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IDENTIFICATION OF A RENIN BINDING SITE IN THE HUMAN PLACENTA

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ABSTRACT

Renin is an aspartyl protease that plays a critical role in the production of angiotensin peptides and therefore in the modulation of blood pressure. Renin participates not only in the circulating renin-angiotensin system (RAS) but may also be taken up by tissues to control the activity of the local RAS. In this study, we provide evidence for the existence of a high affinity, saturable binding site for human renin (estimated K_d of 125 pM) and prorenin, the inactive precursor of renin, in the human placenta. The interesting finding that prorenin could bind to the same site as renin suggests that its role *in vivo* may be to act as a natural antagonist that would limit the local levels of angiotensin II production. It is of further interest to identify the protein regions important in this uptake; here we show that the degree of glycosylation in prorenin and the active site of renin are not essential in this process. In the near future, experiments will involve in-depth studies of the regions implicated in the binding process and long term goals will be to identify and characterize this novel renin and prorenin binding site.

RÉSUMÉ

La rénine est une aspartyl protéase qui joue un rôle essentiel dans la production des angiotensines et ainsi dans la modulation de la pression sanguine. La rénine est impliquée non seulement dans le système rénine-angiotensine circulant mais peut aussi être captée par les tissus afin d'activer les différents systèmes rénine-angiotensine locaux. Dans cette étude, nous démontrons la présence d'un site de liaison saturable et de haute affinité pour la rénine humaine (K_d estimé à 125 pM) et pour la prorénine, le précurseur inactif de la rénine, dans le placenta humain. La découverte que la prorénine lie le même site que la rénine suggère que son rôle *in vivo* pourrait être d'agir comme antagoniste naturel de la rénine afin de limiter les niveaux locaux d'angiotensine II. Il est d'un grand intérêt d'identifier les régions de la protéine impliquées dans cette captation; ici nous démontrons que le degré de glycosylation de la prorénine et le site actif de la rénine ne sont pas essentiels dans cette captation. Il serait important dans les études à venir de définir les régions impliquées dans la reconnaissance de cette liaison et à plus long terme d'identifier et de caractériser ce nouveau site de liaison de la rénine et de la prorénine.

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1. Introduction

Hypertension is a major problem that affects a large proportion of the adult population of North America. Uncontrolled hypertension is associated with high incidences of stroke, coronary heart disease, congestive heart failure and kidney disease. Because of the severe consequences that can arise from sustained high blood pressures, this area of research is of major importance. Understanding the cellular mechanisms involved in blood pressure regulation and also the potential for deviation toward the pathological states will be helpful in finding better treatment for controlling hypertension and possible prevention of disease.

1.1. Overview of the Renin-Angiotensin System

One of the major factors involved in controlling systemic blood pressure is the circulating renin-angiotensin system (RAS) (Figure 1). This system is classically viewed as an endocrine one, with angiotensin II (AII) behaving as the final effector hormone in the highly regulated cascade of events leading to its formation. Briefly, the juxtaglomerular (JG) apparatus of the kidney functions to monitor blood pressure and blood volume. A decrease in either variable causes the cells to respond by secreting the aspartyl protease, renin. Once in the circulation, renin encounters angiotensinogen (Aogen), a glycoprotein released by the liver, which is cleaved by renin to release the decapeptide, angiotensin I (AI). Subsequently, angiotensin converting enzyme (ACE), a zinc metalloprotease present in the membranes of endothelial cells, cleaves two amino acids from the carboxy-terminus of AI to generate the octapeptide, AII (Figure 2). AII controls blood pressure by inducing arteriolar vasoconstriction and stimulates release of aldosterone from the adrenal gland. Aldosterone is responsible for sodium retention and potassium loss from the renal tubules. Na^+ retention in turn causes water retention and therefore corrects blood volume. An increase in Na^+ levels also leads to an increase in sensitivity to vasoactive compounds in arteriolar muscle cells, thereby

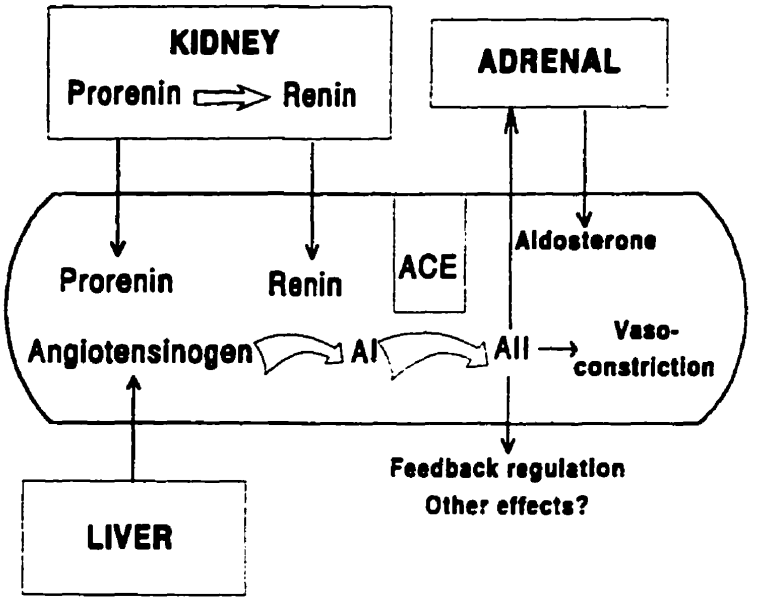


Figure 1: The renin-angiotensin system.

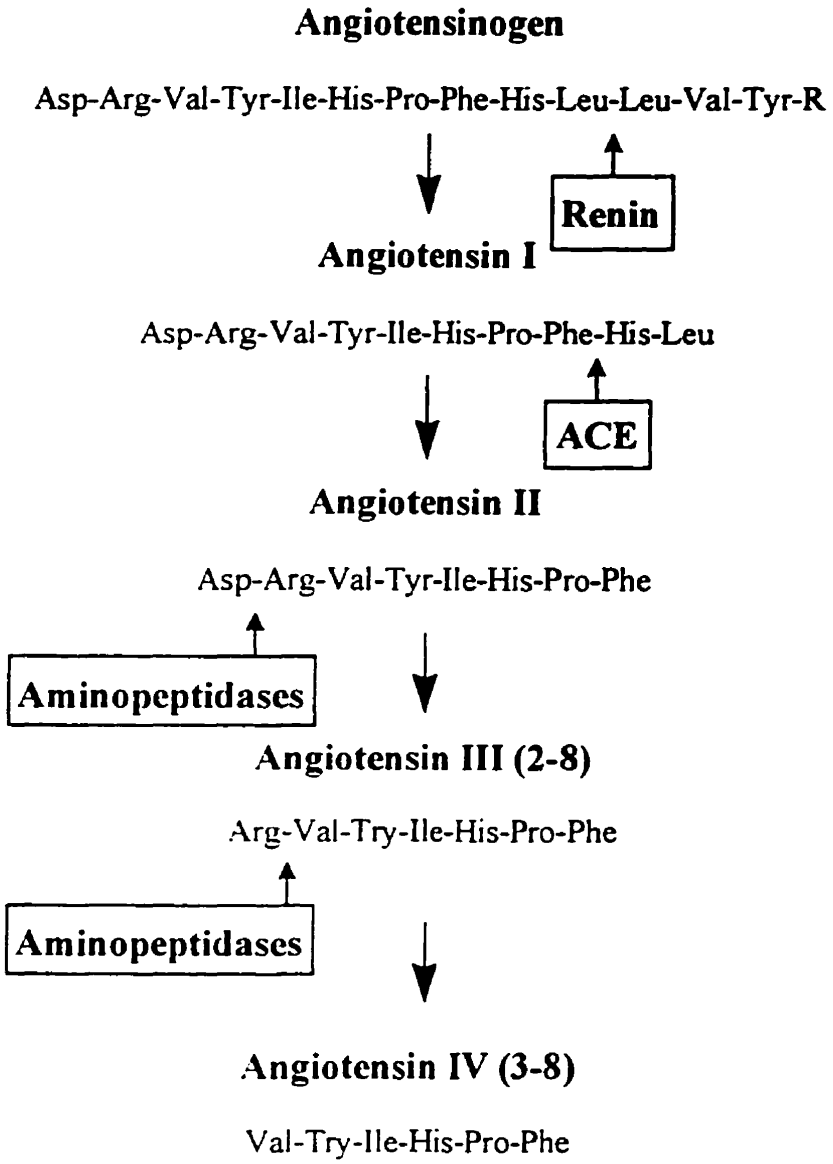


Figure 2: Enzyme cleavage sites in the generation of the angiotensin peptides. R, remainder of the peptide sequence for Angiotensinogen.

maintaining blood pressure. Restored blood pressure and blood volume, as well as increased levels of a number of humoral factors including aldosterone, AII, vasopressin, endothelin, atrial naturetic factor (ANF) and adenosine can negatively feedback to the JG cells to inhibit further renin secretion, thereby down-regulating the system.

