenin levels, suggesting that the increase in plasma prorenin is an aspecific marker for microvascular disease as proposed previously.[19] Yet, probably only the advanced, proliferative stage of retinopathy influences plasma prorenin. This is in contrast with DN, where plasma prorenin increases very early in the course of the disease.[22]

The derangement in renin and prorenin in DN could be connected to a polymorphism in the renin gene, if this polymorphism is associated with DN. DN is weakly associated with the bb-genotype of a Bgl I RFLP in the first intron of the renin gene. This suggests a deleterious recessive effect of the b-allele. The first intron is involved in renin gene transcription regulation[38] and the Bgl I polymorphism may be linked to this regulatory sequence. This may provide a mechanism that explains the association of DN with this marker. It could also explain the findings of Daneman et al. who described a genetic influence on plasma prorenin levels in diabetic patients with microalbuminuria,[21] although no genotyping of the renin gene was performed in this study. From this observation we anticipated a relation between Bgl I renin genotype and plasma renin or prorenin level. However, we could not demonstrate such an association, although a trend towards higher prorenin levels was observed in bb homozygotes. This trend may have failed to reach significance because only prorenin levels in the subgroup of patients not on antihypertensives were included in our analysis.

There was no interaction of the Bgl I marker with variants of other genes of the RAS, although the power of the study was not sufficient to detect any interactions but very strong ones. Three other markers in the renin gene were not associated with nephropathy. There is only one report about polymorphic markers in the renin gene in diabetic patients with nephropathy and their controls.[39] This report did not show an association of renin gene polymorphisms with diabetic nephropathy. However, the report was based on a small group (40 patients) and diabetes duration was shorter (11 years), so that future nephropathic patients may have been present among control subjects.

The polymorphic marker that was first studied in the present study group, the ACE gene I/D polymorphism, was not in Hardy-Weinberg equilibrium in the control group,[23] which might cast doubt on any conclusion on the genetics of DN in this study group. However, polymorphic markers in the renin, angiotensinogen[10] and AT1-receptor[13] genes were in Hardy-Weinberg equilibrium in the control group, which makes selection bias of controls unlikely. The absence of Hardy-Weinberg equilibrium in the ACE gene may be caused by misclassification of alleles,[40] or may be incidental, the ACE-gene not being linked to the other RAS-genes.

Our control group turned out to have better metabolic control. Individuals with the bb-genotype of the renin gene may have been protected from DN if this better metabolic control has been long-term. Whether this may have weakened the association between DN and a renin gene polymorphic marker, we do not know. In subgroup analyses according to glycated hemoglobin levels either matching was lost or groups became very small.

Our study group is unique in that now the renin, angiotensinogen, ACE and AT1R genes have been studied for association with DN. We can conclude that in this group of all RAS-components[10, 13, 23] plasma renin and prorenin and possibly the renin gene Bgl I polymorphism are associated with DN. Plasma ACE has also been found to be increased in DN in this study group,[23] but the ACE gene I/D polymorphism was not associated with DN in these patients. Recent meta-analyses, however, showed that the ACE gene D-allele may confer an increased risk for DN (excess risk up to 56% depending on ethnic background and reliability of genotyping method).[15-17]

Since the effect of the ACE I/D polymorphism and, in our group, the renin Bgl I polymorphism is small and since there is no major interaction between the RAS-gene variants we must assume that the main part of the genetically determined susceptibility to DN lies outside the genes that code for RAS-components. Still, in view of our results on plasma renin and prorenin measurements and in view of the favorable reaction to ACE inhibitors, a pathogenetic mechanism of DN in which the RAS, in particular renin and prorenin, is involved, remains likely.

In conclusion, a direct, validated assay showed that both prorenin and renin are increased in plasma of IDDM patients with diabetic nephropathy. In the present patient group a renin gene variant is the sole genetic factor within the RAS that may contribute, albeit weakly, to the genetic susceptibility to diabetic nephropathy. Renin and prorenin could well play a direct pathogenetic role in DN.

References

1 Borch-Johnsen K, Andersen PK, Deckert T. The effect of proteinuria on relative mortality in type 1 (insulin-dependent) diabetes mellitus. Diabetologia 1985; 28:590-6.

2 Andersen AR, Christiansen JS, Andersen JK, Kreiner S, Deckert T. Diabetic nephropathy in type 1 (insulin-dependent) diabetes: an epidemiological study. Diabetologia 1993; 25:496-501.

3 Seaquist ER, Goetz FC, Rich S, Barbosa J. Familial clustering of diabetic kidney disease: evidence for genetic susceptibility to diabetic nephropathy. N Engl J Med 1989; 320:1161-5.

4 Krolewski AS, Canessa M, Warram JH, Laffel LM, Christlieb AR, Knowler WC et al. Predisposition to hypertension and susceptibility to renal disease in insulin-dependent diabetes mellitus. N Engl J Med 1988; 318:140-5.

5 Viberti GC, Mogensen CE, Groop LC, Pauls JF. Effect of captopril on progression to clinical proteinuria in patients with insulin-dependent diabetes and microalbuminuria. JAMA 1994; 271:275-9.

6 Lewis EJ, Hunsicker LG, Bain RP, Rohde RD, FOR THE COLLABORATIVE STUDY GROUP. The effect of angiotensin-converting enzyme inhibition on diabetic nephropathy. N Engl J Med 1993; 329:1456-62.

7 Weidmann P, Perrari P, Shaw SG. Renin in diabetes mellitus. In: Robertson JIS and Nicholls MG, ed. The Renin-Angiotensin System. Gower Medical Publishing, London 1993: p75.12.

8 Björck S: The renin angiotensin system in diabetes mellitus. Scand J Urol Nephrol 1990; Suppl.126: 1-51.

9 Schmidt S, Giessel R, Bergis KH, Strojek K, Grzeszszak W, Ganten D et al. Angiotensinogen gene M235T polymorphism is not associated with diabetic nephropathy. Nephrol Dial Transplant 1996; 11:1755-61.

10 Tarnow L, Cambien F, Rossing P, Nielsen FS, Hansen BV, Ricard S et al. Angiotensinogen gene polymorphisms in IDDM patients with diabetic nephropathy. Diabetes 1996; 45:367-9.

11 Doria A, Onuma T, Gearin G, Freire MB, Warram JH, Krolewski AS. Angiotensinogen polymorphism M235T, hypertension and nephropathy in insulin-dependent diabetes mellitus. Hypertension 1996; 27:1134-9.

12 Fogarty DG, Harron JC, Hughes AE, Nevin NC, Doherty CC, Maxwell AP. A molecular variant of angiotensinogen is associated with diabetic nephropathy in IDDM. Diabetes 1996; 45 :1204-8.

13 Tarnow L, Cambien F, Rossing P, Nielsen FS, Hansen BV, Ricard S et al. Angiotensin-II type 1 receptor gene polymorphism and diabetic microangiopathy Nephrol Dial Transplant 1996;11:1019-23.

14 Doria A, Onuma T, Warram JH, Krolewski AS. Synergistic effect of angiotensin II type I receptor genotype and poor glycemic control on risk of nephropathy in IDDM. Diabetologia 1997;40:1293-9.

15 Staessen JA, Wang JG, Ginocchio G, Petrov V, Saavedra AP, Soubrier F et al. The deletion/insertion polymorphism of the angiotensin converting enzyme gene and cardiovascular-renal risk. J. Hypertens 1997; 15:1579-92.

16 Fujisawa T, Ikegami H, Kawaguchi Y, Hamada Y, Ueda H, Shintani M et al. Meta-analysis of association of insertion/deletion polymorphism of angiotensin I-converting enzyme gene with diabetic nephropathy and retinopathy. Diabetologia 1998;41:47-83.

17 Tarnow L, Gluud C, Parving H-H. Diabetic nephropathy and the insertion/deletion polymorphism of the angiotensin-converting enzyme. Nephrol Dial Transplant 1998,13:1125-30.

18 Marre M, Jeunemaître X, Gallois Y, Rodier M, Chatellier G, Sert C et al. Contribution of genetic polymorphism in the renin-angiotensin system to the development of renal complications in insulin-dependent diabetes mellitus. J Clin Invest 1997,99:1585-95.

19 Luetscher JA, Kraemer FB, Wilson DM, Schwartz HC, Bryer-Ash M. Increased plasma inactive renin in diabetes mellitus. N Engl J Med 1985;312:1412-7.

20 Franken AAM, Derkx FHM, Man in't Veld AJ, Hop WCJ, van Rens GH, Peperkamp E et al. High plasma prorenin in diabetes mellitus and its correlation with some complications. J Clin Endocrinol Metab 1990;71:1008-15.

21 Daneman D, Crompton CH, Balfe JW, Sochett EB, Chatzilias A, Cotter BR et al. Plasma prorenin as an early marker of nephropathy in diabetic (IDDM) adolescents. Kidney Int 1994; 6:1154-9.

22 Allen TJ, Cooper ME, Gilbert RE, Winikoff J, Skinner SL, Jerums G. Serum total renin is increased before microalbuminuria in diabetes. Kidney Int 1996; 0: 902-7.

23 Tarnow L, Cambien F, Rossing P, Nielsen FS, Hansen BV, Lecerf L et al. Lack of relationship between an insertion/deletion polymorphism in the angiotensin I-converting enzyme gene and diabetic nephropathy and proliferative retinopathy in IDDM patients. Diabetes 1995;44:489-94.

24 Tarnow L, Cambien F, Rossing P, Nielsen FS, Hansen BV, Lecerf L et al. Insertion/deletion polmorphism in the angiotensin I-converting enzyme gene is associated with coronary heart disease in IDDM patients with diabetic nephropathy. Diabetologia 1995;38:798-803.

25 Feldt-Rasmussen B, Dinesen B, Deckert M. Enzyme immunoassay: an improved determination of urinary albumin in diabetics with incipient nephropathy. Scand J Clin Lab Invest 1985;45:539-44.

26 Derkx FHM, de Bruin RJA, van Gool JMG, van den Hoek M-J, Beerendonk CCM, Rosmalen F et al. Clinical validation of renin monoclonal antibody-based sandwich assays of renin and prorenin, and use of renin inhibitor to enhance prorenin immunoreactivity. Clin. Chem. 1996;42:1051-63.

27 Frossard PM, Gonzalez PA, Dillan N, Coleman RT, Atlas SA Human renin (REN) gene locus: Bgl II, Rsa I and Taq I RFLPs. Nucleic Acids Res 1986;16:6778.

28 Naftilan AJ, Williams R, Burt D, Paul M, Pratt RE, Hobart P et al. A lack of genetic linkage of renin gene restriction fragment length polymorphisms with human hypertension. Hypertension 1989;14: 614-8.

29 Edwards A, Hammond HA, Jin L, Caskey CT, Chakraborty R. Genetic variation at five trimeric and tatrameric tandem repeat loci in four human population groups. Genomics 1992;12:241-53.

30 Engelstein M, Hudson TJ, Lane JM, Lee MK, Leverone B, Landes GM et al. A PCR-based linkage map of human chromosome 1. Genomics 1993;15:251-8.

31 Sham PC, Curtis D. Monte Carlo tests for associations between disease and alleles at highly polymorphic loci. Ann Hum Genet 1995;59:97-105.

32 Paulsen EP, Seip RL, Ayers CR, Croft BY, Kaiser DL. Plasma renin activity and albumin excretion in teenage type I diabetic subjects. Hypertension 1989;13:781-8.
33 Robertson JIS, Nicholls MG (1993). Appendix II. Standardization and Standards. In Robertson JIS, Nicholls MG Ed. The renin-angiotensin system; Gower Medical Publishing, London. P.A.7-A.9.
34 Pitarresi TM, Rubattu S, Heinrikson R, Sealey JE. Reversible cryoactivation of recombinant human prorenin. J Biol Chem 1992;267:11753-9.
35 Gadsbøll N, Leth A, Giese J, Nielsen MD, Rasmussen S. Blood pressure response to withdrawal of converting enzyme inhibition. J Hypertens 1986;4 (Suppl. 6):S474-6.
36 Nielsen AH, Poulsen K. Is prorenin of physiological and clinical significance? J. Hypertension 1988;6:949-58.
37 Heinrikson RL, Hui J, Zuercher-Neely H, Poorman RA. A structural model to explain the partial catalytic activity of human prorenin. Am J Hypertens 1989;2:367-80.
38 Germain S, Bonnet F, Philippe J, Corvol P, Pinet F. Molecular regulation of human renin gene expression: cell-specific role of intron A. Hypertension 1997;30:986 (Abstract).
39 Angelico MC, Laffel L, Krolewski AS. Application of denaturing gradient gel electrophoresis to detect DNA polymorphisms in the renin gene in IDDM patients with and without diabetic nephropathy. Current Topics in Diabetes Research. Front Diabetes. Basel, Karger 1993;12:227-30.
40 Shanmugan V, Sell KW, Saha BK. Mistyping ACE heterozygotes. PCR Methods Appl 1993;3:120-1.

Chapter 5 Tissue Prorenin: the Eye

5.1 Renin, Prorenin and Immunoreactive Renin in Vitreous Fluid from Eyes With and Without Diabetic Retinopathy

Summary

Renin, prorenin and immunoreactive renin were present in vitreous and subretinal fluid of eyes from subjects with and without diabetic retinopathy. Renin substrate, albumin, transferrin and immunoglobulin G were also found in these ocular fluids. In many samples renin levels were close to the detection limit of the assay. The levels of renin substrate, albumin, transferrin and immunoglobulin G varied widely among ocular fluid samples, but in each individual sample the levels were, relative to each other, similar to those in plasma. In contrast, the prorenin level in ocular fluid was up to 100 times higher than expected on the basis of the plasma protein content of ocular fluid. Moreover, there was little difference in prorenin concentrations between samples with a low and a high plasma protein content. Prorenin, relative to albumin and other plasma proteins, was higher in vitreous fluid from eyes with proliferative diabetic retinopathy complicated by traction retinal detachment than in eyes of non-diabetic subjects with spontaneous retinal detachment. It appears that prorenin (and possibly renin) in ocular fluid is controlled by an active and specific process, possibly local synthesis within the eye. In view of the vascular actions of angiotensin II, an intraocular renin-angiotensin system may play a role in diabetic retinopathy.

Introduction

The kidney secretes both renin and prorenin, an inactive precursor of renin, into the circulation. Plasma of nephrectomized patients contains little or no renin but it does contain prorenin,[1,2] in concentrations sometimes as high as those in normal individuals. It thus appears that extrarenal production can make a major contribution to the level of prorenin in plasma, whereas most, if not all, renin in plasma is secreted by the kidneys. Synthesis of renin or prorenin and other components of the renin-angiotensin system is known to occur at various extrarenal sites, for instance adrenal,[3,4] pituitary,[3,4] testis,[3,4] brain,[5] and ovary.[6,7,8] Cultured human chorionic cells[9] and ovarian thecal cells[8] release prorenin into the medium and there is good evidence that in women with hyperstimulated cycles and during pregnancy, the ovary, probably the corpus luteum, releases prorenin into plasma.[7,10]

A common feature of the organs in which synthesis of renin or prorenin occurs is their extensive vascularization.[11] The eye, particularly the retina and uveal tract, is such a highly vascularized organ. Angiotensin II-binding sites have been found in retinal blood vessels,[12] and transvitreal infusion of angiotensin I and II produces constriction of the retinal arteries.[13] The retina contains angiotensin converting enzyme activity[14] and this enzyme is also found in aqueous fluid.[15] Here we report measurements of renin, prorenin, immunoreactive renin, renin substrate and various plasma proteins in aqueous, vitreous and subretinal fluid. The ocular fluid samples were obtained at the time of cataract extraction or vitrectomy and the protein concentrations in these samples were compared

with those in simultaneously obtained plasma. Our study included eyes affected by proliferative diabetic retinopathy because the renin-angiotensin system has been implicated in neovascularization.[16]

Subjects and Methods

Non-diabetic Subjects

Aqueous fluid was collected at the time of cataract extraction from 21 subjects (15 women and 6 men; mean age, 68 yr; range, 26-86 yr). Four subjects were receiving a diuretic and six a β-adrenergic antagonist.

Vitreous fluid aspirates were obtained from 16 subjects (8 women and 8 men; mean age, 52 yr; range, 20-82 yr). The samples were collected at the time of pars plana vitrectomy, which was performed because of recurrent retinal detachment due to proliferative vitreoretinopathy. Four subjects were receiving a diuretic, in 3 of them combined with a b-adrenergic antagonist.

Subretinal fluid was obtained from 18 subjects (8 women and 10 men; mean age, 59 yr; range, 8-76 yr), with rhegmatogenous retinal detachment, which is a type of retinal separation precipitated by a hole or a tear in the retina. In this type of detachment fluid accumulates between the retinal pigment epithelial layer and the neural retina. The retinal detachments had occurred between 1 day and 3 months (median, one week) before subretinal fluid collection. Three subjects were receiving a diuretic, in 2 of them combined with a b-adrenergic antagonist.

Diabetic Subjects

Vitreous fluid was obtained from 15 diabetic subjects with proliferative diabetic retinopathy (8 women and 7 men; mean age, 51 yr; range, 28-71 yr). Vitrectomy was performed because of traction retinal detachment. The duration of diabetes ranged from 6-32 yr. Twelve subjects were receiving insulin, 5 were receiving a diuretic and 1 was receiving a b-adrenergic antagonist.

Aqueous fluid can only be collected at the time of cataract extraction. In diabetic subjects, however, this procedure may stimulate proliferative retinopathy. Cataract extraction is therefore not performed in eyes affected by proliferative diabetic retinopathy. Consequently, aqueous fluid could not be collected from such eyes. We also were unable to collect subretinal fluid from diabetic subjects with traction retinal detachment because drainage of subretinal fluid is rarely performed in these subjects and, if it is performed, the approach is via the transvitreal route, so that the sample is heavily contaminated with material from the vitreous. In subjects with a rhegmatogenous retinal detachment, subretinal fluid is removed via the transscleral route, where no such contamination occurs.

Collection of Ocular Fluid Samples

Approximately 0.1 mL aqueous fluid was collected with a tuberculin syringe and a 25-gauge needle. The needle was introduced at the limbus of the cornea through the groove of the cataract incision. A 0.3-1.0 mL sample of vitreous fluid was aspirated before

substitution fluid was infused into the vitreous. Subretinal fluid was aspirated transsclerally, after local diathermic coagulation of the choroid.