1.2. Angiotensin II Receptors

AII exerts its actions by binding to specific receptors (Figure 3) on cell membranes and eliciting intracellular responses. Two pharmacologically distinct receptor isoforms for AII were identified and named AT_1 and AT_2. Both isoforms are seven transmembrane domain receptors with a low degree of homology (32%). Homology is mainly conserved for the hydrophobic amino acids spanning the membrane. Both receptors bind with equally high affinity to AII and peptidic antagonists like (Sar^1,Ile^8)-AngII suggesting involvement of a similar ligand binding domain. The tissue distribution of the receptor subtypes in the rat is varied, with AT_1 predominately found in the liver [1], kidney [2] and aorta [3]. AT_2 is found mainly in the adrenal medulla [4] and there is a mixture of both subtypes in the adrenal cortex [4], uterus [5] and the brain [6,7,8].

1.2.1. AT_1 receptors

Upon AII binding to AT_1 receptors, there is a stimulation of G_q and transient activation of phospholipase CB1 (PLC_{B1}) which cleaves phosphatidyl inositol 4,5P_2 (PIP_2) to inositol triphosphate (IP_3) and diacylglycerol (DAG), leading to increased Ca^{2+} levels derived from intracellular stores, mainly from the endoplasmic reticulum. The rapid rise in Ca^{2+} levels is responsible for the classical effects of AII, namely arteriolar vasoconstriction [9] and synthesis and secretion of aldosterone release from the adrenal gland [10]. More recent evidence shows that tyrosine phosphorylation may play a major role in AT_1 receptor signaling. AII can activate PLC-γ1 via tyrosine phosphorylation [11] and it is thought that a member of the Src family of tyrosine kinases is responsible

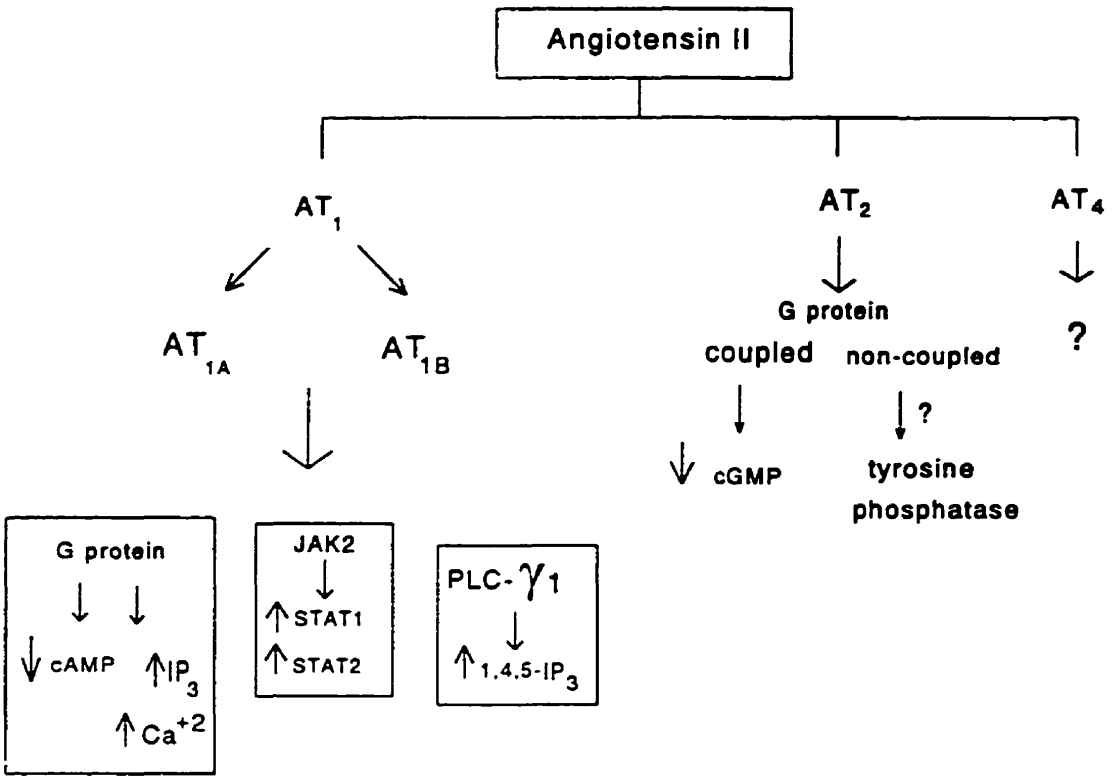


Figure 3: Rodent AII receptors and their postulated intracellular signaling pathways.

for the activation of PLC-γ1 mediated by AII [12]. It is also thought that AII can control initiation of gene transcription through the JAK-STAT pathway [13]. Other studies have shown that binding of AII to the receptor causes an interaction with a G-protein to stimulate phospholipase A2 and production of the arachidonic acids and its metabolic products [14]. Inhibition of adenylate cyclase is also observed and the receptor is rapidly internalized following AII binding. AT_1 has two subtypes in rodents, AT_{1a} and AT_{1b}, which are products of separate genes, and share a 95% amino acid sequence identity. Two non-peptide ligands, TCV-116 and Losartan (DuP753), a tetrazolbiphenylimidazole, were developed as specific AT_1 receptor antagonists.

1.2.2. AT_2 receptors

Much less is known about the structure and function of the AT_2 receptor but preliminary findings suggest an involvement in cell growth. It is unclear what downstream signaling pathways are induced upon agonist binding to AT_2, however, it is possible that the receptor is coupled to a G-protein. This idea stems from experiments conducted in rat adrenal pheochromocytoma PC12w cells which only express the AT_2 receptors [15] and in African green monkey kidney COS-7 cells transfected with the AT_2 receptors [16]. In these studies, AII caused a partial inhibition of phosphotyrosine phosphatase activity in the cell membrane and pertussis toxin treatment was able to reverse this inhibition, suggesting a coupling of the AT_2 receptor to a G_i or G_o protein. The exact role of the AT_2 receptor is uncertain and much controversy exists around its role in control of cell growth. While certain findings suggest that AT_2 does not regulate growth, its mRNA levels are inversely related to the mitogenic activity in some cells [15]. For example, AT_2 is not expressed in cultured rat vascular smooth muscle cells, however, serum depletion in the media induces AT_2 receptor expression; treatment of the cells with growth factors rapidly reverses this effect. It will be interesting to see what future experiments hold in trying to unravel the mystery behind the elusive biological role of this receptor. PD 123177 and

PD123319, both tetrahydroimidazopyridines, are AT_2 receptor antagonists whereas CGP42112A, a pentapeptide analog, is a partial agonist.

1.2.3. AT_4 receptors

A novel receptor, AT_4, has been recently discovered. Its ligand, AIV, is generated when aminopeptidase M cleaves AIII (2-8) to AIV (3-8) (Figure 2). Preliminary data suggests possible involvement in renal development since stimulation of the AT_4 receptor has been shown to increase renal cortical blood flow and similar to AII, can also cause transient increases in intracellular Ca^{+2} levels in proximal tubular epithelial cells [17]. The discovery of this new receptor promises to lead to a new and exciting avenue for AIV function.

1.3. A closer look at renin synthesis and structure

1.3.1. Structure of renin

Mouse [18], rat [19] and human renin genes [20] have been cloned and characterized and a high degree of sequence homology exists among these species. Human preprorenin contains 406 amino acids and a pre and pro segment consisting of 20 and 46 amino acids respectively [20] (Figure 4). Cleavage of the prosegment releases the active protein with a molecular weight of 43 kilodaltons (kDa). The fact that there is a great deal of similarity between renin and other aspartyl proteases (45-65% similarity in primary structure), particularly for the amino acids surrounding the active site of the protein, suggests a similar tertiary structure. X-ray crystallography shows that renin is bilobal, with 2 halves of approximately equal size that fold inward to form a long cleft that forms the substrate binding site. The active site aspartate residues (Asp +38 and Asp +226) are located in the center of this deep cleft. Interaction of angiotensinogen with the active site causes the cleavage of the protein and the first step in the generation of angiotensin peptides.