The ocular fluid samples were free of macroscopically visible blood and were frozen at -70 °C immediately after collection. A peripheral venous blood sample was drawn simultaneously with the collection of ocular fluid. Blood for determination of renin, prorenin, immunoreactive renin, renin substrate, albumin, transferrin and immunoglobulin G (IgG) was collected in tubes containing 0.1 volume of 0.13 mol/L trisodium citrate. The blood was immediately centrifuged at 3000 x g for 10 minutes at room temperature, and 1-mL aliquots of plasma were stored at -70 °C. Blood for determination of angiotensin II was collected in prechilled tubes containing 0.1 volume of 0.06 mmol/L pepstatin-A, 0.125 mol/L disodium EDTA and 0.025 mol/L phenantroline in order to block renin, angiotensin converting enzyme and angiotensinases, respectively. The blood samples were immediately centrifuged at 3000 g for 10 minutes at 4 °C, and 2-mL aliquots of plasma were stored at -70 °C.

Analytical Methods

Renin was measured in duplicate by enzyme-kinetic assay, in which the samples were incubated at 37 °C and pH 7.5 with saturating amounts of sheep renin substrate in the presence of inhibitors of angiotensinases and angiotensin converting enzyme. The generated angiotensin I was quantitated by RIA.[17] For measuring prorenin in plasma, prorenin was converted into renin by incubation with Sepharose-bound trypsin (0.25 mg/mL) for 48 hours at 4 °C. Previous studies, including measurements of total immunoreactive renin (renin plus prorenin), indicated that the prorenin to renin conversion in plasma is complete after incubation with the immobilized trypsin under these circumstances and that destruction of renin or prorenin does not occur.[18] Experiments in which known quantities of purified human kidney renin were added to ocular fluid demonstrated that in some samples destruction of renin did occur with this method. This destruction might be due to the low content of serine protease inhibitors in ocular fluids as compared to plasma. Therefore, in ocular fluid we chose to use plasmin to convert prorenin into renin.[17, 19] For this purpose the sample was incubated with plasmin at a final concentration of 0.5 μmol/L for 48 hours at 4 °C before the assay.

Comparison of the results of the enzyme-kinetic assay in plasmin-activated ocular fluid samples with the results of the assay of total immunoreactive renin in non-activated samples demonstrated that the conversion by plasmin was complete without any loss of prorenin or renin; the specific enzymatic activity of plasmin-activated prorenin in ocular fluid samples was not different from the specific activity of purified kidney renin and plasma renin (see *Results*). Plasmin at the concentration mentioned above cannot be used to activate prorenin in native whole plasma because of its high content of plasmin inhibitors.

The concentrations of renin and prorenin measured by the enzyme-kinetic assay were expressed as milliunits per L using the WHO human kidney renin standard 68/356 (WHO International Laboratory for Biological Standards, London, United Kingdom) as reference standard. The lower limit of detection was 0.5 mU/L and the interassay variability at

low concentrations of renin or prorenin (2-5 mU/L) was 11 % for both renin and prorenin. Immunoreactive renin was measured in duplicate with a sandwich assay[18, 20] using the monoclonal antibodies R 3-27-6 and R 3-36-16 (Ciba-Geigy, Basel, Switzerland). The two monoclonal antibodies recognize different epitopes of the renin molecule and react equally well with human kidney renin and chorionic cell culture prorenin. The assay was carried out in polystyrene tubes (Star Tubes, code 4-70319; Nunc, Roskilde, Denmark). The inner surface of these tubes was coated with antibody R 3-27-621 . Immunoreactive renin in the assay sample is quantitatively bound to this antibody. The amount of solid phase-bound immunoreactive renin was measured with antibody R 3-36-16, which had been radiolabeled with ^{125}I. The results of this assay were expressed as nanograms per L using highly purified human kidney renin (Ciba-Geigy) as a standard. One milliunit of the WHO human kidney renin standard corresponded to 1.41 ng of the Ciba-Geigy standard. The lower limit of detection was 5 ng/L, and the interassay variability was 8 %.

The concentration of renin substrate was determined as the maximum quantity of AngI that was generated during incubation at 37 °C and pH 7.5 with an excess of purified active human kidney renin in the presence of inhibitors of angiotensinases and angiotensin converting enzyme.[18] The lower limit of detection was 1 nmol/L and the interassay variability was 10 %.

Immunoreactive AngII was measured by radioimmunoassay after SepPak (Waters, Milford, MA, USA) extraction of the sample.[22] The lower limit of detection was 2 pmol/L and the interassay variability was 15%.

Albumin, transferrin and IgG were measured by single radial immunodiffusion (LC and NOR-Partigen plates, Behringwerke, Marburg, Germany) according to the method of Mancini et al.[23]

Data Analysis

Plasma proteins enter the vitreous mainly by diffusion. One of the reasons why the concentrations of these proteins are low in vitreous fluid is that they have to cross a relatively impermeable barrier. Breakdown of this so-called blood-retinal barrier leads to increased diffusion of plasma proteins into the eye. The rate of diffusion of a given protein is related to its molecular size and plasma concentration. In accordance with this is the fact that the concentrations of the different proteins relative to each other are similar in plasma and vitreous fluid.[24, 25]

Thus, unless certain specific uptake processes exist, for which in the eye no evidence is available with regard to any of the proteins mentioned in this paper, one would expect a relatively high intraocular albumin concentration (due to partial breakdown of the blood-retinal barrier) to be accompanied by a proportionally high concentration of plasma proteins of comparable size. Therefore, we chose to take the vitreous fluid/plasma concentration ratio of albumin as an index of the integrity of the blood-retinal barrier, an abnormally high ratio being an indication of breakdown of this barrier. By multiplying this ratio with the level of a given protein in plasma, the level of this protein in ocular fluid can be estimated, assuming that, as mentioned above, this

protein is transferred from the blood into the vitreous and *vice versa* by mechanisms that are qualitatively and quantitatively the same as those for the transfer of albumin. For example, for renin substrate the calculation would be as follows:

$$[RS_{oc}] = [RS_{pl}] \times [ALB_{oc}]/[ALB_{pl}],$$

in which RS is renin substrate, ALB is albumin, oc is ocular fluid, pl is plasma, and brackets denote the concentration.

If our assumptions are correct, the calculated concentrations should be equal or at least closely correlated to the actually measured concentrations. Therefore, the two sets of data were analyzed by linear regression.

For analyzing differences between diabetic and nondiabetic subjects unpaired t-tests were performed after logarithmic transformation of the data. Values were considered significant if $p < 0.05$.

Results

Non-diabetic Subjects

The levels of renin in many vitreous and aqueous fluid samples were at or below the detection limit of the assay (0.5 mU/L), which is less than 5 % of the level in plasma. In subretinal fluid the renin level was about 20 % of that in plasma (Table 1). Prorenin was detectable in all samples of vitreous and aqueous fluid; its level in vitreous fluid was about 20 % and in aqueous fluid about 5 % of that in plasma. In subretinal fluid the prorenin level was as high as in plasma. Renin and prorenin concentrations in the fluid compartments of the eye were in the order: subretinal fluid > vitreous fluid > aqueous fluid. The levels of renin substrate in subretinal, vitreous and aqueous fluid were 10, 5 and 0.5 % of those in plasma, respectively. Thus, they too were in the order: subretinal

Table 1. Levels of prorenin, renin, and renin substrate in ocular fluids.

		Prorenin (mU/L)		Renin (mU/L)		Renin substrate (nmol/L)	
	n	eye	plasma	eye	plasma	eye	plasma
Non-diabetic subjects							
Aqueous vs.plasma	21	4.4	163	<0.5	9.8	5.1	1080
		2.0-8.7	36.7-453	ND-0.5	1.5-62.9	1.6-15.4	898-1430
Vitreous vs. plasma	16	34.5	174	<1.0	17.3	54.5	1120
		17.4-61.9	67.0-396	ND-2.8	3.7-51.0	3.0-630	791-3030
Subretinal vs. plasma	18	132	128	2.4	14.3	107	1230
		36.8-305	65.1-251	0.9-6.4	4.3-105	10-1430	841-2250
Diabetic subjects							
Vitreous vs. plasma	15	61.0	357	<2.0	17.7	61.0	1030
		19.0-172	121-679	ND-3.5	4.4-127	7.0-1000	628-2040

Shown are the geometric mean and range. ND, Not detectable. In vitreous fluid and plasma the levels of prorenin, but not those of renin substrate, were higher in diabetic than in non-diabetic subjects ($p < 0.01$).

Table 2. Levels of albumin, IgG, and transferrin in ocular fluids.

		Albumin (g/L)		IgG (g/L)		Transferrin (g/L)	
	n	eye	plasma	eye	plasma	eye	plasma
Non-diabetic subjects							
Aqueous vs.plasma	21	0.19	33.1	ND	ND	ND	ND
		0.06-0.45	28.3-39.4				
Vitreous vs. plasma	16	1.55	33.0	0.19	11.0	0.18	2.44
		0.11-20.2	22.9-42.5	0.03-0.70	6.61-14.4	0.02-1.74	1.60-2.99
Subretinal vs. plasma	18	3.03	35.4	0.48	11.2	0.45	2.57
		0.39-28.5	29.2-41.9	0.07-5.10	7.0-18.2	0.06-3.97	2.08-3.44
Diabetic subjects							
Vitreous vs. plasma	15	1.51	29.2	0.19	9.2	0.14	2.06
		0.32-17.1	21.7-36.8	0.04-0.96	3.88-16.7	0.06-0.37	1.50-2.70

Shown are the geometric mean and range. The levels of albumin, IgG, and transferrin in both vitreous and plasma did not differ between diabetic and non-diabetic subjects. ND, Not done.

fluid > vitreous fluid > aqueous fluid. This was also true for the levels of albumin, transferrin and IgG (Table 2). There was no correlation between the levels in ocular fluid and those in plasma for any of the proteins.

As described under *Data analysis* above, a theoretical concentration in ocular fluid for each protein was predicted based on the albumin content of the sample. For renin substrate, transferrin and IgG the calculated and measured values were linearly correlated in both vitreous and subretinal fluid, and the slopes of these correlation lines were not significantly different from 1.0 (Tables 3 and 4). Renin substrate and transferrin concentrations in ocular fluid could, in fact, be accurately predicted by these calculations. The IgG level measured was systematically about 2 times lower than that calculated, which may be due, at least in part, to its larger molecular size as compared to that of albumin and the other proteins.

For prorenin the findings were different. The prorenin levels in both vitreous and subretinal fluid varied much less than the levels of the other proteins (Tables 1 and 2). Furthermore, calculating prorenin concentrations on the basis of the albumin content of the sample yielded much lower (down to 1/100th) values than those actually measured, particularly in samples with a low plasma protein content (intact blood-retinal barrier). The prorenin level in subretinal fluid was higher than that in vitreous fluid, even when corrections were made for the higher plasma protein content in subretinal fluid samples (Fig. 1). In both vitreous and subretinal fluid the slopes of the regression lines describing the correlation between the measured and calculated prorenin concentrations were significantly different from 1.0, thereby indicating the different behavior of prorenin as compared to albumin and other plasma proteins.

Table 3. Correlations between the measured and calculated concentrations of proteins in vitreous fluid.

Protein	Regression Line	r	p
Prorenin			
Non-diabetic subjects	$y=0.015^{a}x+1.523$	0.05	NS
Diabetic subjects	$y=0.328^{a}x+1.370$	0.64	<0.05
Renin substrate	y=1.024x-0.028	0.97	<0.0001
IgG	y=1.063x-0.344	0.91	<0.0001
Transferrin	y=0.899x+0.066	0.89	<0.0001

y is the log (measured concentration); x is the log(calculated concentration). For renin substrate, IgG, and transferrin, data from diabetic and non-diabetic subjects were combined because no differences were found for these proteins between the two groups (Tables 1 and 2).[a] Slope different from 1.0, p<0.0001

Table 4. Correlations between the measured and calculated concnetrations of proteins in subretinal fluid.

Protein	Regression Line	r	p
Prorenin	$y=0.027^{a}x+1.839$	0.61	<0.01
Renin substrate	y=0.975x+0.064	0.98	<0.0001
IgG	y=0.941x-0.302	0.99	<0.0001
Transferrin	y=0.934x+0.262	0.94	<0.0001

y is the log (measured concentration); x is the log(calculated concentration).[a] Slope different from 1.0, p<0.0001

The data on prorenin shown in Tables 1, 3 and 4 and Figs. 1-3 were obtained by the enzyme-kinetic assay. That prorenin measured by this assay is, in fact, prorenin is supported by the excellent agreement with the measurements of immunoreactive renin (Fig. 4). The mean specific enzymatic activity of in vitro activated prorenin was 0.7 ± 0.2 (± SD) mU/ng (n = 9) in vitreous fluid and 0.6 ± 0.2 mU/ng (n = 10) in subretinal fluid. These values are not different from the specific activity of renin from plasma and kidney.[18]

Immunoreactive AngII was 11.1 ± 1.8 pmol/L in vitreous fluid (n = 12) compared to 17.5 ± 1.3 pmol/L in plasma. In subretinal fluid (n = 15) it was 14.8 ± 1.6 pmol/L compared to 23.9 ± 2.0 pmol/L in plasma.

Diabetic Subjects

The results of the renin substrate, albumin, transferrin, and IgG measurements in vitreous fluid and plasma of the diabetic subjects were similar to those in the non-diabetic subjects

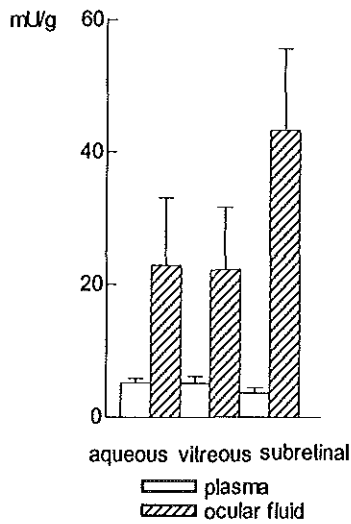


Figure 1. Mean (± SE) prorenin/albumin concentration ratio in ocular fluids and plasma of nondiabetic patients

(Tables 1 and 2). The levels of renin and prorenin in vitreous fluid were higher in the diabetic than in the non-diabetic subjects. Prorenin was also higher when allowance was made for differences in plasma protein content of the samples. In the diabetic subjects the prorenin concentration of vitreous fluid correlated with the plasma prorenin concentration ($r = 0.78$, $n = 15$, $p < 0.001$). In the non-diabetic subjects there was no significant correlation between the levels of prorenin in vitreous fluid and plasma.

As in the non-diabetic subjects, prorenin in vitreous samples with low plasma protein content was much higher (up to 25 times) than expected on the basis of the albumin content of the samples.

Immunoreactive AngII was 9.0 ± 2.5 pmol/L in vitreous fluid ($n = 15$) compared to 9.9 ± 2.9 pmol/L in plasma.

Discussion

The levels of albumin (mol wt, 69 K), transferrin (mol wt, 90 K), IgG (mol wt, 150 K) and renin substrate (mol wt, 65 K) in vitreous fluid differed widely from sample to sample, but in each individual sample the levels were, relative to each other, comparable to those in plasma. The IgG level in vitreous fluid, relative to that of albumin, was systematically somewhat lower than in plasma, probably due to its larger molecular size.[25] These results are in agreement with earlier findings that most soluble protein in the vitreous is derived from plasma.[24-26]

The plasma protein content of normal vitreous fluid has been estimated to be 0.5-2 % of that in plasma.[25,26] In our study vitreous fluid from eyes with recurrent retinal detachment due to proliferative vitreoretinopathy contained higher levels of plasma

proteins. These higher values probably reflect partial breakdown of the blood-retinal barrier in such eyes.[27]

The blood-retinal barrier is formed by the tight junctions between the endothelial cells of the retinal capillaries and the tight junctions between the retinal pigment epithelial cells, the latter restricting the transfer of plasma proteins escaping from the capillaries of the choroid.[28] Proteins from plasma may enter the vitreous as a result of focal cellular necrosis, opening of the intercellular junctions, or vesicular transport and formation of transcellular channels. The rate of diffusion of proteins through such discontinuities in

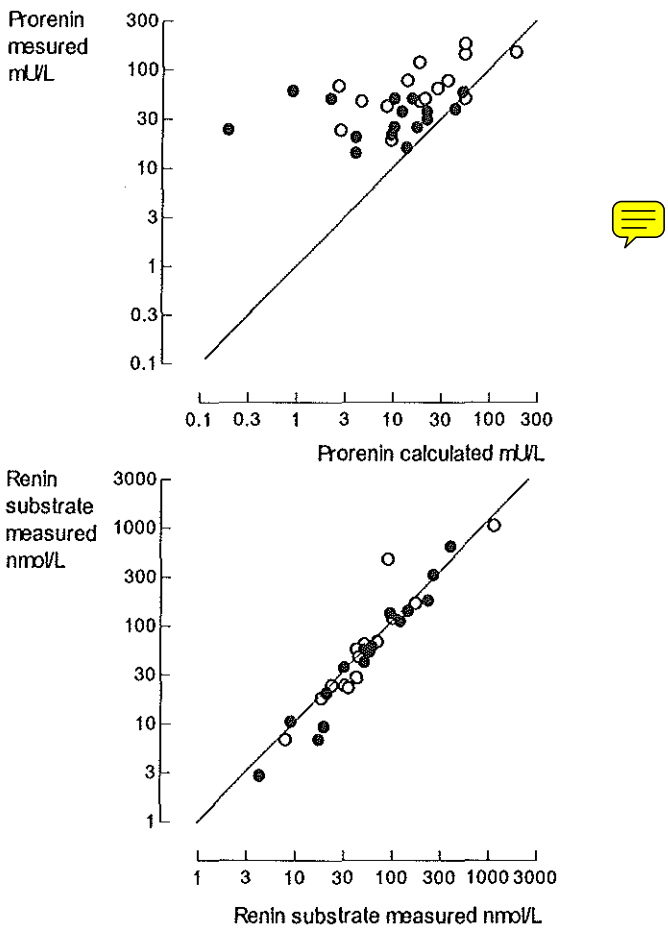


Figure 2. Measured versus calculated concentrations of prorenin (top panel) and renin substrate (bottom panel) in vitreous fluid. For an explanation of the calculation see text (*Data analysis*). The slopes and significance levels of the correlations are given in Table 3. *open symbols:* diabetic, *closed symbols:* non-diabetic.

the blood-retinal barrier depends upon the concentration gradient across this barrier, the molecular size of the proteins and the number and area of discontinuities. Our results are in accordance with the contention that diffusion through these pores is the main mechanism of transfer of plasma proteins to the vitreous.

This process, however, does not appear to hold true for prorenin (M_r 47,000). The concentration of prorenin, relative to that of albumin, was much higher in vitreous fluid than in plasma, and the prorenin level in vitreous fluid also was little influenced by its plasma protein content. Thus, prorenin may enter the vitreous by a mechanism that is different from that of albumin and other plasma proteins. This mechanism is selective

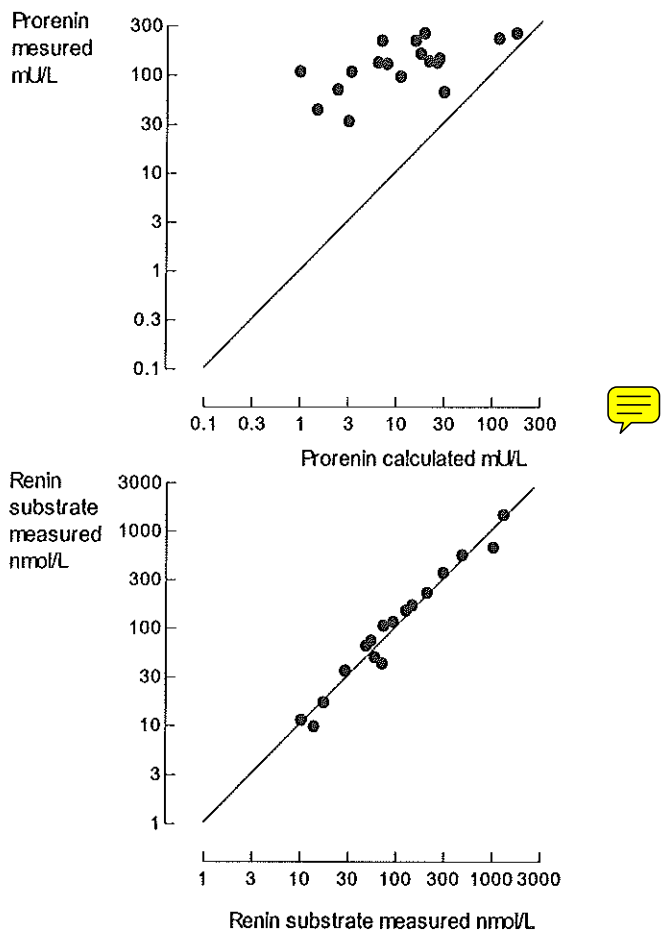


Figure 3. Measured versus calculated concentrations of prorenin (top panel) and renin substrate (bottom panel) in subretinal fluid. For an explanation of the calculation see text (*Data analysis*). The slopes and significance levels of the correlations are given in Table 4.

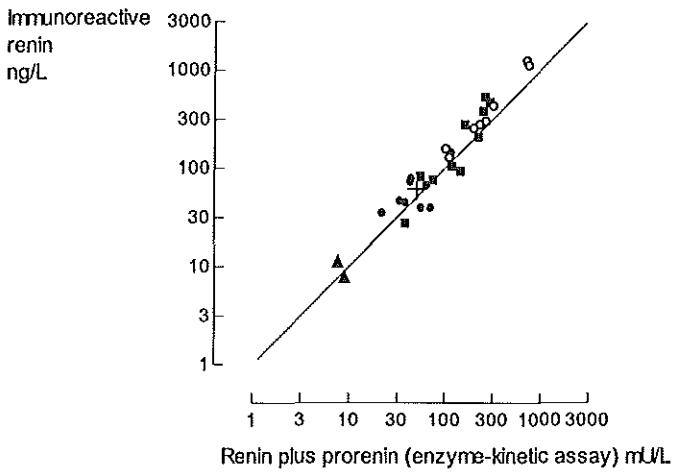


Figure 4. Total renin (prorenin plus renin) measured by enzyme-kinetic assay versus immunoreactive renin ($r=0.97$, $p < 0.0001$). o plasma, ■ subretinal fluid, ● vitreous fluid, ▲ aqueous fluid, ✚ WHO human kidney renin standard.

for prorenin and may involve an active process. Receptor-mediated transcellular transport is such a selective mechanism, but as yet there is no evidence for the existence of this mechanism, although prorenin-binding by cells has been described.[29,30] Our findings raise the possibility that not all the prorenin in the vitreous is derived from plasma but that some of it is produced in the eye.

As described under *Data analysis* above, the vitreous level of prorenin that has crossed the blood-retinal barrier by passive diffusion in the same way as albumin can be estimated by multiplying the vitreous/plasma concentration ratio of albumin by the plasma prorenin level. By subtracting this calculated level of plasma-derived prorenin from the level actually measured, we estimated the level of prorenin that entered the vitreous by some process that is different from diffusion out of the circulation. In most samples of vitreous fluid the estimated level of prorenin that had entered the vitreous by such a diffusion-independent process was more than 4 times higher than the estimated level of prorenin that had entered the vitreous by passive diffusion from blood. Thus, relative to the total amount of prorenin in vitreous fluid, the contribution of plasma-derived prorenin crossing the blood-retinal barrier merely by diffusion appears to be small. The implicit assumption underlying these calculations is that albumin and prorenin leave the vitreous in the same way, that is by free diffusion into the aqueous fluid,[31] where the concentrations of these proteins were much lower than in the vitreous.

The concentrations of albumin, transferrin, IgG and renin substrate were 2-3 times higher in subretinal fluid than in vitreous fluid. This was to be expected, since subretinal fluid from eyes with retinal detachment is more or less a concentrate of vitreous fluid.[27] Vitreous fluid enters the subretinal space through the hole(s) of the retina, and water is actively absorbed from the subretinal space by the retinal pigment epithelium. Again,

the findings for prorenin were different. The concentration of prorenin, relative to albumin, was much higher in subretinal fluid than in plasma, particularly in subretinal samples with low plasma protein concentrations. Moreover, relative to albumin and other plasma proteins, prorenin was 2 times higher in subretinal than in vitreous fluid. If it is assumed, on the basis of the evidence discussed above, that most of the prorenin in the vitreous is not derived from plasma but is produced in the eye, the difference in prorenin content between subretinal and vitreous fluid may suggest that the subretinal compartment is closer to the site of prorenin production.

Not only were the prorenin concentrations of vitreous and subretinal fluid higher than expected, so too were the renin (M_r 48000) concentrations. The data on renin, however, are more difficult to interpret than those on prorenin because in many samples renin was at or below the detection limit of the assay and because some prorenin to renin conversion may have occurred during storage and handling of the samples. Even as little as 1 % conversion will result in a large percentage increase of renin in these samples.

Immunoreactive AngII also was found in samples of vitreous fluid, in concentrations comparable to those in plasma. Further work is needed to answer the question of its origin.

That the levels of albumin and IgG in vitreous fluid from eyes affected by proliferative diabetic retinopathy were higher than the levels in normal eyes can be explained by the increased permeability of the blood-retinal barrier in this condition.[32] The higher vitreous level of prorenin, relative to those of albumin and other plasma proteins, in the diabetic subjects as compared to non-diabetic subjects is more difficult to explain. The same arguments in favor of the hypothesis that, generally, diffusion from the blood contributes little to the total amount of prorenin in the vitreous, apply to both diabetic and non-diabetic subjects. It seems, therefore, unlikely that the higher level of prorenin in vitreous fluid of the diabetic subjects (2 times that in non-diabetic subjects) was caused by the higher level in plasma (also 2 times that in non-diabetic subjects). It might be the other way around; increased release or leakage of prorenin from the eye affected by proliferative diabetic retinopathy may contribute to the increased prorenin level in plasma.

This possibility is further supported by the finding that, in contrast with other proteins, the plasma concentration of prorenin in diabetic subjects correlated significantly with the concomitant vitreous prorenin concentration. Considering the fact that in some diabetic subjects the blood-retinal barrier for plasma proteins was still relatively intact (low vitreous/plasma albumin concentration ratio), whereas in others it was extremely leaky, no such correlation was to be expected, if diffusion from plasma into the vitreous was the main mechanism of transfer of prorenin.

An elevated plasma prorenin level in diabetic subjects has been found to be associated with microvascular complications, including retinopathy.[33] Evidence is accumulating that neovascularization is initiated by diffusible chemical factors arising from ischemic areas of the retina.[34] Renin and angiotensin have been found in cultured neuronal and glial cells from rat brain;[35, 36] both cell types are abundantly present in the retina. AngII acts on vascular tone and has mitogenic and trophic actions on vascular smooth muscle and

other cells.[37] In fact, it has been reported to promote neovascularization.[16] An intraocular renin-angiotensin system may, therefore, play a role in proliferative diabetic retinopathy.

References

1 Sealey JE, Moon C, Laragh JH, Atlas SA. Plasma prorenin in normal, hypertensive, and anephric subjects and its effect on renin measurements. Circ Res 1977;40 (Suppl I):41-5.

2 Derkx FHM, Wenting GJ, Man in 't Veld AJ, Verhoeven RP, Schalekamp MADH. Control of enzymatically inactive renin in man under various pathological conditions: implications for the interpretation of renin measurements in peripheral and renal plasma. Clin Sci Mol Med 1978;54:529-38.

3 Naruse K, Murakoshi M, Osamura RY, Naruse M, Toma H, Watanabe K, Demura H, Inagami T, Shizume K. Immunohistochemical evidence for renin in human endocrine tissues. J Clin Endocrinol Metab 1985;61:172-7.

4 DeSchepper CF, Mellon SH, Cumin F, Baxter JD, Ganong WF. Analysis by immunohistochemistry and in situ hybridization of renin and its mRNA in kidney, testis, adrenal and pituitary of the rat. Proc Natl Acad Sci USA 1986;83:7552-6.

5 Ganten D, Printz M, Phillips MI, Scholkens BA. The renin-angiotensin system in the brain. Exp Brain Res (Suppl 4) 1982;48:3-293.

6 Glorioso N, Atlas SA, Laragh JH, Jewelewicz R, Sealey JE. Prorenin in high concentrations in human ovarian follicular fluid. Science 1986;233:1422-4.

7 Derkx FHM, Alberda AT, De Jong FH, Zeilmaker GH, Makovitz JW, Schalekamp MADH. Source of plasma prorenin early and late in pregnancy. Observations in a patient with primary ovarian failure. J Clin Endocrinol Metab 1987;65:349-54.

8 Do YS, Sherrod A, Lobo RA, Paulson RJ, Shinagawa T, Chen S, Kjos S, Hsueh WA. Human ovarian theca cells are a source of renin. Proc Natl Acad Sci USA 1988;85:1957-61.

9 Acker GM, Galen FX, Devaux C, Foote S, Papernik E, Pesty A, Menard J, Corvol P. Human chorionic cells in primary culture: a model for renin synthesis. J Clin Endocrinol Metab 1982;55:902-9.

10 Itskovitz J, Sealey JE, Glorioso N, Rosenwaks Z. Plasma prorenin response to human chorionic gonadotropin in ovarian-hyperstimulated women: correlation with the number of ovarian follicles and steroid hormone concentrations. Proc Natl Acad Sci USA 1987;84:7285-9.

11 Fernandez LA, Olsen TG, Barwick KW, Sanders M, Kaliszewski C, Inagami T. Renin in angiolymphoid hyperplasia with eosinophilia. Its possible effect on vascular proliferation. Arch Pathol Lab Med 1986;110:1131-5.

12 Rockwood EJ, Fantes F, Davis EB, Anderson DR. The response of retinal vasculature to angiotensin. Invest Ophthalmol Vis Sci 1987;28:676-82.

13 Ferrari-Dileo G, Davis EB, Anderson DR. Angiotensin binding sites in bovine and human retinal blood vessels. Invest Ophthalmol Vis Sci 1987;28:1747-51.

14 Igic RP, Robinson CJG, Erdos EG. Central actions of angiotensin and related hormones. Pergamon Press, Oxford 1977, pp. 23-7.

15 Weinreb RN, Sandman R, Ryder MI, Friberg TR. Angiotensin-converting enzyme activity in human aqueous humor. Arch Ophthalmol 1985;103:34-6.

16 Fernandez LA, Twickler J, Mead A. Neovascularization produced by angiotensin II. J Lab Clin Med 1985;105:141-5.

17 Derkx FHM, Tan-Tjiong HL, Wenting GJ, Boomsma F, Man in 't Veld AJ, Schalekamp MADH. Asynchronous changes in prorenin and renin secretion after captopril in patients with renal artery stenosis. Hypertension 1983;5:244-56.

18 Derkx FHM, Stuenkel C, Schalekamp MPA, Visser W, Huisveld IH, Schalekamp MADH. Immunoreactive renin, prorenin, and enzymatically active renin in plasma during pregnancy and in women taking oral contraceptives. J Clin Endocrinol Metab 1986;63:1008-15.

19 Derkx FHM, Schalekamp MPA, Schalekamp MADH. Prorenin-renin conversion. Isolation of an intermediary form of activated prorenin. J Biol Chem 1987;262:2472-7.

20 Hofbauer KG, Wood JM, Gulati N, Heusser C, Menard J. Increased plasma renin during renin inhibition. Studies with a novel immunoassay. Hypertension 1985;7 (Suppl I) 61-5.
21 Nielsen MD, Rasmussen PH, Giese J. A highly sensitive and reproducible immunoradiometric assay for total human renin using monoclonal antibodies, iodogen labelling and polystyrene star tubes. Clin Exp Hypertension Theory Pract 1987;A9:1391-1414.
Morton JJ, Webb DJ. Measurement of plasma angiotensin II. Clin Sci 1985;68: 483-484
23 Mancini G, Carbonara AO, Heremans JF. Immunochemical quantitation of antigens by single radial immunodiffusion. Immunochemistry 1965;2:235-54.
24 Wurster U, Rise K, Hoffman K. Enzyme activities and protein concentration in the intraocular fluids of ten mammals. Acta Ophthalmologica 1982;60:729-41.
25 Wurster U, Hoffman K. Glaskörper. In: Biochemie des Auges, edited by Hockwin O. Enke-Verlag, Stuttgart 1985, pp. 110-121.
26 Chen CH, Chen SC. Studies on soluble proteins of vitreous in experimental animals. Exp Eye Res 1981;32:381-8.
27 Pederson JE, Toris CB. Experimental retinal detachment IX. Aqueous, vitreous, and subretinal protein concentrations. Arch Ophthalmol 1985;103:835-6.
28 Cunha-Vaz J. The blood-ocular barriers. Surv Ophthalmol 1979;23:279-96.
29 Van Kesteren CM, Danser AHJ, Derkx FHM, Dekkers DHW, Lamers JMJ, Saxena PR, Schalekamp MADH. Mannose 6-phosphate receptor-mediated internalization and activation of prorenin by cultured neonatal rat cardiac myocytes and fibroblasts. Hypertension 1997;30:1389-96.
30 Sealey JE, Catanzaro DF, Lavin TN, Gahnem F, Pitarresi T, Hu LF, Laragh JH. Specific prorenin/renin binding (ProBP). Identification and characterization of a novel membrane site. Am J Hypertens 1996;9:491-502.
31 Maurice DM. Protein dynamics in the eye studied with labeled proteins. Am J Ophthalmol 1959;47:361-8.
32 Cunha-Vaz J, Faria de Abreu JR, Campos AJ, Figo GM. Early breakdown of the blood-retinal barrier in diabetes. Br J Ophthalmol 1975;59:649-56.
33 Luetscher JA, Kraemer FB, Wilson DM, Schwartz HC, Bryer-Ash M. Increased plasma inactive renin in diabetes mellitus. A marker of microvascular complications. N Engl J Med 1985;312:1412-7.
34 Sebag J, McMeel JW. Diabetic retinopathy. Pathogenesis and the role of retina-derived growth factor in angiogenesis. Surv Ophthalmol 1986;30:377-84.
35 Hermann K, Raizada MK, Sumners C, Phillips MI. Presence of renin in primary neuronal and glial cells from rat brain. Brain Research 1987;437:205-13.
36 Hermann K, Raizada MK, Sumners C, Phillips MI. Immunocytochemical and biochemical characterization of angiotensin I and II in cultured neuronal and glial cells from rat brain. Neuroendocrinology 1988;47:125-32.
37 Lever AF. Slow pressor mechanisms in hypertension: a role for hypertrophy of resistance vessels. J Hypertens 1986;4:515-24.

5.2 Identification and Quantification of Renin and Prorenin in the Bovine Eye

Summary

Angiotensin II, the most important biologically active product of the renin-angiotensin system, has been reported to play a role in neovascularization and prorenin has been found in the vitreous of human eyes, particularly in those affected by proliferative diabetic retinopathy, a disease characterized by neovascularization. Prorenin in these eyes was, relative to plasma albumin, higher than in eyes without neovascularization. These findings suggested that an intraocular renin-angiotensin system exists, which might be involved in the development of retinal neovascularization in diabetes mellitus. In this study angiotensin I generating activity was measured in bovine aqueous humor and vitreous and in extracts of bovine retina, pigment epithelium-choroid and anterior uveal tract, before and after subjecting these extracts to procedures known to convert prorenin to renin. The measurements were made by incubation at 37 °C with plasma from nephrectomized rats at pH's ranging form 5.0 to 8.5. 'True' renin in the ocular samples could be separated from non-renin acid protease by a-casein-Sepharose affinity column chromatography at pH 3.5; 'true' renin did not bind to the column, whereas acid protease did. 'True' renin was further identified by its relatively high pH optimum (6.5-7.0) for angiotensin I generation and by its complete inhibition with specific renin antiserum and its high affinity for specific renin inhibitors. More than 75 percent of angiotensin I generating activity of the ocular samples consisted of 'true' renin. Approximately 90 percent or more of 'total' renin (renin plus prorenin) in aqueous humor, vitreous and the ocular tissues could not be explained by trapped plasma. 'Total' renin in aqueous humor and renin in vitreous were near the detection limit of the assay of angiotensin I generating activity. In vitreous prorenin comprised 99 percent of 'total' renin, in retina 81 percent, and in pigment epithelium-choroid and anterior uveal tract less than 50 percent. Prorenin in ocular fluids showed a concentration gradient, posterior vitreous > anterior vitreous > aqueous humor, suggesting that the main source of extracellular prorenin was in the posterior eye. These data support the contention of local renin and/or prorenin synthesis in the eye and are in accordance with the observations in other tissues that extrarenal synthesis of renin is often associated with the release of mainly, or exclusively, prorenin into extracellular fluid.