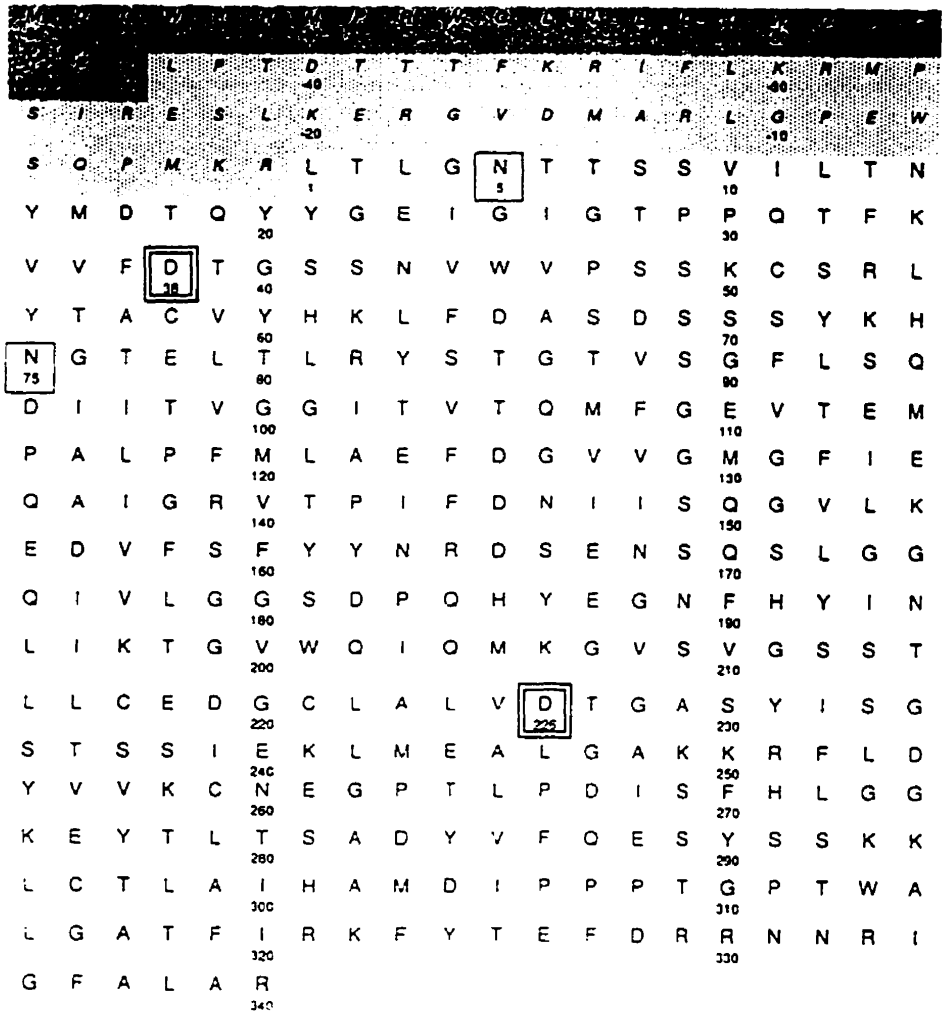


Figure 4: Complete amino acid sequence of human kidney preprorenin. Asparagine residues in the consensus sequences for N-linked glycosylation are boxed and active site aspartate residues are doubly boxed. The mature enzyme begins with position 1, the signal sequence is displayed in dark grey, and the pro sequence is in light grey.

1.3.2. Renin synthesis in the JG cells

Circulating active renin is derived primarily from the JG cells of the kidney. These cells are actually modified smooth muscle cells and account for 0.1% of the cellular mass of the adult kidney [21]. JG cells are rich in dense core secretory granules and hence resemble neuroendocrine cells, however, they differ in two important respects. First of all, in contrast to classical neurocrine and endocrine cells whose proteins tend to aggregate (but do not crystallize) as they are sequestered inside secretory granules, in JG cells, membrane-bound, paracrystalline structures have been shown to pinch off the trans golgi network (TGN). These unorthodox rhomboid structures have been seen during periods of high renin synthesis and it is speculated that these structures are important in the cascade of events leading to active renin (Figure 5). In addition, the secretory granules themselves show similarities to lysosomes; micrographs demonstrate that the granules are capable of pinocytosis and autophagy of other cellular organelles [22]. Furthermore, several lysosomal enzymes have been localized in the granules through use of antibodies [23]. These findings, along with the fact that classical lysosomes have not been discovered in these cells, suggest that the secretory granules of the JG cells are actually adapted lysosomes that function to process prorenin and secrete mature renin upon stimulation [22].

The signal peptide at the amino-terminus directs the nascent polypeptide to the rough endoplasmic reticulum (RER) where the signal peptide is subsequently removed and post-translational modification of the resulting prorenin begins. Protogranules containing prorenin bud off the TGN and fuse together to form amorphous low density structures referred to as immature granules. In this structure, the granule can fuse directly with the plasma membrane, resulting in the basal secretion of the inactive protein [22]. Alternatively, the granules can condense to form mature granules (Figure 5). Evidence indicates that processing of prorenin to renin begins in the immature secretory granules by removal of a 46 amino acid prosegment from the amino terminus [24]. In humans, the cleavage takes place after a lysine-arginine basic pair of amino acids

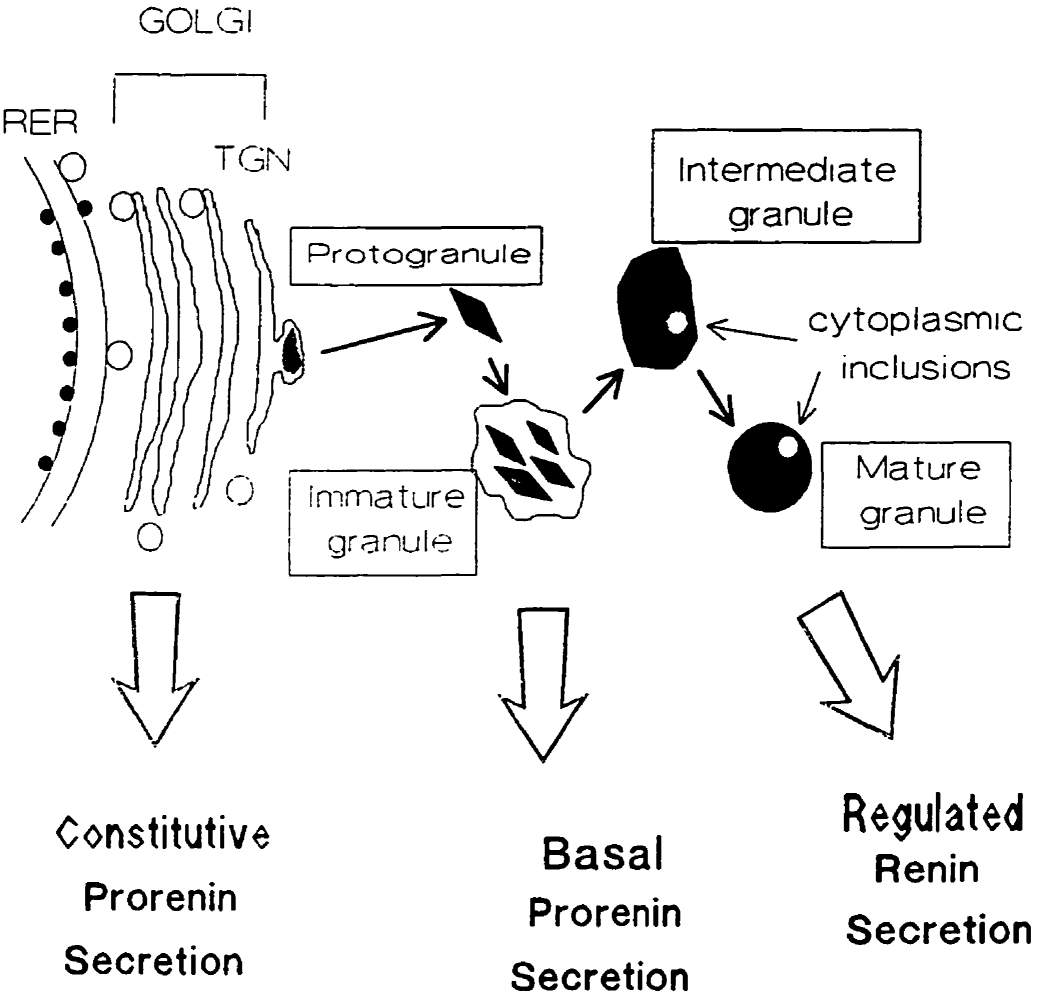


Figure 5: Prorenin and renin secretion in the JG cells. RER, rough endoplasmic reticulum; TGN, trans-golgi network.

(Figure 4). The maturation enzyme responsible for this cleavage is currently under investigation. Suggested candidates are the protein convertase (PC) family, namely PC1(also known as PC3) [25] and PC5 [26]. All have been detected in neuroendocrine and endocrine cells. The ubiquitous lysosomal protease, Cathepsin B, is also a suspect candidate [27].