Introduction

The aspartyl protease renin is the key enzyme in the formation of angiotensin II, an octapeptide with well known functions in blood pressure regulation and fluid and electrolyte homeostasis. Classically, renin is considered a blood-borne enzyme synthesized and secreted by the kidney together with its inactive precursor, prorenin. Plasma from nephrectomized subjects predictably contains little or no renin but it does contain prorenin, in some cases in concentrations as high as in normal individuals.[1] Apparently most, if not all, renin in plasma originates from the kidney, whereas a large proportion of plasma prorenin is produced at extrarenal sites.

Synthesis of renin or prorenin and other components of the renin-angiotensin system (RAS) is known to occur in many organs besides the kidney, for instance adrenal,[2,3] pituitary,[2,3] testis,[2,3] brain[4] and ovary.[5,6,7] In the luteal phase of the menstrual cycle and during pregnancy the ovary, probably the corpus luteum, secretes prorenin into the circulation.[5] Certain renal and extrarenal tumors secrete prorenin in large amounts,[8] and in some patients carrying these tumors plasma renin is elevated as well. Finally, cultured smooth muscle cells,[9] chorionic cells[10] and ovarian follicular theca cells[6] have been shown to secrete prorenin into the medium.

It has been postulated that a correlation exists between the degree of vascularization of tissues and their renin content[11] and there is evidence to suggest that angiotensin II has a role in angiogenesis.[12] Because neovascularization is a hallmark of progressed diabetic retinopathy, we wondered whether renin might also be synthesized in the eye. Previously we described the presence of prorenin in the human eye (see chapter 5.1). Levels of prorenin in vitreous and subretinal fluid obtained during eye surgery were, relative to albumin and other plasma proteins, up to 100 times higher than in plasma. These results lend support to the hypothesis that synthesis of prorenin may occur in the eye. Presumably synthesis takes place in one or more of the surrounding tissues of the vitreous since vitreous itself is virtually acellular. In the present study we attempted to identify and to measure renin and prorenin in extracts of different parts of freshly obtained bovine eyes and in simultaneously taken plasma.

Materials and Methods

Buffers and Reagents

Bovine serum albumin (BSA), 8-hydroxyquinoline (8-OHQ), bovine a-casein, bovine cathepsin D and bovine hemoglobin were obtained from Sigma (St. Louis, MO). Phenylmethylsulfonyl-fluoride (PMSF) and sodium azide (NaN_3) were purchased from Merck (Darmstadt,FRG). EDTA, glycin and polyvinylpyrrolidone (PVP) were purchased from Riedel de Haën (Seelze,FRG), trypsin from Serva (Heidelberg,FRG), N-ethylmaleimide (NEM) from Aldrich (Milwaukee,WIS), sodium tetrathionate (NTT) from Fluka (Buchs, Switzerland), plasmin from Kabi Vitrum (Stockholm,Sweden) and CNBr-activated Sepharose 4B from Pharmacia (Uppsala, Sweden). Aprotinin was purchased from Bayer (Leverkusen, FRG). Antiserum against BSA was obtained from Dakopatts (Glostrup, Denmark). The kit for total protein determination was obtained from Instruchemie (Hilversum, The Netherlands). Rabbit antiserum against bovine pituitary renin was a gift by dr. Murakami, University of Tsukuba, Japan (see ref. 13). The renin inhibitor remikiren was a gift from dr. van Brummelen (Hoffman-La Roche, Basle, Switzerland). The statin containing renin inhibitor CGP 29 287 was a gift from dr. Hofbauer (Ciba-Geigy, Basle, Switzerland). The World Health Organization (WHO) human kidney renin standard 68/356 was obtained from the WHO International Laboratory for Biological Standards (London, UK). For studying the optimal conditions for *in vitro* activation of prorenin the following buffers were used: buffer A of pH 3.3, consisting of 0.05 M glycin, 0.001 M EDTA and 0.095 M NaCl, and buffer B of pH 7.4, consisting of 0.1 M phosphate, 0.001 M EDTA and 0.075 M NaCl. Buffer C of pH 7.4

was used for washing tissues and for homogenization; it consisted of 0.01 M phosphate and 0.15 M NaCl.

Collection of Blood and Ocular Tissues

Blood was obtained from cows at the local slaughterhouse immediately after death. Blood was collected in tubes containing sodium citrate (13 mM, final concentration). At the same time both eyes were enucleated. Eyes and blood were kept on ice and were processed within 60 min. Blood was centrifuged at 4 °C for 10 min at 3000 g, and plasma was frozen at -20 °C. Eyes were dissected as described below. Dissection and extraction procedures were performed at 4 °C.

Aqueous humor was drawn using a tuberculin syringe with a fine needle. The eye was cut equatorially at the ora serrata and the anterior segment was lifted off. The vitreous body was isolated by gently shaking it out of the eye cup. Care was taken to remove all vitreous. The neural retina was cautiously teased away from the pigment epithelium with a thin glass rod and isolated by cutting it at the optic nerve. The choroid with adhering pigment epithelium layer was isolated by dissecting it from the sclera with a pair of fine scissors. The anterior uveal tract, consisting of iris and ciliary body, was isolated by removing the lens from the anterior eye cup, then gently pulling the anterior uveal tract loose from the sclera and blotting it on dry paper to remove any adhering vitreous. Cornea, lens and sclera were discarded.

For the chemical and immunological identification of renin and prorenin in the ocular tissue extracts and in plasma, either the retina, the pigment epithelium-choroid or the anterior uveal tract of 60 bovine eyes, the vitreous bodies of four eyes or 70 ml of bovine plasma were pooled. Tissues were minced with scissors into small pieces and washed three times in two volume weights of ice-cold buffer C, in order to remove as much plasma as possible. Tissues were homogenized in one volume weight of buffer C with a Polytron PT10/35 (Kinematica,Luzern,Switzerland) with 2x15 sec. bursts, level 10. The Polytron was also used to break the gel structure of the vitreous. Homogenates were rapidly frozen and thawed three times and then centrifuged at 4 °C for 1 h at 13000 g. Supernatants were decanted and stored at -20 °C. Pellets were discarded. Preliminary experiments had shown that there was no difference in renin content in extracts that had been made in buffer C alone and those made in buffer C containing the protease inhibitors PMSF (0.5 mM), EDTA (1 mM) and NTT (0.25 mM) (final concentrations).[14] Apparently inadvertent activation of prorenin during the extraction procedures did not take place in these extracts.

The extracts of retina, pigment epithelium-choroid and anterior uveal tract were divided into two portions. One portion of each extract was dialyzed against buffer A to pH 3.3 for 48 h and subsequently against buffer B to pH 7.4 for 48 h, both at 4 °C, after which denatured protein was removed by centrifugation. The resulting supernatants were dialyzed for 6 h in distilled water, lyophilized and stored at -20 °C. The acidification step removed effectively much of the angiotensinase activity, which in preliminary experiments was found to interfere with the renin assay in non-acidified lyophilized extracts despite the use of angiotensinase inhibitors. These lyophilisates were used for the experiments

with renin antiserum or the renin inhibitor remikiren or for α-casein-Sepharose affinity chromatography, as described below. The other portion of each homogenate was dialyzed against buffer B, pH 7.4, only. The dialyzed homogenates were centrifuged and used to establish the optimal method for activating prorenin.

For quantification of renin and prorenin in eyes and simultaneously taken plasma, eyes from eight cows were studied. Isolated parts of one eye were pooled with the corresponding parts of the other eye of the same animal, aqueous and vitreous in equal volumes. No washing procedure to remove plasma was included in order not to lose tissue renin. Tissues were weighed and one volume weight of buffer C was added. Homogenization was performed in the same way as described above.

To examine whether a concentration gradient exists in the vitreous for prorenin and total protein, the vitreous bodies of five eyes that had been snapfrozen in liquid nitrogen immediately after enucleation were divided into a posterior, central and anterior part, after the surrounding tissues had been peeled off. Aqueous of these eyes had been collected rapidly just before freezing.

Bovine kidney was homogenized in one volume weight of buffer C and submitted to pH 3.3 dialysis against buffer A for 48 h; pH was restored to pH 7.4 by dialysis against buffer B for 48 h. Denatured protein was removed by centrifugation. The resulting supernatant contained renin at a concentration of about 20 mg AngI/ml per h, as assessed by incubation at pH 7.4 with plasma from nephrectomized rats as a source of renin substrate. In most experiments this extract was diluted 300-fold in 0.1 M phosphate buffer, pH 7.4. No acid protease activity was detectable at this dilution.

Identification of Renin

Affinity Chromatography on α-casein-Sepharose. Aspartyl proteases bind to α-casein at low pH. Renin, however, is an exception because of its relatively high pH optimum. This property of renin can be used for separating renin from other aspartyl proteases by affinity chromatography on α-casein-Sepharose.[15] Alfa-casein was coupled to CNBr-activated Sepharose 4B,[15] and a column of 10x1cm was prepared. The column was equilibrated with 0.05 M HAc/Ac buffer, pH 3.5, containing 0.075 M NaCl. A lyophilized extract of pigment epithelium-choroid was dissolved in this buffer and 1 ml of this extract was applied to the column and left to stand for 5 h. Elution was started with adsorption buffer at a flow rate of approximately 10 ml/h. After a washout volume of 90 ml, a 0.1 M Tris/HAc buffer, pH 8.6, containing 1.0 M NaCl, was applied and elution was continued at a flow rate of 10 ml/h. Fractions of 3 ml were collected. All procedures were carried out at 4 °C. All fractions were assayed for angiotensin I generating activity at pH 7.4 and for acid protease activity at pH 3.5.

pH Optimum Study. Plasma from nephrectomized rats, which served as a source of renin substrate, and the samples to be tested for angiotensin I generating activity were brought to appropriate pH by overnight dialysis in separate dialysis bags at 4 °C in 0.1 M phosphate buffers ranging from pH 5.0 to pH 8.5. Angiotensin I generating activity was assessed at these pH's, incubation conditions being the same as in the renin assay

described below except for pH. The pH of the incubation mixture did not change more than 0.05 pH unit during incubation. The following samples were tested: 1) pool of bovine plasma, which was treated with trypsin-Sepharose for activating prorenin *(vide infra)*, 2) dilute bovine kidney extract, 3) bovine vitreous fluid, which was treated with acid and plasmin for activating prorenin *(vide infra)*, 4) renin from bovine pigment epithelium-choroid purified by α-casein-Sepharose chromatography *(vide infra)*, and 5) purified bovine cathepsin D.

Inhibition of Renin by Renin Antiserum or Renin Inhibitors. The rabbit antiserum against bovine pituitary renin at 1:320 dilution caused 50 % inhibition of the angiotensin I generating activity at pH 7.4 of a bovine kidney extract with a renin concentration of 70 ng AngI·ml^{-1}·h^{-1}.

A 1:40 dilution of antiserum was made in 0.1 M Tris/HCL, pH 7.4, containing 0.1% BSA and 0.02% NaN_3. Equal volumes of test sample and dilute antiserum were mixed and left to stand for 20 h at 4 °C. The following samples were tested: 1) bovine plasma, after treatment with trypsin-sepharose for activating prorenin *(vide infra)*, 2) vitreous fluid, which was treated with acid and plasmin for activating prorenin *(vide infra)*, 3) lyophilized extracts of retina, pigment epithelium-choroid and anterior uveal tract diluted in distilled water, 4) bovine kidney extractand 5) renin from pigment epithelium-choroid, purified by α-casein-Sepharose affinity chromatography *(vide infra)*.

To assess the specificity of the antiserum, inhibition experiments were also conducted with bovine cathepsin D. Renin assays that followed preincubation with the antiserum were performed not only at pH 7.4, but also at pH 5.5 because cathepsin D has little angiotensin I generating activity at neutral pH, whereas renin does have angiotensin I generating activity at neutral pH as well as at pH 5.5. Controls were samples incubated with preimmune rabbit serum. Results were corrected for the presence of renin in the antiserum and preimmune serum (approximately 3 ng Ang I.ml^{-1}.h^{-1}).

Inhibition of angiotensin I generating activity by two renin inhibitors was assessed by performing the renin assay in the presence of different inhibitor concentrations, ranging from 10^{-11} to 10^{-5} mol per liter (final concentration). One inhibitor was remikiren from Hoffman-La Roche, a substrate analogue, which is claimed to be enzyme-specific for renin. The concentration at which it causes 50 % inhibition (IC_{50}) of human renin is 0.5 $x10^{-9}$ M in the plasma renin activity assay, in which plasma renin is allowed to react with endogenous renin substrate at pH 7.4. The IC_{50} of this inhibitor for bovine cathepsin D is $3.7x10^{-5}$ M, when assessed by proteolysis of denatured hemoglobin at pH 3.5 (information provided by the manufacturer). The other renin inhibitor was CGP 29 287 from Ciba-Geigy, also a substrate analogue, which has been described in reference.[16] CGP 29 287 is a potent inhibitor of human renin but appears to be less effective against dog or rat plasma renin, with an IC_{50} of $1x10^{-9}$, $2x10^{-7}$ and $3x10^{-5}$ M respectively, in the plasma renin activity renin assay.[16] Its IC_{50} for bovine cathepsin D is $4x10^{-5}$ M, again assessed by proteolysis of denatured hemoglobin at pH 3.5. The samples tested with the renin inhibitors were the same as those used for the antiserum inhibition experiments,

except for renin purified from ocular tissues by α-casein-Sepharose chromatography, which was only used in the antiserum experiments.

In order to assess the specificity of the inhibitors for bovine renin, the effect of the inhibitors on angiotensin I generating activity of bovine kidney renin was compared with their effect on the angiotensin I generating activity of bovine cathepsin D. As in the antiserum inhibition experiments, this was done at pH 5.5. The effect of the inhibitors on the angiotensin I generating activity of human kidney renin in our renin assay was also tested and this was done both at pH 5.5 and 7.4.

Assays of Renin and Activated Prorenin. Different procedures were attempted for activating prorenin in bovine plasma, vitreous and extracts of retina, pigment epithelium-choroid and anterior uveal tract. We used three methods that were proven to be successful in activating human prorenin. One can either make use of endogenous proteolytic activators of prorenin, such as factor XII/kallikrein, plasmin, or pepsin-like proteases, or one can add activators, for instance plasmin or trypsin. In two of our methods we used an acidification step. Acidification has several effects: 1) prorenin undergoes a reversible conformational change of the molecule by which it becomes more susceptible to limited proteolysis necessary for irreversible activation,[17] 2) acidification destroys inhibitors of proteases that activate prorenin at neutral pH, like kallikrein or plasmin,[17] and 3) pepsin-like acid proteases, capable of activating prorenin, may become active at low pH, like for example in human amniotic fluid.[17] The first method of activation we tested in this study consisted of acidification followed by neutralization; no exogenous activator was added. Two ml of sample were dialyzed in buffer A for 48 h at 4 °C. Then the dialysis buffer was changed to buffer B, which after 36 h was replaced by buffer B containing 6 % PVP to prevent dilution due to colloid-osmosis. Dialysis in the presence of PVP proceeded for another 12 h after which the content of dialysis bags was collected by rinsing the interior with buffer B. Volumes were adjusted to 2 ml with the same buffer. The second method involved incubation at 4 °C with immobilized trypsin as previously described.[18] Trypsin had been coupled to CNBr-activated Sepharose 4B. The final concentration of trypsin was 4.4 x10^3 Na-Benzoyl-L-Arginine Ethyl Ester (BAEE) units per 500 μl sample. Activation of prorenin in plasma with this method does not require prior acidification. After 48 h incubation trypsin was removed by centrifugation. This method is similar to the often used trypsin-soy bean trypsin inhibitor (SBTI) combination,[14] with the advantage that destructive proteolysis of prorenin and renin is less likely to occur. The third method was an acidification step followed by incubation with plasmin at pH 7.4. After plasmin inhibitors have been removed at low pH, exogenous plasmin will cause activation of prorenin at neutral pH without destruction of prorenin or renin, a problem which is sometimes encountered with trypsin and acid proteases. After dialysis for 48 h at 4 °C in buffer A, samples were rapidly adjusted to pH 7.4 with 1 M NaOH. Then 0.1 volume of plasmin solution in 0.15 M NaCl was added to a final concentration of 1 Casein unit per ml. Incubation with plasmin was for 48 h at 4 °C. With each sample all three procedures were performed in duplicate.

Renin and activated prorenin were assayed in duplicate by measuring the rate of AngI generation at pH 7.4 and 37 °C in the presence of the protease inhibitors PMSF (2.4 mM), EDTA (5 mM), 8-OHQ (3.4 mM), aprotinin (2.2 nM) and NEM (5 mM) (final concentrations).[14,18] The incubation mixture consisted of one volume of 0.1 M phosphate buffer, one volume sample, two volumes of renin substrate and 0.14 volume of inhibitor solution. Plasma from nephrectomized rats was the source of renin substrate. The renin activity of this plasma was less than 0.1 ng AngI.ml^{-1}.h^{-1}. The final substrate concentration in the incubate was 2.9 ng AngI equivalents per ml, which was about four times K_m. The mixtures were incubated for 1,2,3 and in some cases 6 h at 37 °C. Angiotensin I was measured by radioimmunoassay.[18] Only incubations in which angiotensin I generation was linear with time were used for renin assay. Recovery of angiotensin I added to the incubation mixture was 98 % after 6 h incubation, demonstrating effective inhibition of angiotensinase activity.

Assays of Serum Albumin and Acid Protease. Albumin, as a measure of trapped plasma in the ocular tissue extracts, was determined with a single radial immunodiffusion technique, as described by Mancini et al.[19] One ml of the BSA antiserum precipitated 900 mg of BSA. Dilutions of samples were made in 0.07 M barbital, 0.003 M calcium lactate and 0.3% Tween 20, pH 8.6. Standard BSA solutions ranged from 5 to 100 mg/L. Diffusion into the agar gel plates was allowed to proceed for two days in humidified air at room temperature. The agar plates were then stained with coommassie blue. Recovery of added albumin ranged from 90-100% in the ocular tissue extracts.

The acid protease assay was a modification of the method by Anson.[20] Briefly, 200 µl sample, which had been brought to pH 3.5 with 1 M HCl, was added to 1 ml hemoglobin (1 %) in 0.1 M glycine/HAc, pH 3.5, at 37 °C. At t = 0 and t = 20 min 500 µl of this mixture was mixed with 500 µl ice-cold 10% trichloroacetic acid (TCA). Denatured protein was removed by centrifugation and the absorption at 280 nm of the supernatant was read in an Uvikon 810 spectrophotometer (Kontron,Zurich,Switzerland). The difference in absorption at 280 nm between 20 and 0 min at 37 °C was taken as a measure of acid protease activity. Acid protease activity is expressed in units; one unit is the proteolytic activity that causes a change in extinction of 1.000 per min at 280 nm by TCA-soluble products at pH 3.5 and a temperature of 37 °C, with denatured hemoglobin as a substrate.

Results

Affinity Chromatography

The protein concentration (absorption at 280 nm), the acid protease activity at pH 3.5 and the angiotensin I generating activity at pH 7.4 of the eluted fractions from the α-casein-Sepharose column are depicted in **Fig. 1**. Renin-like activity was eluted later than the bulk of protein. Most angiotensin I generating activity was found in a broad peak eluted at pH 3.5 (peak I). A small amount of angiotensin I generating activity (peak II) was eluted after changing the pH to 8.6. Peak I contained renin activity but no acid protease activity. Peak II contained acid protease activity. Fractions 13-24 (peak I) were

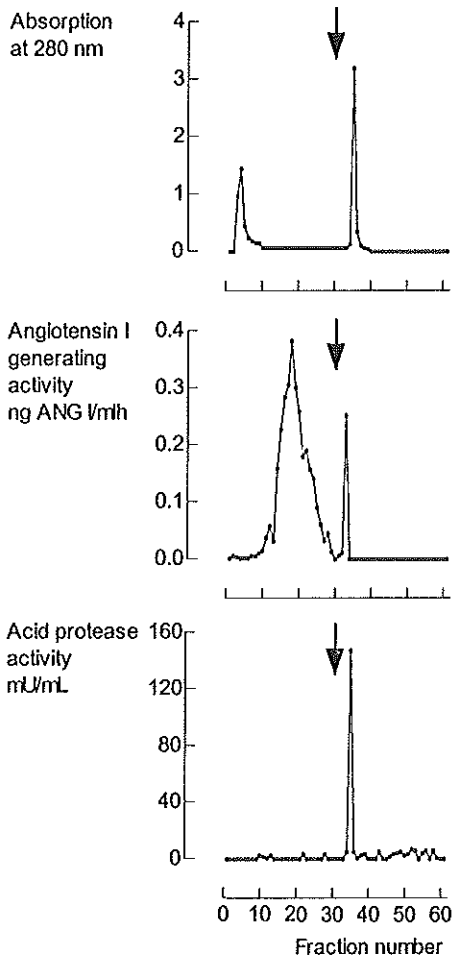


Figure 1. Separation of renin from non-renin acid protease in extract of bovine pigment epithelium-chroid by affinity chromatography on α-casein-Sepharose. Arrow indicates change of 0.05 M HAc-Ac⁻, containing 0.05M NaCl, pH 3.5, to 0.1 M Tris-HAc buffer, containing 1M NaCl, pH 8.6

dialyzed against distilled water for 6 h and lyophilized. This preparation was used for determining the pH optimum of angiotensin I generation and for inhibition with renin antiserum. Peak II was not further evaluated because of its small volume and low renin-like activity.

Identification of Renin

For preparing the extracts that were used for identification studies the tissues were washed in order to remove trapped plasma. Measurement of albumin in these extracts showed that washing with buffer C had removed 70-85 % of plasma. More than 90 % of the angiotensin I generating activity of these extracts could not be accounted for by contamination with plasma or by simple diffusion of renin from plasma.

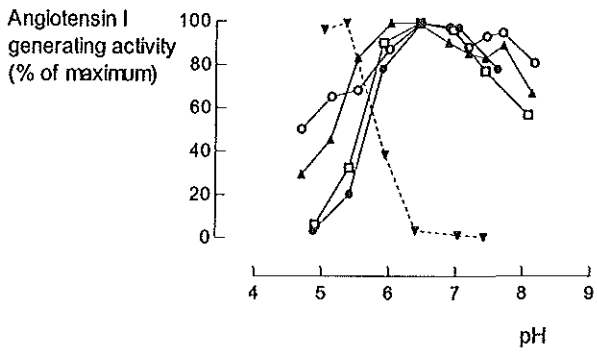


Figure 2. pH optimum curves for angiotensin I-generating activity of purified renin from bovine pigment-epithelium-choroid (□), activated prorenin from bovine vitreous (▲), activated prorenin from bovine plasma(○), bovine kidney extract (●) and bovine cathepsin D (▼)

The pH optimum curves of angiotensin I generating activity for kidney extract, activated prorenin of vitreous and plasma, and purified pigment epithelium-choroid renin (peak I of α-casein-Sepharose column) are shown in Fig. 2. During incubation pH did not shift more than 0.05 pH-unit. Results are expressed as a percentage of maximal

Table 1. Angiotensin I-generating activity of various bovine specimens and its inhibition by renin antiserum.

	Ang I generation (ng AngI/mlh)		% inhibition	
Sample	pH 7.4	pH 5.5	pH 7.4	pH 5.5
Plasma	5.8		71	
Vitreous	6.4		70	
Retina	23.4		83	
Pigment-epithelium-choroid	40.4		83	
Anterior uveal tract	76.7		93	
Kidney	70.1	20.1	97	94
Purified ocular tissue renin	10.6	2.9	83	95
Cathepsin D	ND	3.8		3

Plasma was treated with trypsin-Sepharose to activate prorenin. Vitreous was treated with plasmin after an acidification step in order to activate prorenin. Extracts of pools of bovine retina, pigment-epithelium-choroid and anterior uveal tract were dialyzed to pH 3.3 for 48 h and then to pH 7.4 for 48 h, before being lyophilized. Bovine kidney extract was treated in a similar manner as the eye tissue extracts, but was not lyophilized. Purified ocular tissue renin was from pigment-epithelium-choroid (peak I of α-casein column). For details on preparation of samples, see text. Renin assays were performed at pH 7.4 or pH 5.5, with and without preincubation with antiserum at pH 7.4. Experiments were carried out in duplicate. ND, not detectable

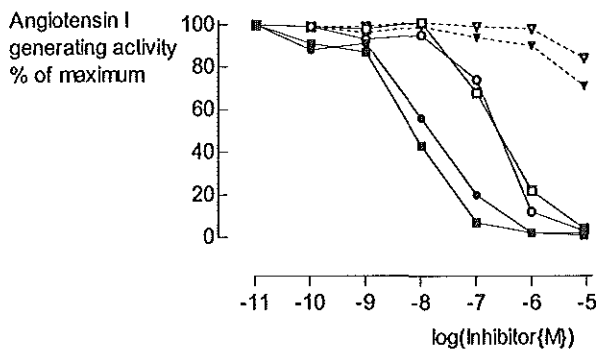


Figure 3. Inhibition of angiotensin I-generating activity of extract of bovine anterior uveal tract (■ and □), bovine kidney extract (● and ○) and bovine cathepsin D(▼ and ▽) by the renin inhibitors remikrien (closed symbols) and CGP 29,287 (open symbols).

angiotensin I generating activity. Curves were similar, with a pH optimum of 6.5-7.0. Activated prorenin in plasma and in vitreous appears to have a shoulder in the curve at pH 8.0. pH optimum curves were clearly different from the curve of cathepsin D angiotensin I generating activity. Angiotensin I generating activity in kidney, retina, pigment epithelium-choroid and anterior uveal tract extracts and in vitreous as well as purified pigment epithelium-choroid renin was inhibited for. 97, 83, 93, 97, 70 and 82 % respectively by the antiserum against bovine pituitary renin (Table 1). No inhibition was observed when samples were incubated with preimmune rabbit serum. Cathepsin D was not inhibited by the antiserum at pH 5.5, whereas purified pigment epithelium-choroid renin was. This demonstrates that the inhibitory activity of the antiserum was retained at pH 5.5 and that the antiserum was specific for bovine renin.

For the sake of clarity only the inhibition curves for kidney renin, anterior uveal tract extract and cathepsin D for each of the two renin inhibitors at pH 5.5 are depicted in Fig. 3. Samples of the other ocular tissues and vitreous showed identical curves. The

Table 2. Renin in bovine plasma and various ocular tissue extracts after different procedures for activating prorenin.

Sample	Renin activity (ng Ang I/mLh)			
	No activation	pH 3.3→7.4	Trypsin-Sepharose	PH3.3→7.4 + plasmin
plasma	1.6	3.0	5.9	4.1
Vitreous	0.04	4.7	4.8	6.1
Retina	0.3	1.6	1.2	2.1
Pigment-epithelium-choroid	3.0	3.1	3.2	4.4
Anterior uveal tract	1.5	2.9	2.2	3.0

For details on the activation procedures of prorenin, see text. Experiments were carried out in duplicate.

IC_{50} of remikiren for bovine renin was approximately 8.3×10^{-9} M, whereas for bovine cathepsin D the IC_{50} appeared to be well over 10^{-5} M. There was no difference in inhibitory activity of remikiren on human kidney renin at pH 5.5 nor at pH 7.4, proving that in this range pH did not influence the inhibitory activity of remikiren. CGP 29 287 was clearly less potent than remikiren, with an IC_{50} of 2.6×10^{-7} M, which is still much lower than the IC_{50} for bovine cathepsin D (4×10^{-5} M). Apparently, CGP 29 287 is more species-specific than remikiren.

Activation of Prorenin

Acidification followed by rapid neutralization and subsequent plasmin treatment resulted in the highest renin activity in the ocular tissues and in vitreous (Table 2). Not so, however, in plasma, in which treatment with trypsin-Sepharose was the more effective to activate prorenin. Activation with trypsin-Sepharose at pH 7.4 (without prior acid treatment) and activation with plasmin at pH 7.4 (after acid-treatment) reached a plateau after 48 h at 4 °C in all samples tested (results not shown). Hence for quantification of 'total' renin, *i.e.* renin plus prorenin, in the various compartments of the eye, we chose the acidification-plasmin method for the eye tissue extracts and for plasma we used the trypsin-Sepharose method. From these maximal activation values a percentage of prorenin can be calculated. In vitreous prorenin comprised 99 % of 'total' renin, in retina 81 %, whereas in pigment epithelium-choroid and anterior uveal tract less than 50 % of 'total' renin was prorenin.

Renin and Prorenin Levels in Different Parts of the Eye

Table 3 shows the 'total' renin (renin plus prorenin) levels of plasma, aqueous humor, vitreous, retina, pigment epithelium-choroid and anterior uveal tract of eight cows. In none of the eye samples tested, renin could be accounted for by trapped plasma or by

Table 3. Total renin (renin plus prorenin) and serum albumin in bovine plasma and different parts of the bovine eye.

Sample	Total renin	Total renin per albumin	Total renin, attributable to plasma
	ng Ang I/g tissue·h	ng Ang I/mg albumin·h	%
Plasma	6.3±0.5	0.21±0.02	100
Aqueous	0.3±0.04	2.36±0.39	11±2
Vitreous	5.7±0.4	26.8±3.6	1±0.2
Retina	5.1±0.7	16.3±2.0	1±0.2
Pigment epithelium-choroid	14.5±0.8	2.26±0.19	10±1
Anterior uveal tract	10.1±0.7	6.18±0.50	4±0.3

The percentage of total renin attributable to contamination with or diffusionfrom plasma was calculated as follows: $(alb_{tissue}/alb_{plasma}) \times (renin_{plasma}/renin_{tissue}) \times 100\%$. Results were obtained from 8 cows and are means ± SEM.

simple diffusion from plasma, since the renin/albumin ratio was much higher in the eye compartments than in plasma. In pigment epithelium-choroid 10 % of renin could be explained by contamination with plasma. In retina and anterior uveal this was only 1 and 4 % respectively. 'Total' renin levels, expressed as enzymatic activity per ml of fluid, were approximately equal in vitreous and plasma. In pigment epithelium-choroid and anterior uveal tract the 'total' renin concentration per gram tissue even surpassed that of plasma.

From **Fig.4** it can be seen that a concentration gradient exists for prorenin in the vitreous, with levels being highest in the posterior vitreous. There was a large difference in prorenin between anterior vitreous and aqueous, which was not apparent for total protein.

Discussion

A critical problem in studying renin in tissue extracts is the distinction between renin and other aspartyl proteases that are present in the tissues. These pepsin-like acid proteases act optimally at a pH lower than the pH optimum of 'true' renin. Nevertheless they can influence the renin assay in which renin-like activity is detected by angiotensin I generation from renin substrate. The presence of acid proteases other than renin can cause an overestimation of renin content, even when the reaction is performed at pH 7.4.[21] That the renin-like activity we detected at pH 7.4 in ocular tissues was due to true renin is demonstrated by the following findings. We separated ocular tissue renin from acid

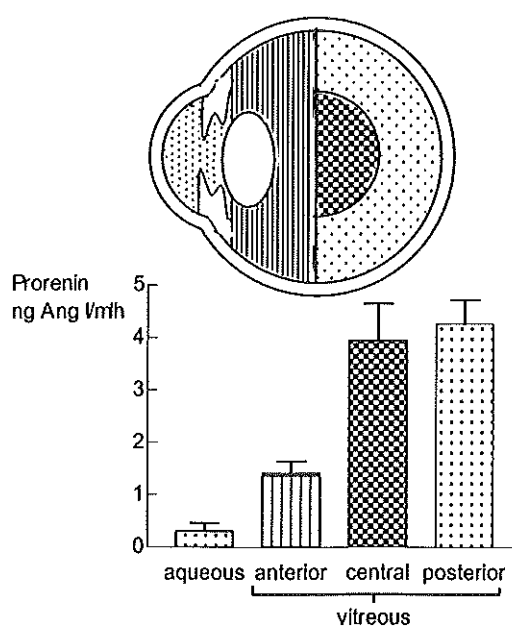


Figure 4. Prorenin in aqueous and in anterior, central and posterior vitreous of the bovine eye

protease by a-casein-Sepharose affinity chromatography. The peak that contained renin activity but not acid protease activity, showed a pH optimum of 6.5-7, which was similar to that of activated prorenin from vitreous and also similar to the pH optimum of kidney or plasma renin and very different from the pH optimum of purified cathepsin D, which is the most important of the angiotensin I generating acid aspartyl proteases in tissues. This almost neutral pH optimum already indicates that we are dealing here with 'true' renin, since renin is the sole AngI generating aspartyl protease known to have a near neutral pH optimum. Definitive proof that the renin-like activity in the eye extracts is true renin, was obtained by inhibition of enzymatic activity with a specific antiserum and with two experimental renin inhibitors, remikiren and CGP 29287, whereas purified bovine cathepsin D was inhibited by neither antiserum nor the renin inhibitors.

Another problem in the present study is that the eye is a highly vascularized organ so that ocular tissue extracts are heavily contaminated with plasma. Renin-like activity in tissue extracts may therefore be due to kidney-derived renin present in plasma. Obviously we could not perform nephrectomy to eliminate this source of renin, so we had to correct for plasma renin in the tissues. We did not wash the tissues for quantification studies in order not to lose any tissue renin. Determination of albumin content of the ocular speciments and of simultaneously taken plasma showed that plasma renin was of no or minor importance, since the renin/albumin ratio in eye structures was much higher than in plasma. In the experiments aimed at the identification of renin in the ocular tissues we washed the tissues first gently in buffer and in this way we were able to remove some 80% of the plasma, as estimated by measurement of the albumin concentration. Consequently some 80% of plasma renin was removed. The remainder of plasma renin in the resulting extracts was negligible relative to tissue renin, i.e. extravascular renin, since in the extracts that had not been washed, tissue renin already exceeded plasma renin by far.

Little is known about activation of bovine prorenin. Activation is possible with trypsin[22] but in tissues this has never been proven to be the optimal method. Moreover we were dealing with two kinds of specimens, namely plasma and tissue extracts, which were very different in composition and may require different procedures for activating prorenin. We examined different methods in order to establish optimal activation conditions. Together they cover the activation mechanisms that are known for human prorenin. From our results it appears that in bovine materials endogenous activators play a less important role in the in-vitro activation of prorenin than in human plasma. In bovine plasma it was not possible to activate prorenin maximally by acidification followed by a neutral phase. In human plasma this occurs via the factor XII/kallikrein pathway.[23] In tissue extracts acidification is supposed to activate acid proteases that in turn will activate prorenin.[17] However in the bovine ocular tissue extracts acidification alone did not yield optimal results. These were obtained when plasmin was added during the neutral phase after the acidification step. Plasmin is known to be an effective activator also of human prorenin. Acid-pretreatment was a prerequisite for the activation of prorenin by plasmin; plasmin alone did not give activation in the ocular tissue extracts. In vitreous,

however, acidification was not necessary (results not shown), probably because of the low content of plasmin inhibitors.

Some remarks can be made on the observed renin/prorenin ratio's. Little activation of prorenin seems to occur during the preparation of the ocular tissue extracts; the addition of inhibitors acting on different groups of proteases during preparation of these extracts had no effect on the renin/prorenin ratio. Our finding of bovine vitreous containing almost exclusively prorenin is consistent with our measurements in human vitreous, in that also human vitreous contains much more prorenin than renin. Furthermore the renin/prorenin ratio in the various parts of the eye differs from that in plasma. This difference suggests that a separate regulation mechanism exists for renin in plasma and in the eye and, possibly, that renin in the eye is produced locally. A striking analogy exists with the situation in the bovine ovary.[22] In follicular fluid the prorenin/renin ratio is greater than 30, like in vitreous, whereas in the surrounding theca cells this ratio is approximately one, as compared to 0.7-4.0 in retina and uveal tract.