1.4. Existence of a local RAS?

The idea of a local RAS emerged as a number of the components of the system as well as their corresponding mRNAs were discovered in the peripheral tissues. It has long been accepted that the circulating RAS is responsible for assuring immediate hemodynamic stability by controlling blood pressure and blood volume. In contrast, it is hypothesized that the local tissue RAS (tRAS) may be implicated in the maintenance of long term hemodynamic equilibrium through local generation of AII which can in turn affect surrounding tissues or cells in an autocrine or paracrine manner (Figure 6). For example, the tissue system may be involved in mediating structural changes that can influence vascular tone and cardiac contractility and mass and hence may be implicated in the development of diseases such as hypertension, cardiac hypertrophy, coronary artery disease and atherosclerosis. It is important to pursue further studies into this area in order to better characterize the function and mechanism by which these tRAS maintain overall general homeostasis and moreover, its potential contribution to pathological states.

1.4.1. Evidence of a local RAS

As mentioned previously, mRNA encoding the proteins involved in the RAS have been localized in various tissues including in the kidney, adrenals, pituitary, heart, vasculature and reproductive organs, suggesting a distinct and separate system from the circulating RAS. For example, while the bulk of the systemic angiotensinogen is derived from the liver, mRNA has been detected in various other tissues and can

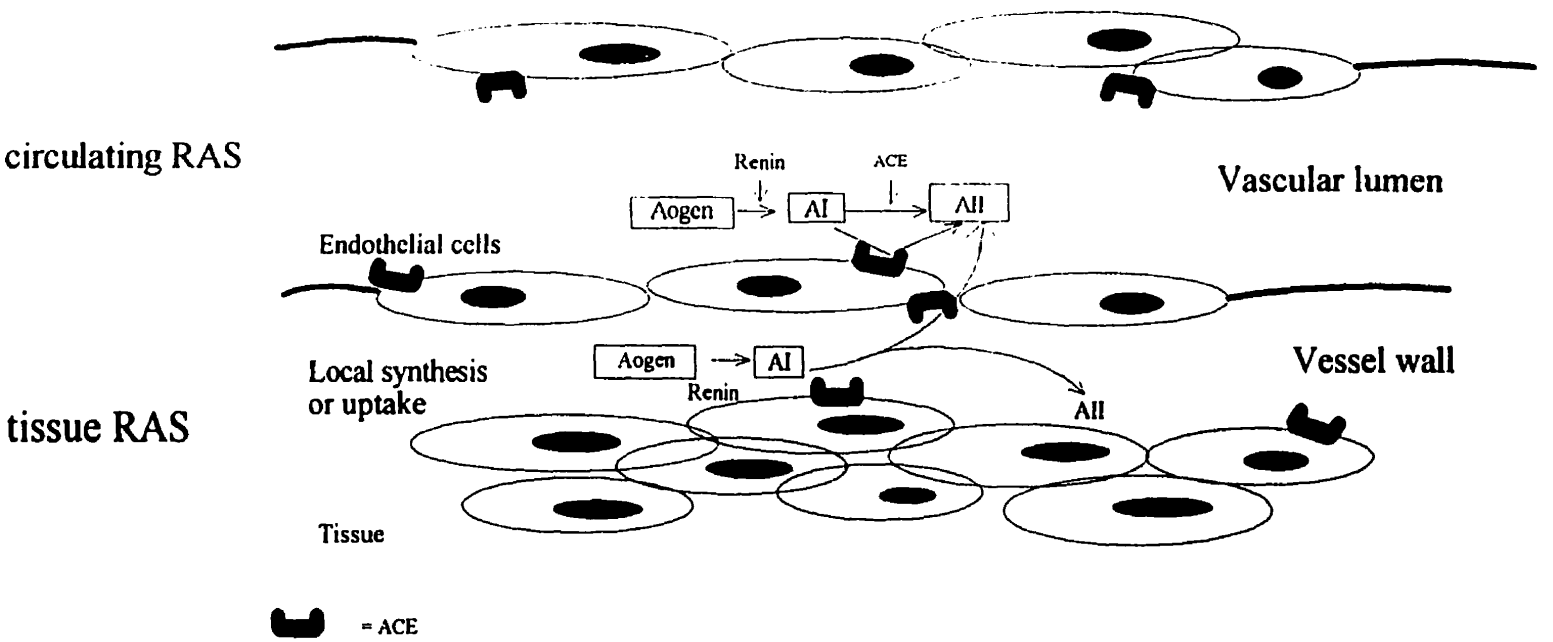


Figure 6: The relationship between the circulating and the tissue RAS.

therefore participate in local AII production. In addition, ACE can be found in many tissues throughout the body, anchored to endothelial membranes in the lung, kidney, gastrointestinal tract and reproductive tract, choroid plexus and the placenta. Use of ACE inhibitors in the treatment of hypertension and congestive heart failure suggests that its effectiveness is due to suppression of local systems rather than the systemic RAS. In the spontaneously hypertensive rat (SHR), a renin-independent model, ACE inhibitors are capable of reducing blood pressure [28] and in other studies, chronic ACE inhibitor therapy leads to a return to normal circulating AII levels, however, lower blood pressures are maintained [29]. Finally, Unger et al. [30] as well as several other groups have demonstrated that the blood pressure lowering effect of inhibitors is in better agreement with inhibition of tissue ACE rather than plasma ACE. In one such study, SHR were chronically treated with ACE inhibitors and upon withdrawal, blood pressure remained lowered for a few days, correlating with an inhibition of tissue ACE in the aorta, mesenteric vasculature and the kidneys.

1.4.2. The kidney RAS

The idea of a tRAS was first proposed in the kidney where the mRNA encoding all the proteins involved in the RAS were localized. It is postulated that a kidney RAS may function in the regulation of single nephron glomerular filtration rate, tubular function, glomerulotubular feedback, and intrarenal regional blood flow [31]. By in situ hybridization, renin mRNA was detected primarily in the JG cells [32], angiotensinogen in the proximal renal tubule [33] and ACE on the brush border facing the tubule lumen and on the basolateral membrane using immunohistochemistry [34]. Transport of filtered renin into the proximal tubules may initiate the cascade of angiotensin formation through renin interaction with local angiotensinogen. The resulting AI could be cleaved by local ACE to generate AII which could then activate proximal tubular AII receptors. Indeed, sodium transfer in the proximal tubules is increased by AII through a mechanism involving stimulation of Na^+/H^+ antiporter via G-protein-coupled

decrease in intracellular cAMP [35]. Hence this finding suggests the existence of a functional paracrine RAS in the proximal tubules of the kidney.

1.4.3. The cardiac RAS

The concept of a cardiac RAS has been developed since the mRNA encoding all the components of the RAS was detected in this tissue. As mentioned previously, the benefits of ACE inhibitors in the treatment of chronic congestive heart failure as well as acute myocardial ischemia implies involvement of a cardiac RAS in cardiovascular diseases. Arguably, the usefulness of these drugs may not be entirely due to the inhibition of the RAS but may be partly due to the blockade of the bradykinin/nitric oxide synthase (NOS) system. As a working hypothesis, bradykinin would upregulate endothelial NOS activity, an enzyme which cleaves arginine into NO and citrulline. Increased NO levels would stimulate soluble guanylate cyclase (GC) and the corresponding increase in cGMP levels would mediate vascular relaxation. Since ACE is able to metabolize bradykinin, increased ACE levels and activity would lower endothelial NO availability thereby preventing vasorelaxation and potentiating AII vasoconstrictive activity. Preliminary *in vitro* results have failed to show a correlation with bradykinin and upregulation of NOS in cultured endothelial cells. Nonetheless, it has been clearly demonstrated that increased ACE activity causes a decrease in endothelial NO availability and whether bradykinin is responsible for this effect has yet to be confirmed.

Furthermore, locally generated AII in the heart may behave as a growth factor, as several groups have reported stimulation of nuclear proto-oncogenes such as c-*fos* and c-*myc* via the protein kinase C (PKC) pathway as well as increased expression of growth factors such as platelet derived growth factor (PDGF) and fibroblast growth factor (FGF) [36]. In addition, when cultured smooth muscle cells were incubated with AII, an increase in RNA and protein synthesis was observed [37]. In *in vivo* experiments, rats with severe cardiac hypertrophy were treated with levels of ACE

inhibitors insufficient to cause hypotension, however, the drugs were responsible for a regression in cardiac hypertrophy through a reduction in cardiac mass [38].

1.4.4. The vascular tissue RAS

Finally, the vasculature is also a major area where the tRAS is active. Renin, angiotensinogen and ACE have been detected in the vasculature using immunohistochemical and biochemical studies. Acute effects mediated by locally synthesized AII include arteriolar contraction and facilitation of sympathetic nervous transmission whereas long term effects include growth and mitogenesis of surrounding cells. It has been shown that AII increased mRNA levels of c-*fos*, c-*myc* and PDGF in smooth muscle cells [39]. In addition, local generation of AII may be involved in angiogenesis. In studies conducted in the chick embryo, Le Noble [40] was able to show an AII-stimulated microvascular growth.