From our results it cannot be said whether renin in the eye is located intra- or extracellularly. Nor is it possible to conclude whether renin is synthesized locally in the eye or sequestered from plasma. Binding of prorenin to cells has been reported recently[24] and this would be compatible with sequestration from plasma. However, binding was followed by internalization and activation of prorenin. Although this would explain the presence of prorenin and renin in the tissues, it would not account for the presence of prorenin in the vitreous. The distribution of renin and prorenin at various sites in the eye (Table 1) suggests local synthesis. Vitreous, being the large extracellular space in the eye, has a high percentage of prorenin, which is in agreement with findings of others that extrarenal renin synthesis is accompanied by secretion of exclusively prorenin into the extracellular fluid.[7-9, 15] Evidence for expression of the renin gene in human choroid and retina,[25] and in rat anterior uveal tract,[26] has been obtained indeed. The higher concentration of prorenin in the posterior segment of the vitreous (Fig.4) suggests that the origin of vitreous prorenin is located either in the posterior retina or in the posterior uveal tract, analogous to the total protein in the vitreous.[27] One problem with the uveal tract as a source of vitreous prorenin is that, in order to explain the high levels in vitreous, prorenin has to be transferred across the retinal pigment epithelium which, by virtue of its tight junctions, is part of the blood-retinal barrier. This barrier is little permeable to proteins. Still, the high renin concentrations in the anterior and posterior uvea make this tissue a candidate for production of renin. Production in the retina is also conceivable. In the brain the same cell types that are present in the retina, both neuronal and glial cells, have been shown to synthesize renin.[28] Very recently a renin variant has been described in neuronal cells that is the result of translation from a renin mRNA variant.[29] This variant (designated as renin b) results from different transcription initiation with alternative splicing and lacks the pre- and part of the propeptide of prorenin. Apparently it is not secreted and hence, again, the presence of prorenin in the vitreous is not explained by this renin variant. Immunohistochemical studies have suggested that the retinal Muller cells are the source of renin.[30] Renin was predominant in the anterior part of these macroglial cells that is in intimate contact with the retinal capillaries. Prorenin was located

at the posterior pole of the cells. Another group detected prorenin in human ciliary body by immuno-staining,[31] rather than in the retina. This raises the possibility that prorenin produced in the ciliary body is secreted into the aqueous. However, we found a large difference between prorenin in anterior vitreous and aqueous. From there it may diffuse into the vitreous. However, we found prorenin in aqueous to be much lower than in the vitreous, and it is difficult to conceive how prorenin could be transported from a lower concentration in aqueous to a higher concentration in the vitreous. Since vitreous can be considered the sink of products originating from the retina,[32] its high level of prorenin might best be explained by production in and secretion from the retina. The renin/prorenin ratio is quite different in uveal tissues on one hand and in retina and vitreous on the other hand, suggesting a third option that two distinct renin-producing systems exist in uvea and in retina separated by the blood-retinal barrier.

Reports have been published on components of the renin-angiotensin system in the eye. Angiotensin converting enzyme has been found in bovine retina, in both vascular and neural fractions.[33] Binding sites of angiotensin II are present in eye-vessels,[34] apparently also on the abluminal side, not in reach of systemic angiotensin II, since intravitreal administration of this peptide causes vasoconstriction.[35] Angiotensinogen has been detected in the ciliary-epithelium by the same group that described the presence of prorenin in this tissue.[36] Angiotensin II type 1 receptor mRNA appears to be present in rat retina.[37] Finally, direct angiotensin measurements have been performed.[17, 18] Both angiotensin I and II are present in ocular tissues at concentrations that could not be explained by admixture of blood, suggesting that both peptides are produced locally. In normal porcine eyes virtually no angiotensin was present in vitreous and aqueous fluid, which is evidence for the eye being a highly compartmentalized organ.

The function of a renin-angiotensin system in the eye is not clear. The higher concentration of renin in the uveal tract suggests that its major function is exerted there. Since the uveal tract is extremely vascular and angiotensin II is a vasoactive peptide it may serve a role in local blood supply. Reports are contradictory: one describes an important role for angiotensins in the control of the ophthalmic microcirculation[40] in perfused porcine eyes. The other presents data that angiotensin II is not a major determinant of ocular blood flow in healthy human subjects.[41] Topical administration of an angiotensin converting enzyme inhibitor to the eye lowers intraocular pressure.[42, 43] The renin-angiotensin system may therefore play a role in the control of intraocular pressure, possibly, in view of the presence of prorenin in the ciliary body,[31] through an effect on the production of aqueous humor. The iris sphincter muscle of dogs, located near the ciliary body, appears to be responsive to angiotensin II.[44] Angiotensin II has trophic and mitogenic actions on vascular smooth muscle and other cells.[45] We found prorenin to be increased in human vitreous from patients with proliferative diabetic retinopathy (see preceding chapter). In view of possible angiogenic effects of angiotensin II[46] the renin-angiotensin system may be a causative factor in proliferative retinopathy. In the ovary such a role of renin-angiotensin II has been proposed for the rapid vascularization of the developing corpus luteum which occurs after ovulation.[47] Inhibition of angiotensin converting enzyme in the isolated perfused feline eye causes changes in

the electrophysiological response of the retina to light,[48] suggesting that angiotensin II may have some neuromodulatory role in the retina. This was also suggested by a study in humans.[49] Finally, angiotensin II may have an angiotensin II receptor type 2-specific neurotrophic action.[50]

References

1 Sealey JE, Moon C, Laragh JH, Atlas SA. Plasma prorenin in normal, hypertensive and anephric subjects and its effect on renin measurements. Circ Res 1977;40(suppl I);41-5.

2 Naruse K, Murakoshi M, Osamura RY, Naruse M, Toma H, Watanabe K, Demura H, Inagami T, Shizume K. Immunohistochemical evidence for renin in human endocrine tissues. J Clin Endocrinol Metab. 1985;61:172-7.

3 DeSchepper CF, Mellon SH, Cumin F, Baxter JD, Ganong WF,. Analysis by immunohistochemistry and in situ hybridization of renin and its mRNA in kidney, testis, adrenal and pituitary of the rat. Proc natl Acad Sci USA 1986;83:7552-7.

4 Ganten D, Printz M, Phillips MI, Scholkens BA. The renin-angiotensin system in the brain. Exp brain Res 1982 (Suppl 4);3-293.

5 Derkx FHM, Alberda AT, de Jong FH, Zeilmaker GH, Makovitz JW, Schalekamp MADH. Source of plasma prorenin early and late in pregnancy. Observations in a patient with primary ovarian failure. J Clin Endocrinol Metab 1987;65:349-54.

6 Do YS, Sherrod A, Lobo RA, Paulsen RJ, Shinagawa T, Chen S, Kjos S, Hsueh WA. Human ovarian theca cells are a source of renin. Proc Natl Acad Sci USA 1988;85:1957-61

7. Glorioso N, Atlas SA, Laragh JH, Jewelewicz R, Sealey JE. Prorenin in high concentrations in human ovarian follicular fluid. Science 1986;233:1422-24.

8 Corvol P, Pinet F, Galen FX, Plouin PF, Chatellier G, Pagny JY, Corvol MT, Menard J. Seven lessons from seven renin-secreting tumors. Kidney Int 1988; 34(Suppl 25):S38-S44.

9 Ohashi H, matsunaga M, Kuwahara T, Pak CH, kawai C. Production and release of inactive renin by human vascualr smooth muscle cells. Clin Exp Hypertension 1985;A7:1395-1407.

10 Pinet F, Corvol MT, Bourguignon J, Corvol P. Isolation and characterization of renin-producing human chorionic cells in culture. J Clin Endocrinol Metab 1988;67:1211-20.

11 Fernandez LA, Olsen TG, Barwick KW, Sanders M, Kaliszewski C, Inagami T. Renin in angiolymphoid hyperplasia with eosinophilia. Its possible effect on vascular proliferation. Arch Pathol Lab Med 1986;110:1131-35.

12 Fernandez LA, Twickler J, Mead A. Neovascularization produced by angiotensin II. J Lab Clin Med 1985; 105: 141-5.

13 Hirose S, Ohsawa T, Inagami T, Murakami K. Brain renin from bovine anterior pituitary. J Biol Chem 1982;257: 6316-21.

14 Howard RB, Pucell AG, Bumpus FM, Husain A. Rat ovarian renin: Characterization and changes during the estrous cycle. Endocrinology 1988;123:2331-40.

15 Hirose S, Yokosawa H, Inagami T. Immunochemical identification of renin in rat brain and distinction from acid proteases. Nature 1978;274:392-93.

16 Wood JM, Gulati N, Forgiarini P, Fuhrer W, Hofbauer KG. Effects of a specific and long-acting renin inhibitor in the marmoset. Hypertension 1985;7:797-803.

17 Derkx FHM, Schalekamp MPA, Schalekamp MADH. Prorenin-renin conversion. Isolation of an intermediary form of activated prorenin. J Biol Chem 1987;262: 2472-77.

18 Derkx FHM, Tan-Tjiong HL, Wenting GJ, Boomsma F, Man in 't Veld AJ, Schalekamp MADH. Asynchronous changes in prorenin and renin secretion after captopril in patients with renal artery stenosis. Hypertension 1983;5:244-56.

19 Mancini G, Carbonara AO, Heremans JF. Immunochemical quantification of antigens by single radial immunodiffusion. Immunochemistry 1965;2:235-54.

20 Anson ML. The estimation of cathepsin with hemoglobin and the partial purification of cathepsin. J Gen Physiol 1934;20:565-74.

21 Hackenthal E, Hackenthal R, Hilgenfeldt U. Isorenin, pseudorenin, cathepsin D and renin. A comparative enzymatic study of angiotensin-forming enzymes. Biochim Biophys Acta 1978;522:574-88.

22 Schultze D, Brunswig B, Mukhopadhyay AK. Renin and proreninlike activities in bovine ovarian follicles. Endocrinology 1989;124:1389-98.

23 Derkx FHM, Bouma BN, Schalekamp MPA, Schalekamp MADH. An intrinsic factor XII-prekallikrein-dependent pathway activates the human renin-angiotensin system. Nature 1979;280:315-16.

24 Van Kesteren CM, Danser AHJ, Derkx FHM, Dekkers DHW, Lamers JMJ, Saxena PR, Schalekamp MADH. Mannose 6-phosphate receptor-mediated internalization and activation of prorenin by cultured neonatal rat cardiac myocytes and fibroblasts. Hypertension 1997;30:1389-96.

25 Wagner J, Danser AHJ, Derkx FHM, De Jong PTVM, Mullins JJ, Schalekamp MADH, Ganten D. Demonstration of renin mRNA, angiotensinogen mRNA, and angiotensin converting enzyme mRNA expression in the human eye: evidence for an intraocular renin-angiotensin system. Br J Ophthalmol 1996;80:159-63.

26 Brandt CR, Pumfery AM, Micales B, Bindley CD, Lyons GE, Sramek SJ, Wallow. Renin mRNA is synthetized locally in rat ocular tissues. Curr Eye Res 1994;13:755-63.

27 Swann DA, Constable IJ. Vitreous structure I. Distribution of hyaluronate and protein. Invest Ophthalmol 1972;11:159-63.

28 Hermann K, Raizada MK, Sumners C, Phillips MI. Presence of renin in primary neuronal and glial cells from rat brain. Brain Res 1987;437:205-13.

29 Lee-Kirsch MA, Gaudet F, Cardosos MC, Lindpaintner K. Distinct renin isoforms generated by tissue-specific transcription initiation and alternative splicing. Circ Res 1999;84:240-6.

30 Berka JL, Stubbs AJ, Wang DZ, DiNicolantinio R, Alcorn D, Campbell DJ, Skinner SL. Renin-containing Muller cells of the retina display endocrine features. Invest Ophthalmol Vis Sci 1995;36:1450-8.

31 Sramek SJ, Wallow IHL, Day RP, Ehrlich EN. Ocular renin-angiotensin: Immunohistochemical evidence for the presence of prorenin in eye tissue. Invest Ophthalmol Vis Sci 1988;29:1749-52.

32 Wurster U, Hoffmann K. Glaskörper. In: Biochemie des Auges, edited by Hockwin 0. Enke-Verlag, Stuttgart 1985, pp. 114, 119 and 121.

33 Ferrari-Dileo G, Ryan JW, Rockwood EJ, Davis EB, Anderson DR. Angiotensin-converting enzyme in bovine, feline and human ocular tissues. Invest Ophthalmol Vis Sci 1988;29:876-81.

34 Ferrari-Dileo G, Davis EB, Anderson DR. Angiotensin binding sites in bovine and human retinal blood vessels. Invest Ophthalmol Vis Sci 1987;28:1747-51.

35 Rockwood EJ, Fantes F, Davis EB, Anderson DR. The response of retinal vasculature to angiotensin. Invest Ophthalmol Vis Sci 1987;28:676-82.

36 Sramek SJ, Wallow IH, Tewksbury DA, Brandt CR, Poulsen GL. An ocular renin-angiotensin system. Immunohistochemistry of angiotensinogen. Invest Ophthalmol Vis Sci 1992;33:1627-32.

37 Murata M, Nakagawa M, Takahashi S. Expression and localization of angiotensin II type 1 receptor mRNA in rat ocular tissues. Ophthalmologica 1997;211:384-6.

38 Osuskey R, Nussberger J, Amstutz C, Flammer J, Brunner HR. Individual measurements of angiotensin II concentrations in aqueous humor of the eye. Eur J Ophthalmol 1994;4:228-33.

39 Danser AHJ, Derkx FHM, Admiraal PJ, Deinum J, de Jong PTVM, Schalekamp MADH. Angiotensin levels in the eye. Invest Ophthalmol Vis Sci. 12994;35:1008-18.

40 Meyer P, Flammer J, Luscher TF. Local action of the renin-angiotensin system in the porcine ophthalmic circulation: effects of ACE-inhibitors and angiotensin receptor antagonists. Invest Ophthalmol Vis Sci. 1995;36:555-62.

41 Matulla B, Streit G, Pieh S, Findl O, Entlicher J, Graselli U, Eichler HG, Wolzt M, Schmetterer L. Effects of losartan on cerebral and ocular circulation in healthy subjects. Br J Clin Pharmacol 1997;44:369-75.

42 Constad WH, Fiore P, Samson C, Cinotti AA. Use of an angiotensin converting enzyme inhibitor in ocular hypertension and primary open-angle glaucoma. Am J Ophthalmol 1988;105:674-77.

43 Abrams KL, Brooks DE, Laratta LJ, Barnhill MA, Frazier D. Angiotensin converting enzyme system in the normal canine eye: pharmacological and physiological aspects. J Ocul Pharmacol 1991;7:41-51.

44 Okamura T, Wang Y, Toda N. Local generation and action of angiotensin II in dog iris sphincter muscle. Exp Eye Res 1992;55:563-8.

45 Lever AF. Slow pressor mechanisms in hypertension: A role for hypertrophy of resistance vessels? J Hypertension 1986;4:515-24.
46 Ariza A, Fernandez LA, Inagami T, Kim JH, Manuelidis EE. Renin in glioblastoma multiforme and its role in neovascularization. Am J Clin Pathol 1988;90:437-41.
47 Palumbo A, Jones C, Lightman A, Carcangiu ML, DeCherney AH, Naftolin F. Immunohistochernical localization of renin and angiotensin 11 in human ovaries. Am J Obstet Gynecol 1989;160:8-14.
48 Jacobi PC, Osswald H, Jurklies B, Zrenner E. Neuromodulatory effects of the renin-angiotensin system on the cat electroretinogram. Invest Ophthalmol Vis Sci 1994;35:973-80.
49 Jurklies B, Eckstein A, Jacobi P, Kohler K, Risler T, Zrenner E, The renin-angiotensin system-a possible neuromodulator in the human retina? Ger J Ophthalmol 1995;4:144-50.
50 Lucius R, Gallinat S, Rosenstiel P, Herdegen T, Sievers J, Unger T. The angiotensin II type 2(AT2) receptor promotes axonal regenaration in the optic nerve of adult rats. J Exp Med 1998;188:661-70.

6 Summary

Prorenin, the enzymatically inactive precursor of the aspartic protease renin, circulates in blood plasma at a concentration that, in normal humans, is on average 9 times that of renin. It consists of a single polypeptide chain, which, three-dimensionally, is arranged into a bilobal renin part covalently connected with the N-terminal 43 amino acid-propeptide. The two lobes of the renin part are separated by a deep cleft containing the enzyme's active site. The propeptide is likely to be folded in the enzymatic cleft, thereby obstructing the active site. *In vitro*, prorenin acquires enzymatic activity, not only by limited proteolysis cleaving the propeptide from the renin part, but also non-proteolytically and reversibly by incubation at low pH or low temperature. It is unknown whether this non-proteolytic activation occurs *in vivo*.

Proteolytic activation takes place in the kidney mainly in the juxtaglomerular cells, but probably also in some extrarenal tissues and cells. In recent years the notion has emerged that renin and/or prorenin may exert their actions at the tissue level rather than in the circulation. The present thesis adresses some issues concerning the structural aspects of the non-proteolytic activation of prorenin, its implications for renin and prorenin measurements, the aberrant regulation of circulating levels of prorenin in diabetes mellitus, and finally the presence of prorenin in extrarenal tissues.

Central in chapter 2 is our discovery that remikiren, an active site-directed renin inhibitor, is capable of binding to prorenin. By doing so it induces a conformation which is recognized by a monoclonal antibody that is specific for an epitope expressed on renin but not on native, enzymatically inactive, prorenin. Although the prorenin-remikiren complex lacks enzymatic activity, we designate this remikiren-induced process as "activation". The observation that the active site of prorenin is accessible to remikiren and our study of the kinetics of remikiren-induced prorenin activation indicated the existence of an intermediate form of prorenin, which is in rapid equilibrium with the native species (chapter 2.1).

Recently we obtained monoclonal antibodies that were directed at epitopes of the prorenin-propeptide. These epitopes are not expressed on native prorenin but are readily recognized in prorenin treated by remikiren or incubated at low pH. Proteolytic removal of the propeptide by plasmin abolished binding of these monoclonals. Since the process of activation at 4 °C is very slow compared to the binding rates of the monoclonals, it was possible to follow the kinetics of activation. Enzymatic activity developed at the same rate as the unmasking of propeptide and renin epitopes and the maximal activation rates were similar with exposure to remikiren and with exposure to low pH, suggesting a similar activation mechanism. We constructed a model of non-proteolytic prorenin activation that was based on these observations and on published data on other aspartic proteases. In this model prorenin may changes into an intermediate form in which the active site is accessible to small ligands but not to natural angiotensinogen. In this intermediate conformation the propeptide is presumably still located in the enzymatic cleft, since there was no binding to specific propeptide-monoclonals and no enzymatic

activity. From this intermediate state prorenin slowly progresses to its active state, in which the active site is freely accessible and the propeptide is totally detached from the enzymatic cleft, though still covalently connected with the renin part (chapter 2.2).

Site-directed mutagenesis studies reported by others indicated that substitution of the proline residue in the propeptide at position –4 of the start of the renin sequence by alanine abolished the proteolytic processing of prorenin into renin in a cell line that, after transfection with a wild-type prorenin cDNA containing plasmid, produced renin as well as prorenin. It is possible that proline is essential for the recognition of the activation site by the (unidentified) processing protease. An alternative hypothesis is that the proline residue permits a conformational change of the prosegment through *cis-trans* isomerization, which is typical for proline residues. This isomerization might be a necessary first step for subsequent processing by the activation protease. We speculated that in this case that this conformational change caused by isomerization is the same as the change into the intermediate form postulated in the preceding two chapters. This hypothesis predicts that this prorenin mutant cannot be activated by remikiren or by cold or by incubation at low pH. However, we found that non-proteolytic activation of the mutated prorenin occurred at about the same rate as wild-type prorenin. We therefore conclude that the proline residue at position –4 is not involved in the first step of prorenin activation *in vitro*, as observed in our experiments (chapter 2.3).

Remikiren was also used to study the kinetics of reversible prorenin activation and inactivation at different temperatures. Pure recombinant prorenin acquires up to 15% of maximal enzymatic activity upon prolonged (days) incubation at low temperature (4°C). This activation is reversible upon incubation at 37 °C. We used simple models to describe inactivation and inactivation. Enzyme-kinetic assays and immunoradiometric assays were used to detect active prorenin. Remikiren promoted activation and retarded inactivation. As with remikiren, binding of the natural ligand, angiotensinogen, retarded the inactivation of prorenin. Angiotensinogen, however, was not capable of promoting prorenin activation. Our kinetic experiments indicate that the activation rate of prorenin is relatively independent of temperature and that the inactivation rate is highly temperature-dependent (chapter 2.4).

Chapter 3 applies some of the insights obtained by the results of chapter 1 to the methodology of renin and prorenin measurements. An immunoradiometric assay we often use for renin measurement overestimates renin level when renin levels are low and prorenin levels high, as is often the case in patients with diabetes mellitus. This is explained by co-measurement of 1.5% of prorenin as renin. We hypothesized that this is due to activation of prorenin during the long incubation time (24h) of the assay and the incubation temperature (22°C). Incubation for 6 hours at 37°C indeed abolished the co-measurement of prorenin as renin. Performance of the assay was not adversely affected by incubation at 37 °C, and the assay proved adequate for renin measurement in the clinical setting (chapter 3.1). We also describe a new, immunoradiometric assay (IRMA) of prorenin. It is a direct assay, as opposed to indirect assays that first measure renin, then measure

renin plus prorenin after activation of prorenin. In such indirect assays the prorenin level is calculated as the difference between the results before and after prorenin activation.

The new IRMA employs a monoclonal directed at the propeptide of prorenin, which is expressed after incubation of prorenin with remikiren, as described in chapter 2. This antibody is biotinylated and immobilized by linkage to avidin-coated plastic beads. Remikiren-treated plasma prorenin is specifically bound by the immobilized antibody and subsequently incubated with a radiolabeled antibody against active renin, which is the same monoclonal we used in the IRMA of renin. Results of the prorenin IRMA were identical to those obtained by the indirect enzyme-kinetic and immunological assays. The standard for the direct prorenin IRMA is remikiren-treated recombinant prorenin and since this preparation expresses specific renin and prorenin epitopes it can serve as a universal standard for both renin and prorenin assays. Performance of the new direct prorenin IRMA is excellent in clinical samples, though not superior to the indirect IRMA employing remikiren. Its use will be indispensable to monitor renin and prorenin levels in plasmas of patients treated with renin inhibitors, since prorenin is measured by the indirect IRMA as renin in these patients (**chapter 3.2**).

Chapter 4 aims to investigate the deranged prorenin physiology in diabetes mellitus. It is now well established that patients with insulin-dependent diabetes mellitus and microvascular complications like retinopathy and nephropathy, have high levels of plasma prorenin. We measured serum prorenin in yearly obtained serum samples during a 10-year follow-up in 199 normoalbuminuric patients with type 1 diabetes mellitus. In 29 patients who developed microalbuminuria the prorenin level, but not the renin level, increased before the onset of microalbuminuria. Microalbuminuria precedes the development of diabetic nephropathy. Apparently, renin/prorenin physiology is disturbed very early in the course of events that lead to nephropathy. This, together with the clinical observation that pharmacologic interference with the renin-angiotensin system (through ACE inhibition) has a beneficial effect on progression of nephropathy, suggests that prorenin, either itself or through the formation of renin, is involved in the pathogenesis of this diabetic complication. A practical implication is the fact that the measurement of prorenin can be used to predict future microalbuminuria. This has potential clinical value in the management of diabetic patients (**chapter 4.1**).

Our next study was inspired by the fact that only a subset of diabetic patients develops nephropathy and by the many reports that predisposition to nephropathy is genetically determined. The patient group we studied was from Denmark and has been studied by others for association of variation in the angiotensinogen, ACE and angiotensin type 1 receptor genes with nephropathy. Here we studied plasma renin and prorenin and variation in the renin gene in this study group. Of the various components of the renin-angiotensin system, renin and prorenin are altered. Renin and prorenin are increased, but the increase in prorenin is disproportionate with respect to that in renin. The rise in renin is somewhat unexpected, since older literature often reported normal or even decreased renin levels in diabetic nephropathy. One polymorphic marker in the first intron of the renin gene was weakly associated with nephropathy and could have a recessive deleterious effect.

This strengthens the notion that perhaps increased renin or prorenin is instrumental in the development and progression of diabetic nephropathy (**chapter 4.2**).

Chapter 5 is concerned with tissue prorenin and renin. We measured renin and prorenin in two distinctive tissue fluids, namely subretinal and vitreous fluid obtained during eye surgery. Prorenin and renin were present at levels that could not be explained by plasma contamination. Apparently some mechanism exists that causes high local levels of prorenin and renin. Whether this mechanism is local synthesis or uptake from plasma is not known (**chapter 5.1**). These findings from pathologic human specimens were corroborated by the findings in normal bovine eye tissues. Prorenin was present in chorioid and retina tissue, as well as in vitreous and aqueous fluid, at levels that were substantially higher than the levels in plasma. Again the mechanism leading to these high concentrations remains unidentified, although various studies report local expression of the renin gene (**chapter 5.2**). Other components of the renin-angiotensin also have been identified in the eye, most notably high levels on AngII in the eye tissues. Messenger RNA of angiotensinogen, ACE and AT1R, has been found suggesting local synthesis. The function of a local renin-angiotensin system in the eye is unknown. Evidence has been obtained for a role of AngII in blood flow and intraocular pressure regulation, but AngII may be a neuromodulatory factor too in the retina. The findings of chapter 5 support the concept of local RASs and confirm the predominance of prorenin in these local systems. However, whether prorenin has a direct role in these systems remains to be determined.

Where do the results described in this thesis leave us? First, the question of whether prorenin fulfills any physiologic role other than being the intrarenal precursor of renin remains unanswered and gnawing. In the absence of evidence for proteolytic or non-proteolytic activation of prorenin in the circulation, the most likely site for a role of prorenin from blood plasma is at the tissue level, either in the interstitial fluid or at the cell surface or in the cells after prorenin has been taken up by the cells. The results of chapter 5 and many reports in the literature show that prorenin and renin are present at considerable concentrations at diverse tissue sites. We know from chapter 2 that non-proteolytic activation is possible at physiologic pH and temperature, provided that inactivation of prorenin is precluded. Thus, factors that might cause activation of prorenin by inhibiting inactivation should be looked for if non-proteolytic activation is ever to be proven to be physiologically important. Recently our group described the binding of prorenin to cardiomyocytes.[1] After its binding, prorenin is internalized and activated, most probably by limited proteolysis. It has been shown that prorenin is more readily activated if it is in the active state with the propeptide out of the enzymatic cleft[2]. It is possible that binding to the putative receptor induces or fixates the enzymatically active conformation of prorenin and that this conformation is the substrate for subsequent, irreversible, proteolyic activation.

These new developments may ultimately lead to the acceptance of a hybrid paradigm, which implies that 1) the renin-angiotensin system is an endocrine system as far as renin is

concerned, 2) prorenin acts both in an endocrine and paracrine fashion, and 3) angiotensin II acts in an autocrine or paracrine fashion. The internalization of (pro)renin and the emerging reports on intracellular AngII[3-6] may even lead to a concept of an intracrine renin-angiotensin system. All in all it is possible that the term *prorenin-renin-angiotensin* system will prove to be more appropriate than the term renin-angiotensin system. In my view it is essential for acceptance of this concept that the role of angiotensinogen, until now somewhat neglected, be elucidated.

Our future research on prorenin will be directed at cellular binding and activation of prorenin. The monoclonal antibodies that have recently become available and have already been useful in the studies of this thesis, will enable us to identify the nature and extent of activation steps that occur during and after cell binding. This may ultimately provide an answer to the question of whether reversible activation of prorenin is more than an *in-vitro* phenomenon. Interest in the reported connections between the plasma contact activation system and the activation of prorenin (see first chapter) may be revived, because many protease inhibitors of the contact activation system in plasma are probably not present at the tissue level. More insight in the phenomenon of (pro)renin binding may also teach us about the significance of high prorenin levels at the very early stages of diabetic nephropathy. As mentioned above, the role angiotensinogen also needs clarification. Although somewhat alien to our expertise we would also like to address this issue. Essential for this work is good measurement technology. With the IRMAs described in this thesis we are confident that we are well equipped to measure the various conformations of prorenin and renin in physiological and pathological conditions.

The development of a new universal prorenin/renin standard, consisting of recombinant human prorenin, treated with remikiren, that expresses specific epitopes of both renin and prorenin should be undertaken in an international collaboration. If this standard can be established, the gain is obvious: after one century of renin research there will be at last an opportunity to rigorously compare the results of different laboratories.

The results presented in chapter 4.1 need confirmation in other diabetic populations, because they may have important consequences for clinical management. Currently we are starting a new project in which we will study a group of patients with type 1 diabetes mellitus that have been followed from the start of diabetes to see whether in this group elevated prorenin and glycated hemoglobin levels precede microalbuminuria. In this group it can also be tested whether the same holds true for the development of retinopathy, which, in its proliferative form, is also associated with increased prorenin levels. If the findings of chapter 4.1 are confirmed, a study can be envisaged in which prorenin and glycated hemoglobin levels are used to identify normoalbuminuric, normotensive, patients at risk of microalbuminuria. These can then be allocated for ACE inhibitor treatment.

Studying the biochemistry of prorenin activation by direct observation through spectroscopy is attractive and potentially more powerful than the methodology we have used thus far. Raman spectroscopy may be suitable for this goal. Furthermore, in a reductionist's view, life's events occur at the cell membrane, with the actions of the renin-

angiotensin system probably no exception to that. Why not, then, study life or the RAS at that level? Again, Raman spectroscopy, combined with confocal microscopy may permit to study the processes during the aforementioned cell binding and internalization of prorenin. However, we shall have to await further development of these techniques, which are still in their infancy for this kind of application.

In the genetic engineering field, an animal in which the renin gene can be knocked-out selectively in any organ at any moment in life provides an opportunity to study (pro)renin deprivation in intact, normally developed, animals. The Cre-lox approach[7] allows to construct such animals (please note the connotation of a deity-like faculty of mankind 'to construct an animal'). The intrarenal processing of prorenin to renin is still not understood. After the identification of the processing enzyme –let us hope it is only one– and the cloning of its gene, one may 'construct' knock-out animals for this enzyme. If this can be achieved it will be possible to study animals without renin but with 'normal' prorenin physiology. Such studies may also indicate whether the prorenin processing enzyme is an attractive target for drug therapy in, for instance, hypertension. Only then perhaps, we shall be able to refute the statement that prorenin is not worth bothering...

1 Van Kesteren CAM, Danser AHJ, Derkx FHM, Dekkers DHW, Lamers JMJ, Saxena PR, Schalekamp MADH. Mannose 6-phosphate receptor-mediated internalization and activation of prorenin by cultured neonatal rat cardiac myocytes and fibroblasts. Hypertension 1997; 30:1389-1396.

2 Derkx FHM, Schalekamp MPA, Schalekamp MADH. Two-step prorenin-renin conversion: isolation of an intermediary form of activated prorenin. J Biol Chem 1987 262:2472-7.

3 Sadoshima J, Xu Y, Slayter HS, Izumo S. Autocrine release of angiotensin II mediates stretch-induced hypertrophy of cardaic myocytes in vitro. Cell 1993;95:977-84.

4 Dostal DE, Rothblum KN, Conrad KM, Cooper GR, Baker KM. Detection of angiotensin I and II in cultured rat cardiac myocytes and fibroblasts. Am J Physiol 1992;32:C851-63.

5 Haller H, Lindschau C, Erdmann B, Quass P, Luft FC. Effects of intracellular angiotensin II in vascular smooth muscle cells. Circ Res 1996;79:765-72.

6 Brailoiu E, Filipeanu CM, Tica A, Toma CP, de Zeeuw D, Nelemans SA. Contractile effects by intracellular angiotensin II via receptors with a distinct pharmacological profile in rat aorta. Br J Pharmacol 1999;126:1133-8.

7 Marth J. Recent advances in gene mutagenesis by site-directed recombination. J Clin Invest 1996;97:1999-2002.

Acknowledgements

The work described in these thesis would not have been possible without the assistance of many people from outside our department. To all of them I am very grateful. In particular many thanks to Salima Mathews and Walter Fischli from Hoffmann-LaRoche, who provided us with recombinant human prorenin, remikiren and the antibodies against the propeptide of prorenin. Drs. Jeanette Wood and Marc de Gasparo from Novartis were so kind to provide us with the antibodies against renin. Dr. Tim Reudelhuber, who is one of my hopes for discovery of the prorenin-processing enzyme, gave us his prorenin mutants. Geurt helped me with the mathemathics, so clear and easy to him, but so cumbersome to me.

Lise Tarnow gently bore my questions on the Steno study group and did not blame me when I caused her a lot of trouble by a foolish mistake in a spreadsheet. Hans-Henrik Parving animated the same awe in me that my ancestors must have felt when they met his forebears when they came from the North. I am most grateful to Lise and Professor Parving for their willingness to collaborate and I hope this cooperation will continue.

Elisabeth Mathiesen and Brigitte Rønn, also at Steno at the time, allowed us to explore their treasure: a rare, huge follow-up study of patients with type 1 diabetes. Wim Hop, from the department of Epidemiology and Biostatistics at our university was a wizard to me with his skills in Cox' regression analysis.

Professor P. de Jong and Dr. E. Peperkamp took great care to provide us with the human ocular fluid samples. Professor Kazuo Murakami gave us the antibody against bovine renin, which was very helpful in identifying (pro)renin in the eye.

Kort Verslag AAN MIJN OMA, MIJN OVERIGE FAMILIE, en de belastingbetaler

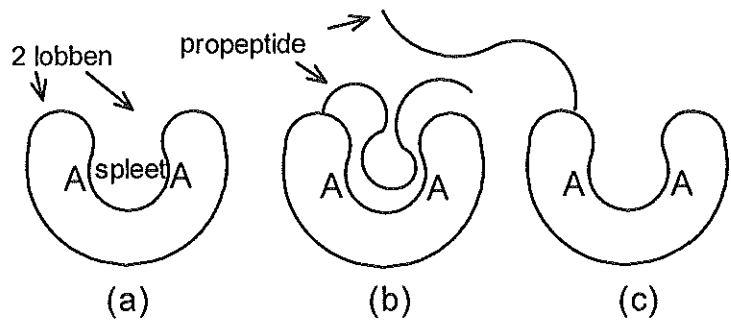
Hart- en vaatziekten zijn tegenwoordig goed te voorkomen en behandelen. Toch blijft een gestoorde bloedsomloop, veroorzaakt door hart-en vaatziekten, één van de belangrijkste oorzaken van lijden en sterven. Een belangrijke rol in het handhaven, maar soms ook in het verstoren van een goede bloedsomloop speelt het renine-angiotensine systeem. Hoewel artsen al wel beschikken over succesvolle medicijnen die gericht zijn op dit systeem, weten we nog maar weinig van dit systeem af. In het nu volgende zal ik u verslag doen van het onderzoek dat we uitgevoerd hebben naar een raadselachtig onderdeel van dit systeem, prorenine.
Prorenine is de voorloper van het eiwitsplitsende enzym (protease) renine, dat wordt gemaakt in de nier en daarna in het bloed wordt uitgescheiden. Renine splitst in het bloed van het eiwit angiotensinogeen een stukje af, angiotensine I, dat vervolgens door een ander enzym, angiotensin converting enzyme (ACE), wordt omgezet in angiotensine II. Angiotensine II speelt een belangrijke rol bij het handhaven van de bloeddruk en bij het regelen van de hoeveelheid zout en water in het lichaam.

We weten niet of in het lichaam prorenine net als renine enzymatisch actief is, dat wil zeggen een rol speelt bij de vorming van angiotensine II. In een reageerbuisje met prorenine en angiotensinogeen gebeurt in ieder geval niets bij 37 °C en een normale zuurgraad. Waarom dan onze belangstelling voor prorenine?

Allereerst, niet alleen renine wordt uitgescheiden in het bloed, maar ook prorenine. De concentratie van prorenine in het bloed van normale mensen is zelfs ongeveer 9 keer zo hoog als die van renine. Als dat prorenine wél betrokken zou raken bij angiotensine II-vorming, zou dat een forse stijging van het angiotensine II kunnen betekenen. Dit zou tot een misschien gevaarlijke verhoging van de bloeddruk kunnen leiden. Maar ook als prorenine maar een klein beetje enzymatische activiteit heeft zou dat belangrijk voor de bloeddruk kunnen zijn.

Een ander rechtvaardiging voor onze belangstelling is dat alle zoogdieren die onderzocht zijn, prorenine in hun bloed hebben. Dat is waarschijnlijk niet voor niets ('onze Lieve Heer heeft daar vast een bedoeling mee gehad').