1.5. Maturation of prorenin into renin occurs exclusively in the JG cells of the kidney

Extra-renal mRNA for renin has also been detected in various tissues including the adrenals, reproductive organs and the pituitary. However, the extent to which these extra-renal tissues are able to process the inactive enzyme prorenin into the active form, renin, is questionable. There are several pieces of evidence that suggest that the source of circulating active renin is derived from the kidneys. For example, in humans, circulating renin levels become undetectable within two days after nephrectomy [41]. In contrast, prorenin levels drop considerably, but can still be detected years later [42]. Furthermore, results from experiments performed on isolated rat hind limbs indicate generation of angiotensin peptides [45]. However, if nephrectomy is performed prior to limb isolation and perfusion, no angiotensin peptides are generated. The data from these experiments strongly suggest that the kidney secretes prorenin as well as the bulk of the circulating active renin. Non-renal sources hence secrete predominantly the

inactive precursor, prorenin. Moreover, there is no evidence that prorenin is ever converted to renin in the circulation. To demonstrate this point, large amounts of recombinant human prorenin were injected into monkeys, where no increases in blood pressure or AII were observed [49]. The overall activity of the RAS is therefore related to the amount of active renin that is processed in the kidney and secreted into the circulation.

1.6. The beginnings of a theory for renin uptake by tissues

The absence of local synthesis of mature renin does not refute the idea of a local RAS. Instead, it fosters the notion of renin uptake by tissues from the circulation where it can act upon angiotensinogen to contribute to local angiotensin formation. This stems from the findings that the circulating RAS cannot possibly account for the total production of plasma AI and that therefore, a major part of it must be generated in the periphery [43]. Furthermore, Admiraal's group showed that local renin levels may reach concentrations higher than that of plasma renin. It is with this reasoning that this study was conducted such that we could address the question of uptake more directly and examine the existence of a renin binding site in human tissues.

1.6.1. Evidence of renin uptake

1.6.1.1. Perfused rat hindlimb studies

Since the early 1980's, several groups have demonstrated the likelihood of renin uptake in the vasculature by perfusion studies conducted on isolated rat hindlimbs. The original studies conducted by Oliver and Sciacca [44] showed that the synthetic renin substrate, tetradecapeptide (TDP) could cause vasoconstriction in isolated rat hindlimbs. These preliminary results implied that renin in the vascular wall was responsible for the cleavage of the TDP and generation of the vasoactive compounds. However, subsequent studies showed that enzymes other than renin could also cleave

this synthetic substrate [45] and hence in the early 1990's, natural angiotensinogen, known to be metabolized solely by renin at physiological pH, was used in further perfusion studies. Hilgers et al. [46] injected purified rat angiotensinogen into isolated perfused rat hindquarters and observed a 14-fold increase in AII over controls. When the rats were nephrectomized 24 hours prior to isolation and preparation of the hindlimbs, AI and AII dropped to almost non-detectable levels after angiotensinogen infusion. These findings strongly suggested that the renin present in the vessel walls was of renal origin and had been taken up from the bloodstream where it could then act on the perfused angiotensinogen to release the angiotensin peptides.

1.6.1.2. Renin uptake in the heart

Since then, numerous studies have been able to show that renin may be taken up by tissues to participate in the local RAS. In 1994, Schalekamp's group measured cardiac and plasma renin levels from normal and bilaterally nephrectomized pigs [47]; the direct correlation of renin levels in cardiac tissue with that in the circulating plasma, along with the practically undetectable levels of renin in the heart and plasma 30 hours after nephrectomy, strongly support the notion of renin uptake from the circulation into the heart. Similarly, it has been documented that rat aortic tissue renin progressively declined after bilateral nephrectomy [48]. Also, in early experiments, uptake of renin in vascular tissues was demonstrated when radiolabelled renin was injected into monkeys. Radioactivity accumulated in the liver and kidney (thought to represent clearance), with smaller amounts present in the heart and blood vessels [49]. In another study conducted by Swales et al. [48] mouse renin was injected into rats and detected in the aorta and intrasplenic arteries by immunofluorescent studies. In a similar experiment, hog renin was administered to hypertensive rabbits for several days and uptake occurred in the aorta, carotid arteries and the kidney [50].

1.7. Insights into the mechanism of renin uptake

1.7.1. Identification of 2 renin binding proteins in rat tissues by cross-linking experiments

In studies aimed at further addressing the mechanism of renin uptake, Danser et al. found renin in purified membrane fractions, suggesting that renin could be a membrane-bound enzyme [47]. In agreement with this idea, Campbell et al. [51] identified 2 vascular renin-binding proteins in rat tissues using ^{125}I-labelled renin. Binding was examined by cross-linking the labeled protein to the membranes and analysis by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. After accounting for the molecular weight of renin, the binding proteins had a molecular mass of 70 kDa and 40 kDa. The distribution was varied, with largest amounts present in the mesenteric artery and lower levels reported in the aorta, lung and renal medulla. The kidney cortex, heart, adrenal capsule, adrenal medulla, peri-aortic brown adipose tissue, uterus and pituitary did not contain these renin binding proteins. The 2 proteins identified did not seem to be covalently linked through disulfide bonds, however, the possibility of non-covalent interactions in the membrane could not be ruled out. Interestingly, renin enzymatic inhibitors were able to block renin binding to mesenteric arteries suggesting that occupation of the active site might induce unfavorable conformational changes within the protein, thereby abolishing recognition and association with the membrane proteins. It is possible that the mechanism by which renin inhibitors are effective *in vivo* may be by preventing renin binding to the vasculature and thus limiting the local formation of angiotensin peptides. Angiotensinogen, which also binds to the renin active site, prevented renin binding to the membrane preparations as well. Because membrane- associated renin was still able to generate AI from angiotensinogen, Campbell's group argued that the binding of renin did not depend on its active site, nor on the degree of renin deglycosylation, as rat renin and mouse submandibular (SMG) renin both had similar membrane binding affinities. (Mouse SMG renin does not contain potential

glycosylation sites whereas rat renin has three consensus sequences for N-glycosylation). No studies were performed with prorenin, so it is unknown as to whether prorenin binds to the same site.

1.7.2. Identification of a renin and prorenin binding site in rat tissues through binding studies

In similar efforts to identify renin and prorenin binding sites in rat tissues, Sealey et al. used ^{35}S methionine-labelled recombinant rat prorenin as a probe in binding studies [52]. Renin and prorenin bound to the same site, with prorenin having a slightly higher binding affinity than renin (200 versus 900 picomolar respectively). Binding capacity was greatest in the renal cortex (where Campbell found no binding), followed by the liver, testes, lung, brain, renal medulla, adrenal, aorta, heart and skeletal muscle. The discovery that rat tissues have different binding capacities may reflect the extent to which the tRAS is active. The fact that prorenin effectively competes with renin for the same binding site without contributing to peripheral angiotensin formation suggests that its role may be to antagonize the local RAS by preventing or limiting renin uptake. Under this proposal, prorenin uptake would not affect the circulating renin levels and the systemic RAS. This hypothesis would be in agreement with the reported vasodilated state and the high levels of prorenin found in pregnancy and diabetes mellitus [53]. Furthermore, Sealey's group argues that the tissue distribution for renin binding sites agrees with predicted prorenin function. For example, high prorenin levels are associated with increase blood flow to the kidneys and reproductive organs and hence, as expected, prorenin and renin binding was high in the kidneys and testes. In addition, studies involving prorenin infusion had only a small blood pressure lowering effect [38], suggesting low prorenin and renin binding in the resistance vessels. Consistent with this idea is the minimal binding observed in the aorta and skeletal muscle.

The difference observed in tissue distribution of binding proteins between Campbell and Sealey's group may be that two different renin binding proteins were being studied. Alternatively, the different experimental methods employed may be responsible for the variation in the results obtained. Campbell's group used extrinsically labeled renin which may have had adverse effects on protein structure, whereas Sealey's group used intrinsically labeled prorenin, thereby minimizing alteration of protein conformation. Furthermore, binding experiments were performed at 37°C for Campbell's group. At these elevated temperatures, Sealey's group found high levels of nonspecific binding. Also, Campbell's group lacked sufficient amounts of unlabelled rat renin necessary for effective competition in assessing non-specific binding and were therefore only able to show saturation binding indirectly. Finally, Sealey's group removed endogenous renin from the membrane preparations prior to binding experiments, whereas Campbell's group did not; endogenous renin in the Campbell study may have prevented radiolabelled renin binding, hence possibly accounting for the lower binding observed in tissues where Sealey's group found high levels of binding.

1.8. The basis for our study of renin uptake in the human placenta

The exciting possibility of a specific prorenin and renin binding site involved in directing a localized RAS led our group to examine if such a site existed in human tissues. We thereby began our search in the human placenta through a collaboration with Dr. Jean St. Louis and Dr. Yan Ping Shi at the Hôpital Ste. Justine. Our results have indeed demonstrated the presence of a high affinity, saturable binding site for renin and prorenin in these tissues. Furthermore, we have shown that neither the carbohydrate residues nor the active site of renin is essential in the uptake process. Future experiments will involve identifying and characterizing the binding site with possible long term goals of blocking the sites for potential treatment of hypertension and related pathologies involving the RAS.