Renine is een eiwit en bestaat uit een keten van aminozuren. Deze keten is zo opgevouwen dat een tweelobbig molecuul ontstaat. Bij prorenine zit aan dit renine-molecuul een keten van 43 aminozuren, die we het propeptide noemen. Dit propeptide wordt in de nier afgeknipt zodat renine ontstaat. Tussen de twee lobben van het renine-molecuul bevindt zich een diepe enzymatische spleet, waarin het actieve centrum zich bevindt. Dit is de plaats waar het enzym zijn eiwitsplitsende taak uitvoert. Er bevinden zich in dit actieve centrum twee zg. aspartyl aminozuren. We rekenen renine dan ook tot de zogenaamde aspartyl proteases. Deze hebben alle twee lobben met een diepe enzymatische spleet ertussen met de twee aspartyl groepen. De bekendste voorbeelden van andere aspartyl proteasen zijn wel chymosine, dat in het zure stremsel zit waarmee kaas wordt gemaakt, en pepsine, dat in het zure maagsap voor de vertering van eiwitten zorgt. In prorenine is het propeptide waarschijnlijk op zodanige wijze in het aktieve



De verschijningsvormen van renine en prorenine. (a) laat renine zien. De enzymatische spleet ('spleet') tussen de twee lobben is open en toegankelijk voor angiotensinogeen. (b) laat prorenine zien. Het propeptide sluit de toegang tot het actieve centrum af. (c) laat actief prorenine zien. Het propeptide zit nog wel aan het molecuul vast, maar sluit het actieve centrum niet meer af. A is een aspartyl groep van het actieve centrum

centrum gevouwen dat angiotensinogeen niet bij het actieve centrum kan komen. Andere aspartyl proteasen kennen ook zo'n inactieve voorloper met een propeptide.

In een reageerbuis kan prorenine wel enzymatisch actief worden, niet alleen door het propeptide af te knippen, maar ook door prorenine bij lage pH (d.w.z. een zure omgeving) en lage temperatuur te laten staan. In dat laatste geval blijft het propeptide wel aan het molecuul vast zitten maar zwaait het waarschijnlijk naar buiten zodat angiotensinogeen wel bij het actieve centrum kan komen. Bij normale pH en een hoge temperatuur verdwijnt de activiteit van prorenine weer. Deze eigenschap, dat prorenine onder *speciale,* met name zure omstandigheden ook eiwitsplitsende activiteit kan hebben, is ook bekend van voorlopers van andere aspartyl proteasen. Bij die andere aspartyl proteasen is het zelfs zo dat zo'n geactiveerde voorloper bij zichzelf het propeptide kan afknippen. Dit kan prorenin echter niet. Het verschijnsel doet wel weer de vraag rijzen of in het lichaam omstandigheden voorkomen waarbij prorenine actief is en versterkt onze belangstelling voor prorenine.

Er is in de laatste jaren duidelijk geworden dat prorenine niet alleen in de nieren gemaakt wordt maar ook elders in het lichaam. Men denkt ook dat de angiotensine II vorming voornamelijk buiten de bloedstroom plaatsvindt in de weefsels. Het zou heel goed kunnen zijn dat prorenine, dat ter plaatse wordt gemaakt, daarbij een grote rol speelt.

Een laatste reden voor onze belangstelling voor prorenine is dat bij sommige aandoeningen prorenine in het bloed erg hoog is. Dit is bijvoorbeeld zo bij suikerziekte, waarbij de nieren aangetast zijn. Waarom prorenin zo hoog is, is onbekend.

In dit proefschrift is een aantal aspecten van prorenine onderzocht, met als doel erachter proberen te komen of prorenine, behalve als voorloper van renine, nog een andere rol speelt in het renin-angiotensine systeem. Ten eerste (hoofdstuk 2) hebben we getracht meer inzicht in de structuur of vorm van prorenine te verkrijgen. Hoe en onder welke omstandigheden verandert die vorm en wat zijn de gevolgen voor de

enzymatische activiteit van prorenine? Wij hebben ontdekt dat remikiren, een medicijn dat renine remt door in de enzymatische spleet te gaan zitten, ook aan prorenine kan binden. We denken dat remikiren in plaats van het propeptide in de spleet gaat zitten. Het propeptide zwaait dan uit de enzymatische spleet, net als wanneer prorenine wordt aangezuurd of in de kou wordt weggezet (Figuur). Alleen gebeurt de remikiren binding ook bij 37 °C en neutrale pH. Verder moeten we aannemen dat onder deze normale omstandigheden er een tussenvorm van prorenine bestaat, waarin er nog geen enzymatische activiteit bestaat, maar al wel het propeptide een beetje aan de kant geschoven is, zodat remikiren kan binden. Prorenine verandert na binding van remikiren dan zodanig dat het herkend wordt als renine door antistoffen die alleen maar renine en niet prorenine kunnen herkennen. Wij hebben dit door remikiren veroorzaakte verschijnsel gebruikt om de vormverandering van prorenine die gepaard gaat met activering, te bestuderen onder verschillende omstandigheden. Het was daardoor mogelijk een model te maken van de activering van prorenine. Verder hebben we aannemelijk gemaakt dat ook bij normale lichaamstemperatuur en -zuurgraad prorenine actief kan worden. Er is dan alleen een extra factor nodig die voorkómt dat prorenine zijn enzymatische activiteit weer verliest. Of deze factor bestaat en welke dat is, is niet bekend. In het reageerbuisje kan angiotensinogeen in geringe mate ervoor zorgen dat actief gemaakt prorenine (door aanzuren) actief blijft. Dat is wel voor te stellen: als angiotensinogeen in de enzymatische spleet zit. kan het propeptide er niet in.

In hoofdstuk 3 wordt een aantal methoden beschreven hoe renine en prorenine het beste gemeten kunnen worden. Dit is van belang om een indruk te krijgen hoe het lichaam prorenine en renin reguleert. Door de resultaten van hoofdstuk 2 begrijpen we beter hoe prorenine en renine eruit zien en hoe ze van elkaar onderscheiden kunnen worden. We doen dat met antistoffen die alleen maar kunnen binden aan óf renine én prorenine, óf aan renine alleen óf aan prorenine alleen. Onze nieuwe methoden zijn zeer geschikt voor metingen in het bloed. Bovendien kunnen ze gemakkelijk gestandaardiseerd worden, wat betekent dat elk laboratorium op de wereld dezelfde (juiste) uitslagen geeft voor dezelfde bloedmonsters. Dat laatste is al een eeuw lang een groot probleem in de renine-wereld: een reninewaarde in een bepaald monster kan in het ene laboratorium totaal anders uitpakken dan in een ander.

Het vierde en vijfde hoofdstuk gaan over de regulatie van prorenine. Hoofdstuk vier behandelt het verband tussen suikerziekte en en prorenine. Bij suikerziekte met nieraantasting (diabetische nefropathie) is het prorenine-gehalte van het bloed meestal sterk verhoogd. Het Steno Diabetes Centrum, een groot ziekenhuis in Denemarken, behandelt alleen mensen met suikerziekte. Deze patienten worden intensief gecontroleerd en gegevens en bloedmonsters van deze patienten worden zorgvuldig bewaard. We werden in de gelegenheid gesteld ook een grote groep van deze patienten te bestuderen. We laten in hoofdstuk 4.1 zien dat prorenine al stijgt voordat het de dokter duidelijk wordt dat de patient een diabetische nefropathie ontwikkelt. Het lijkt er zelfs op dat je prorenine kunt gebruiken om het risico op het ontwikkelen van de diabetische nefropathie te bepalen. Als prorenine verhoogd is, zou je zelfs kunnen zeggen dat je de suikerziekte nog strenger moet proberen te behandelen en zelfs zou je dan kunnen overwegen

medicijnen te geven waarvan we weten dat het de nieraantasting door suikerziekte voorkomt of vertraagt.

In hoofdstuk 4.2 hebben we gekeken naar het verband tussen het gen (erfelijke informatie voor een eiwit op een chromosoom) voor prorenine en renine en het ontstaan van diabetische nefropathie. Dit werd ingegeven door het sterke vermoeden dat erfelijke factoren een belangrijke rol spelen bij het ontstaan van diabetische nefropathie en door het afwijkende prorenine gehalte. Het bleek dat één bepaalde variant van het renine-gen een geringe invloed had op het ontstaan van diabetische nefropathie. Die invloed was echter zo gering dat je deze variant waarschijnlijk niet kunt gebruiken om nieraantasting bij suikerziekte te voorspellen. Verder bleek dat naast prorenin ook het reninegehalte verhoogd was. De heersende mening in de medische wereld is echter dat renine aan de lage kant is bij suikerziekte. We denken dat deze mening misschien wel herzien moet worden. Voorheen werden gebrekkiger methoden gebruikt om renine te meten en de resultaten waren toen wellicht minder betrouwbaar. Tenslotte, of (pro)renine de nieraantasting *veroorzaakt* is (nog) niet bekend.

Het laatste hoofdstuk gaat over renine en prorenine in een weefsel, namelijk het oog. De keuze van dit orgaan lijkt misschien wat ongewoon, maar bij oogoperaties is vrij gemakkelijk weefselvocht te verkrijgen, dat een afspiegeling is van het vocht dat de cellen van het weefsel omringt. Verder hebben we ook koeienogen gebruikt, die gemakkelijk in het slachthuis zijn te krijgen. Het blijkt dat renine en prorenine beide voorkomen in oogweefsels, in concentraties die niet zijn te verklaren door bijmenging van bloed. In het binnenste, heldere, glasvochtlichaam van het oog komt voornamelijk prorenine voor, hetgeen zou kunnen bevestigen dat prorenine ter plaatse wordt gemaakt. Welke functie prorenin (en renine) zouden kunnen hebben in het oog is nog volstrekt onduidelijk. Veel van de patienten hadden suikerziekte met aantasting van de ogen. Het is niet uitgesloten dat (pro)renine een rol speelt bij deze aandoening.

Al met al is de vraag of prorenine 'iets doet' nog steeds niet met zekerheid beantwoord. Hoe zou het onderzoek naar prorenine nu verder moeten? Voor de patientenzorg lijkt verder onderzoek naar het belang van prorenine metingen bij patienten met suikerziekte van belang. Als men namelijk in een vroeg stadium al het risico op een complicatie van suikerziekte kan bepalen, ontstaat ook de mogelijkheid al in een vroeg stadium in te grijpen met medicijnen en een striktere regeling van de bloedsuikers. Of dit werkelijk zo werkt, dient bevestigd te worden.

Dit onderzoek bij patienten met suikerziekte zal echter de hamvraag niet oplossen: doet prorenine nu iets of niet? In proefdieren en mensen blijft het moeilijk om onderzoek te doen naar prorenine, omdat ook altijd renine aanwezig is. Een mogelijke oplossing is, muizen te scheppen (let op de pretentie van dat woord) die niet meer in staat zijn om renine en prorenin te maken of muizen die in de nier van prorenine geen renine meer kunnen maken. Het enzym dat voor dat laatste zorgt is nog niet bekend. Heeft men deze muizen, dan kunnen de volgende vragen beantwoord worden: Kan een muis zonder renine? Is een muis dan wel levensvatbaar? Welke afwijkingen treden op? Wordt er nog angiotensine gemaakt als er alleen maar prorenine is? Dit soort onderzoek, met genetisch

gemanipuleerde dieren, kan veel informatie opleveren, maar is maatschappelijk omstreden.

Tenslotte is het theoretisch mogelijk meer inzicht te verkrijgen in de structuur en het activatie-mechanisme van prorenine met behulp van spectroscopische technieken. U moet zich hierbij voorstellen dat moleculen met licht of straling worden gebombardeerd. Moleculen hebben de eigenschap dat ze dan afhankelijk van hun structuur een ander soort licht of straling ('spectra') terugzenden. Dit kan met speciale apparatuur gemeten worden. Ook de recente waarnemingen in ons laboratorium dat prorenine aan cellen gebonden kan worden, zou hiermee onderzocht kunnen worden. Deze technieken staan voor deze vraagstelling echter nog in de kinderschoenen.

Renine is al in 1898 ontdekt, prorenine in 1971. In de tussenliggende tijd is veel bekend geworden over renine en prorenine, maar het moge duidelijk zijn dat na een eeuw, respectievelijk bijna 30 jaar, nog vele vragen onbeantwoord blijven. Ik ben benieuwd of dat nog zo zal zijn in 2098 of 2071.

Nawoord

Beleidsmakers geven de indruk dat wetenschappelijk onderzoek zich goed leent voor planning, in grote onderzoeksscholen moet plaatsvinden en in ieder geval maatschappelijk relevant moet zijn. In dat licht dien je voor een succesvol promotie-onderzoek bij voorkeur op een rijdende trein te springen, liefst op een TGV. Het traject een aantal malen afleggen, telkens in een andere coupé, en je arriveert als doctor. Hogesnelheidslijnen zijn rendabel, stipt en hebben belangrijke bestemmingen; er is geen twijfel over nut. Het onderzoek dat heeft geleid tot dit proefschrift had niets van zo'n TGV-reis. Eerder was het een stoomboemeltje in een onbekend oerwoud, waar de reiziger zelf nog het spoor moest aanleggen. Langzaam is de weg van A naar B afgelegd, in terugblik zeker niet de kortste afstand tussen twee punten. Vaak was er langdurig oponthoud: geen druk op de ketel, boom op het spoor, een excursie, machinist malaria, geen rails op voorraad, lange aarzelingen bij wissels. En al die tijd stapelden de volgeschoten fotorolletjes zich op. Er zijn er nu een paar ontwikkeld en de afdrukken zijn in dit album geplakt. De kleren zijn gewassen, vrouw en kinderen gekust. Maarten Schalekamp heeft als reisleider en directeur van de spoorwegmaatschappij de reis tot een avontuur gemaakt, met gelukkig weinig aandacht voor de dienstregeling. Geen comfort van 300 km/uur met air-conditioning, maar een daadwerkelijke ervaring van het landschap. Frans Derkx, de mede-reiziger en -spoorwegingenieur, Sjors van Kats, Lies Tan, Jeanette van Gool, René de Bruin, Jan Danser, Ton van den Meiracker, Frans Boomsma, Ellen Maurik, Arie Man in't Veld en al die anderen die ik tijdens de tocht tegenkwam: U was aangenaam en behulpzaam gezelschap. Mijn dank daarvoor. Dank ook aan de leescommissie die zo snel de spoorbomen sloot en het sein op groen zette.

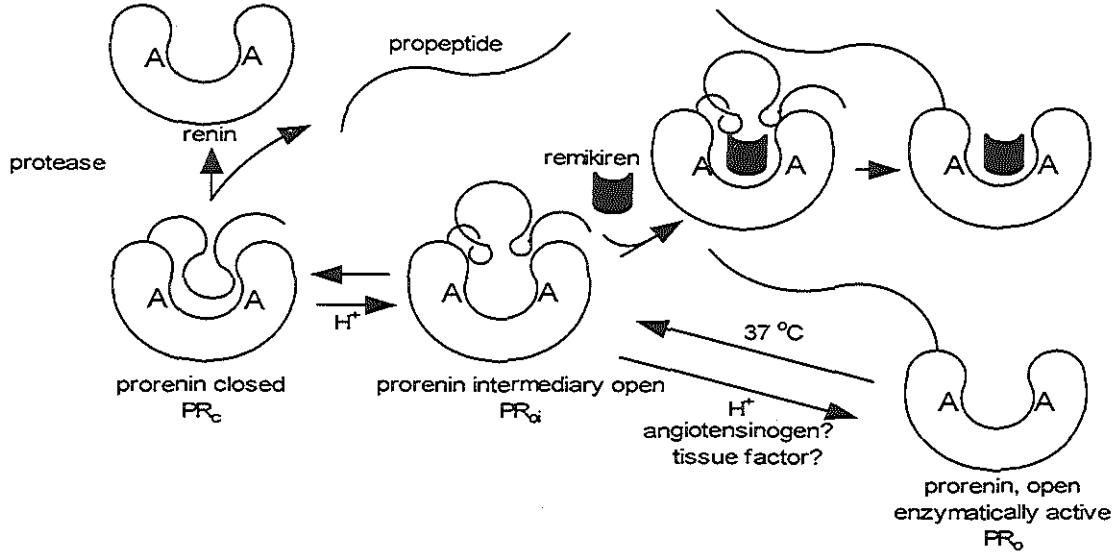
En nu? Promoveren is niet aankomen op een station, maar de mogelijkheid verkrijgen om weer op weg te gaan. Nee, niet met een TGV.

Curriculum vitae

De schrijver van dit proefschrift werd geboren in Ede. Na het doorlopen van de middelbare school volgde hij het propedeutisch jaar van de Vrije Hogeschool te Driebergen. In 1980 besloot hij, om onnavolgbare redenen, medicijnen te gaan studeren. In 1987 legde hij het artsexamen af aan de Erasmus Universiteit te Rotterdam. Na een vervangende dienstplicht op de afdeling Interne Geneeskunde I van het Dijkzigt Ziekenhuis te Rotterdam, volgde hij van 1989 tot 1995 op deze afdeling de opleiding tot internist (opleider: Prof. Dr M.A.D.H. Schalekamp). Sinds 1995 is hij als staflid verbonden aan deze afdeling. Hij is getrouwd met Ellen van der Wal en is vader van Sanne, Bouke en Fenna.

Afkortingenlijst

AngI, II	angiotensin I, II
AT1,2R	angiotensin type 1,2 receptor
AER	albumin excretion rate
ACE	angiotensin converting enzyme
aHT	antihypertensive therapy
BSA	bovine serum albumin
CHO	chinese hamster ovary
CV	coefficient of variation
IC_{50}	concentration at which 50% inhibition occurs
CI	confidence interval
DN	diabetic nephropathy
EKA	enzyme-kinetic assay
IRMA	immunoradiometric assay
IRP	international reference preparation
K_m	Michaelis constant
M_r	molecular weight
mAb	monoclonal antibody
PMSF	phenylmethylsulfonyl fluoride
PBS	phosphate buffered saline
PRA	plasma renin activity
PRC	plasma renin concentration
PCR	polymerase chain reaction
PVP	polyvinylpyrrolidone
PI	prognostic index
PR	prorenin
RIA	radioimmunoassay
RAS	renin-angiotensin system
NaN_3	sodium azide
NTT	sodium tetrathionate
SBTI	soy bean trypsin inhibitor
SD	standard deviation



Schema van de structuur en activatie van prorenine zoals gepresenteerd in dit proefschrift. 'A' staat voor een aspartylgroep in de spleet tussen de twee lobben van het renine-molecuul. H^+ staat voor een zuur milieu. (Aangepast vanuit Deinum & Schalekamp Renin and prorenin. *In* Hypertension. Eds. Weber & Oparil, New York, 1999, met toestemming)

ERRATUM

A mistyping occurred in two figures of chapter 5.1. In Figure 2 (page 137) and Figure 3 (page 138) the title of the *x*-axis of the lower panel should read 'Renin substrate *calculated* nmol/L'

Structure and Regulation of Prorenin/J. Deinum/1999