2. Materials and Methods

2.1. Plasmid constructions for placental perfusion studies and membrane binding experiments

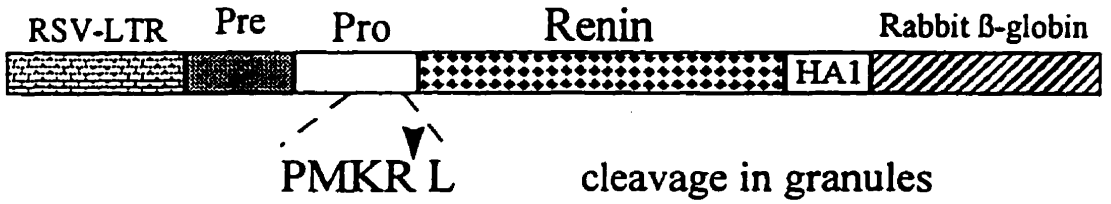
2.1.1. Plasmid construction of renin and prorenin

In previous studies, the cDNA for human renin was cloned into the pRHR plasmid under transcriptional control of the Rous sarcoma virus long terminal repeat (RSV-LTR) (Figure 7). In addition to native prorenin, a similar plasmid was used to encode a cleavage-modified prorenin (Figure 7, furin-cleaved prorenin). In this expression vector, the native cleavage site was mutated such that in transfected GH_4Cl cells (a rat somatomammotrophic cell line devoid of secretory granules), proteolytic removal of the prosegment (resulting in active renin) can occur in the endoplasmic reticulum or golgi apparatus by the ubiquitous enzyme, furin. This allows for a high level, constitutive secretion of active renin from transfected cells. From here on, this furin-cleaved prorenin will be referred to simply as renin.

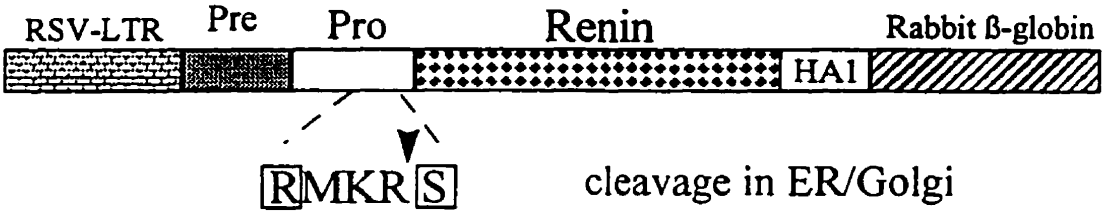
2.1.2. Construction of renin active site mutants:

One of the aspartates (Asp +38 or Asp +226) in the active site of human renin was mutated to asparagine (Asn) to render the enzyme inactive. The Asp +38/Asn mutation was accomplished using site-directed mutagenesis with the forward oligonucleotide 5' GTCGTCTTTAACACTGGTTC 3' (oligo 466) and the reverse oligonucleotide 5' GAACCAGTGTTAAAGACGAC 3' (oligo 467). The forward oligonucleotide 5' GCA TTG GTA AAC ACC GGT GC 3' (oligo 468) and the reverse oligonucleotide 5' GC ACC GGT GTT TAC CAA TGC 3' (oligo 469) was used for the construction of the Asp+226/Asn mutation. Polymerase chain reactions (PCR) were performed on a ERICOMP INC Easy Cycler™ series machine using the

Prorenin



Furin-cleaved prorenin (active renin)



Deglycosylated renin

4	5	6	7	74	75	76	77	
...Gly	Asn	Thr	Thr	.....His	Asn	Gly	Thr...	Native
GGC	AAC	ACC	ACC	CAC	AAT	GGA	ACA	
... Gly	*Ser*	Thr	Thr...					Asn +5 → Ser
GGC	*TCC*	ACC	ACC					
				...His	*Ser*	Gly	Thr...	Asn +75 → Ser
				CAC	*TCC*	GGA	ACA	

Renin active site mutant

36	37	38	39	40		224	225	226	227	228	
... Val	Phe	Asp	Thr	Gly...	Native	...Leu	Val	Asp	Thr	Gly...	
GTC	TTT	GAC	ACT	GGT		TTG	GTA	GAC	ACC	GGT	
...Val	Phe	*Asn*	Thr	Gly...		...Leu	Val	*Asn*	Thr	Gly...	
GTC	TTT	*AAC*	ACT	GGT		TTG	GTA	*AAC*	ACC	GGT	
Asp+38 → Asn						Asp +226 → Asn					

Figure 7: Plasmid constructions for prorenin, furin-cleaved prorenin (active renin), deglycosylated prorenin and renin active site mutants.

following conditions: Step 1 (1X): (3 min, 94°C), Step 2 (24X): (30 sec, 94°C; 1 min, 50°C; 2 min, 72°C), Step 3 (1X): (10 min, 72°C). The two pieces of DNA were put together using overlap extension PCR with oligos 28 and 322 (see below) and the same conditions as given above. PCR amplified products were run on a 0.8% agarose gel and DNA bands were obtained by Gene Clean II kit (Bio/Can Scientific), followed by a 37°C overnight digestion with Hind III and BamH1. Samples were precipitated and quantified on gel before ligation in RSV-LTR/ß-globin-pRHR vector at 16°C overnight. Competent XL1-Blue cells were transformed and clones were amplified in 5 ml of LB containing 100 µg/ml ampicillin, minipreps performed and plasmid DNA digested with Hind III and BamH1. Positive clones were subject to sequencing by dideoxy chain termination method to verify that the site-directed mutagenesis had been successful.

2.1.3. Construction of deglycosylated prorenin

Deglycosylated prorenin was previously constructed using site-directed mutagenesis. Briefly, the two consensus sequences for N-linked glycosylation, asparagine (Asn)+5 and Asn +75 were mutated to serine (Ser) residues such that glycosylation was no longer possible. The forward oligonucleotide used for the Asn+5/Ser mutation was 5' CAGTTGGCTCCACCACCTCC 3' and that used for the Asn+75/Ser mutation was 5' CTACAAGCACTCCGGAACAGAA 3'. The PCR amplified products were then cloned into the RSV-LTR/ß-globin-pRHR vector and sequenced as described above.

2.1.4. Antigen Tagging of human prorenin, deglycosylated prorenin, renin and active site mutated renin

A DNA cassette encoding an epitope of the hemagglutinin (HA1) protein of the influenza virus (YPYDVPDYA) recognized by a commercially available monoclonal antibody (mouse IgG_{2b} 12CA5, Boehringer Mannheim) was inserted at the carboxy terminus of human prorenin, deglycosylated prorenin, renin and active site mutated

renin (see above) by overlap extension PCR. All constructs were PCR amplified from RSV-LTR/ß-globin-pRHR vector (Figure 8) using the forward primer 5' CGCCATTTGACCATTCAC 3' (oligo 28), containing a unique Hind III site and the reverse oligonucleotide 5' CGGGATCCTAGGCGTAGTCGGGCACGTCGTAGGGGTAGCGGGCCAAGGCG AA 3' (oligo 322) which contains a unique BamH1 site, HA1 epitope and stop codon. PCR conditions were as described above except that the annealing temperature for the oligonucleotides was decreased to 48°C. The PCR amplified products were then cloned into the RSV-LTR/ß-globin-pRHR vector as described above. Positive clones were sequenced by dideoxy chain termination method using the 3' oligonucleotide 5'AAAGAACAATCAAGGGTC 3' (oligo 27) present in the ß-globin portion of the expression vector to verify the HA1 tag.

2.2. Preparing stable cell lines expressing the HA1-tagged proteins

GH_4C1 cells (from a rat somatomammotrophic cell line) were maintained in monolayer culture in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (FCS), 0.1% SerXtend (Irvine Scientific, CA) and 10 µg/ml Gentamycin (complete media) at 37°C with 5% CO_2, 95% air. Cells were stably transfected with the plasmid DNA and a neomycin-resistant plasmid. Briefly, $2X10^6$ cells were seeded in 10 cm plates one day before transfection and the media changed 4 hours prior to transfection the following day. A transfection cocktail was prepared containing 20 µg of plasmid DNA, 2 µg of the neomycin-resistant plasmid and 0.5 ml of 250 mM $CaCl_2$. The cocktail was slowly added over a period of 1 minute to 0.5 ml of 2X HBS (270 mM NaCl, 10 mM KCl, 2.7 mM $Na_2HPO_4 \cdot 7H_2O$, 5.6 mM dextrose, 21 mM HEPES) pH 7.05. This solution was being constantly agitated by bubbling compressed air through it using a sterile plugged 2 ml pipette. The precipitate was allowed to stand for 30 minutes at room temperature and then distributed evenly to the cells. Twenty-four hours later, the media was aspirated and replaced with 5 ml of DMEM (without serum) containing 5% glycerol for 4 minutes. The cells were then washed once with

DMEM (complete media) and allowed to grow overnight in this media. For the next 5 days, cells were selected with 0.8 mg/ml G418 in complete media. Cells were then trypsinized, centrifuged for 5 minutes at 500 rpm, re-plated, and maintained in 0.2 mg/ml G418 Sulfate (Gibco). Cells were allowed to grow to confluency and supernatant was collected every 24 to 48 hours. Supernatants were cleared by centrifugation for 5 minutes at 1000 rpm and a radioimmunoassay (RIA) was performed to determine renin activity (see below). Aliquots were frozen at -20°C until required for perfusion studies and binding experiments (see below).

2.3. Radioimmunoassay for prorenin, deglycosylated prorenin, renin and active site mutated renin activity in the supernatants of stably transfected GH_4C1 cells

Enzymatic activity in tissue culture supernatants were determined by the Angiotensin I generation assay (DuPont) (Figure 8). Samples (40 µl) were treated with trypsin (3 mg/ml in 5X trypsin activation buffer (TAB) (250 mM Tris pH 8, 0.5 M NaCl, 15 mM EDTA, 50 mM Benzamidine HCl, 2.5% BSA) for total renin content or without for active renin content for 1 hour at room temperature. A mixture containing renin substrate and converting enzyme inhibitors (to prevent conversion of AI to AII by any endogenous ACE that may be present in samples such as membrane preparations) and angiotensinase inhibitors (to avoid degradation of AI) was added to the samples (0.49 µl 100 mM PMSF in isopropanol, 49 µl maleate buffer pH 6, 0.49 µl dimercaprol, 0.49 µl 8-Hydroxyquinoline solution and 5 µl of semipurified sheep angiotensinogen) and incubated for 1 hour at 37°C. Fifty µl of ^{125}I-AI (approximately 360 cpm/µl) and 50 µl of AI rabbit antiserum were added and a competition was set up for primary antibody binding between the hot and cold AI. After a 90 minute incubation at room temperature, the antigen-antibody complexes were precipitated using 250 µl of anti-rabbit serum and the reaction allowed to proceed for 30 minutes. Tubes were centrifuged at 3000 rpm for 10 minutes in an IEC Centra-8R centrifuge. The supernatants were aspirated and the pellets counted on an LKB-Wallac CliniGamma 1272 automatic gamma counter. Counts were compared to a standard AI curve.

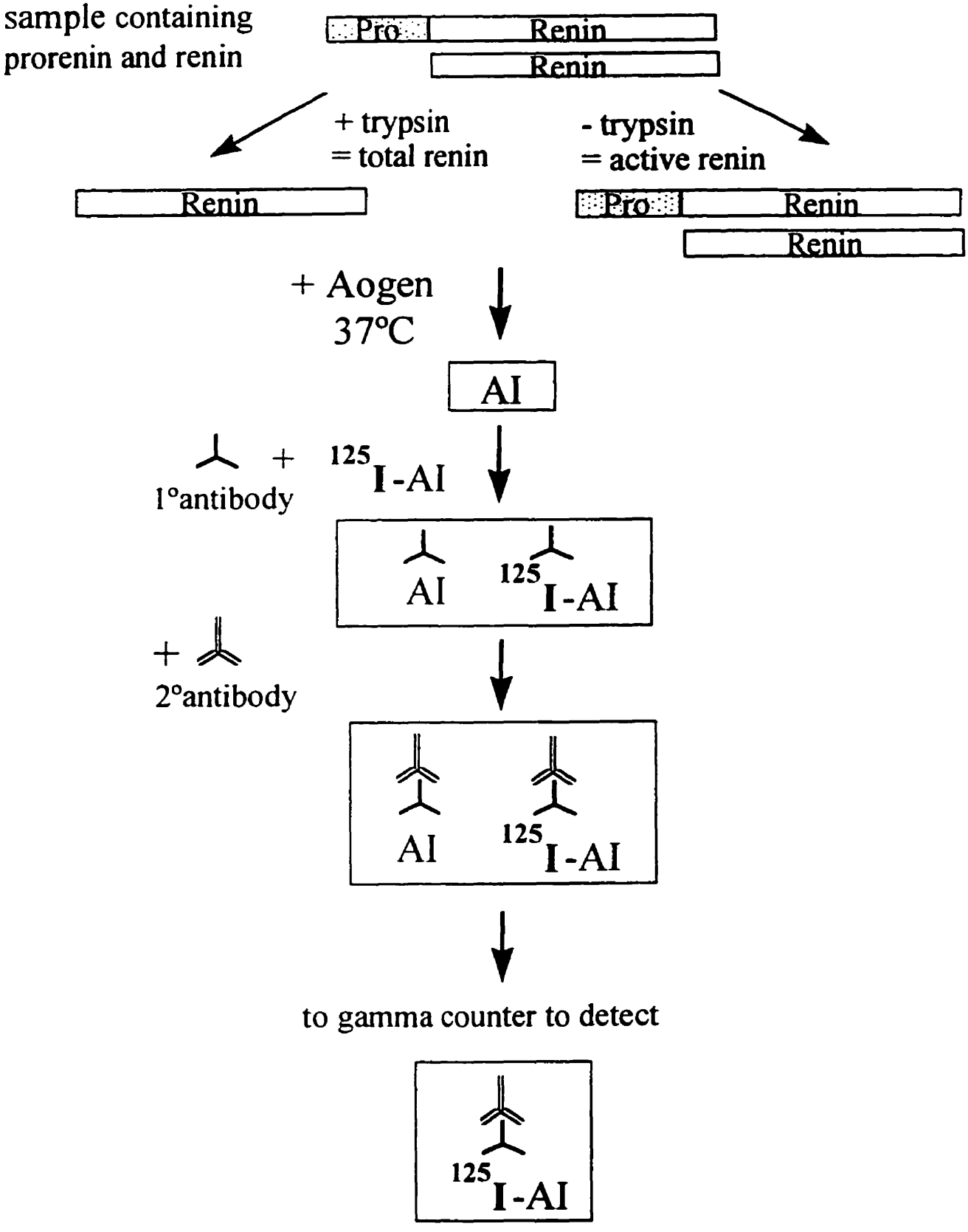


Figure 8: RIA protocol for AI determination. Treatment of samples with trypsin gives the total renin content. Treatment without trypsin gives the active renin content. Prorenin = total renin-active renin.

Prorenin was calculated by subtracting active renin from the total renin content. Renin and prorenin activities were expressed as nanograms of AI produced per milliliter per hour (ng AI/ml/hr).

2.4. Western blotting to quantitate amounts of prorenin, deglycosylated prorenin, renin and renin active site mutants in the supernatants of stably transfected GH_4Cl cells

Concentration of prorenin, deglycosylated prorenin, renin and active site mutated renin in the supernatants of stably transfected GH_4Cl cells were quantified as follows. Briefly, known amounts (2-8 ng) of purified recombinant human renin (a generous gift from Daniel Lamarre, Bio-mega), were fractionated by SDS-PAGE (12% polyacrylamide gel) along with 10 µl of culture supernatant containing the renin variants and electrophoresed at 200 volts for 40 minutes. Proteins were transferred onto a 0.2 µm supported nitrocellulose membrane (Bio-Rad) by running at 100 volts for 1 hour. The blot was then washed in TBS-T (50 mM Tris, 150 mM NaCl, 0.05% Tween) for 10 minutes at room temperature and subject to blocking with 5% Carnation instant skim milk powder in TBS (50 mM Tris, 150 mM NaCl) pH 7.4 in a rotating hybridization oven. The blots were then quickly rinsed twice with TBS-T and washed one time with TBS-T for 15 minutes in a shaker at 65 rpm. The TBS-T was replaced with fresh buffer and the blot was subject to 2 further washes for 5 minutes each. The blot was then incubated with a polyclonal anti-renin antibody (Babco lot 25) at a 1:600 dilution and placed in a rotating apparatus at 4°C overnight. The following day, the washes were repeated and the blot probed with an anti-rabbit serum conjugated to horse-radish peroxidase (made from donkey) for 45 minutes at room temperature. The washes were repeated, with two additional 5 minute washes and the reaction detected by placing the blot in a solution containing equal volumes of luminol and stable peroxide buffer (PIERCE) for 1 minute. The blot was rinsed briefly in TBS-T, wrapped in cellophane and exposed to film for 1 second to 2 minutes, depending on the intensity of the reaction. Protein amounts in the supernatant were

determined by densitometry of exposed X-ray films using a standard curve fitting program (IS-1000 version 2.00 from the Alpha Innotech Corporation).

2.5. Placental perfusion studies

2.5.1. Cotyledon Isolation and Perfusion

Human placentas from normotensive term pregnancies (38-40 weeks) were obtained from the Hôpital Ste. Justine and used in these experiments. The placentas were obtained within 15 minutes after vaginal or cesarian section deliveries and immediately transported on ice to the laboratory. Two cotyledons roughly 4 to 6 cm in diameter with the decidual plate intact were chosen and the chorionic artery and vein were cannulated using polyethylene catheters (PE-90, Clay Adams). The artery was perfused with heparin (1000 i.u./ml) at a flow rate of 0.1 ml/min using a pump Model 2400-006 (Harvard Apparatus). The perfusate was allowed to flow freely out of the corresponding cannulated chorionic vein. The two cotyledons were then dissected from the placenta and placed in separate glass jacketed perfusion baths maintained at 37°C. The arterial cannula was connected to an in-line perfusion circuit where the placenta was perfused with Krebs-Hanseleit solution (pre-oxygenated with 95% O_2, 5% CO_2, pH 7.4, 37°C) at a rate of 2 ml/min using a Gilson Mini Pulse 2 peristaltic pump (Mandel Scientific Co.) for one hour (Figure 9). Previous experiments had demonstrated that 98% of the perfusate was recuperated from the venous return (J. St. Louis, personal communication). A Gould P23 pressure transducer linked to a chart recorder was connected to a side arm of the inflow cannula to record the perfusion pressure. Basal perfusion pressure was 21.0 ± 0.20 mm Hg. The cotyledons were then perfused with DMEM for 10 minutes. In the experimental cotyledon, fifty ml of culture supernatant from GH_4Cl cells secreting active human renin (activity approximately 5000 ng AI/ml/hr) in complete DMEM was perfused at a flow rate of 2 ml/min followed by a 100 ml wash with complete DMEM devoid of human renin. Next, 50 ml of a solution of semipurified sheep angiotensinogen at a concentration of

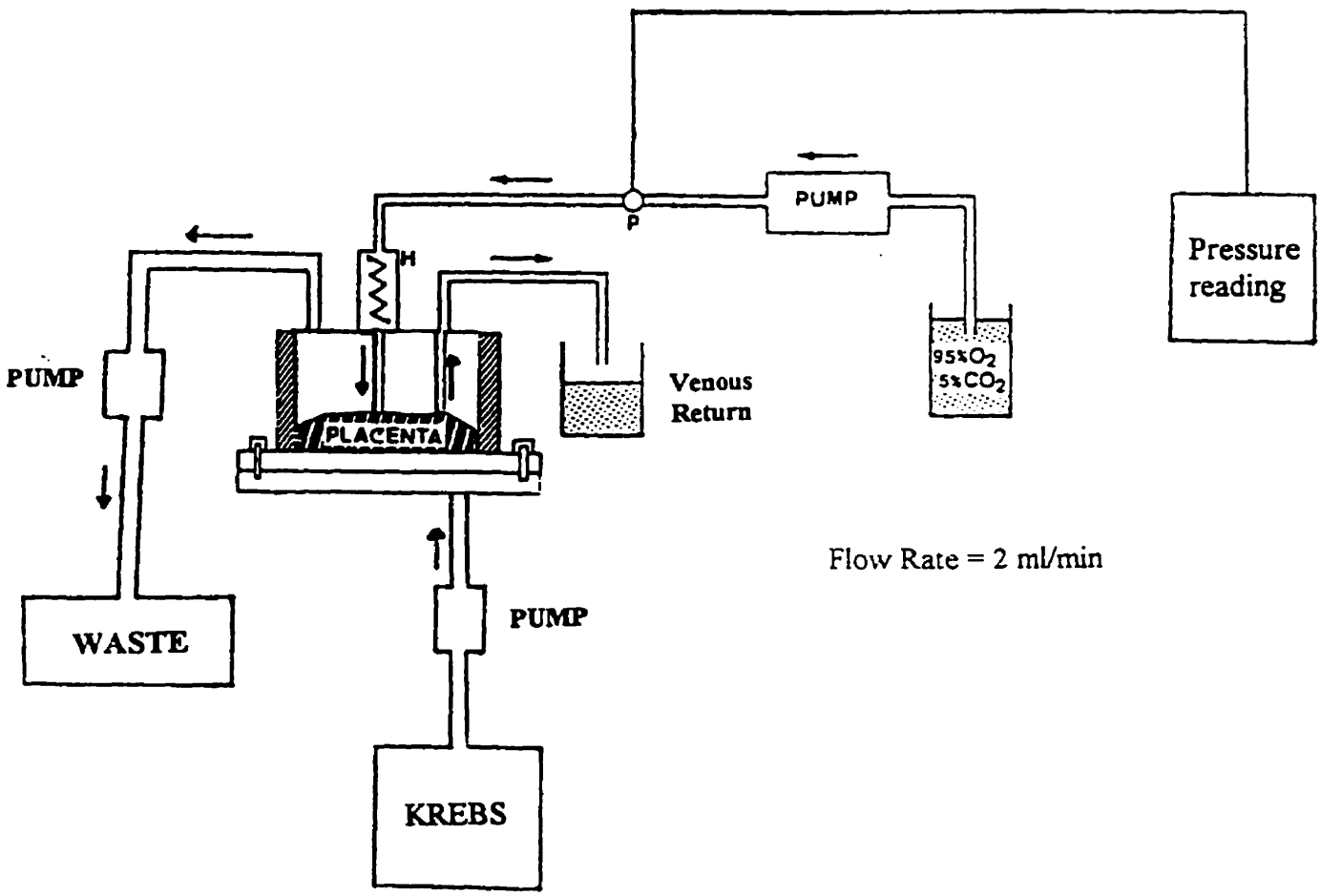


Figure 9: System for cotyledon perfusion. There are two systems running in parallel; only one is illustrated. H, heater; P, pressure recorder.

roughly 1μM in complete DMEM was injected followed by a final wash with 20 ml of complete DMEM devoid of human renin. RIAs were performed on venous returns for analysis of renin activity (40 μl) and quantitation of the angiotensins produced (400μl). Samples assayed for AI and AII were collected in 4% glacial acetic acid to avoid peptide degradation. A cotyledon perfused with complete DMEM devoid of human renin was used as a control where all other perfusion steps were the same. Solutions were oxygenated and prewarmed to 37°C prior to the perfusion step. This perfusion study was repeated two times on different days and the cotyledons were obtained from two different placentas.

2.5.2. Treatment of venous return samples for determination of AI and AII content

Five ml fractions obtained from the venous return were collected in tubes containing 200 μl of glacial acetic acid (final concentration of 4% acetic acid) and 400 μl samples lyophilized overnight in a Speed Vac Concentrator (Savant). Samples were then resuspended in 1ml of 100mM Tris pH 8.0.

2.5.2.1. RIA for AI

For determination of AI content, a similar RIA was used as described above for renin except that the incubation with angiotensinogen was omitted. All other steps were the same.

2.5.2.2. RIA for AII

AII content was determined by addition of 100 μl of I^{125}AII (80 cpm/μl) and 200 μl of an antiserum against AII (designated CD4) at a 1:200 000 dilution added to a 250 μl aliquot of sample and incubation at 4°C overnight. The next day, 1 ml of charcoal-dextran was added, the tubes vortexed and then centrifuged for 20 minutes at 3000 rpm. The charcoal was discarded and supernatants counted on an LKB-Wallac

CliniGamma 1272 automatic gamma counter and results compared to a standard AII curve.

2.6. Immunohistochemical studies

2.6.1. Immunohistochemistry on GH_4Cl cells transfected with HA1-tagged renin

GH_4Cl cells were plated at a density of $2X10^6$ cells per 10 cm plate and transfected transiently as described above with HA1-tagged renin. Cells were then rinsed with TBS pH 7.4 and fixed with 4% formaldehyde for 10 minutes at room temperature. After another rinse with TBS, the cell membranes were permeated with pre-chilled methanol for 10 minutes at room temperature and rinsed again with TBS. The cells were then pre-incubated for 30 minutes at room temperature with 1% donkey serum in TBS to reduce non-specific signals. The cells were incubated with 1 µg/ml of anti-HA1 primary antibody (Boehringer Mannheim) in 5% Carnation instant skim milk powder at 4°C overnight. The following day, cells were rinsed with TBS, and incubated with an anti-mouse IgG biotinylated from sheep (Amersham) at a 1:200 dilution for 1 hour at room temperature. Streptavidin-horseradish peroxidase (Amersham) was added at a 1:300 dilution for one hour at room temperature. Presence of the HA1 tag was revealed using diaminobenzidine (Sigma) and H_2O_2 as a chromogen. A drop of 90% glycerol in TBS was added and a coverslip placed over the cells and sealed with nailpolish.

2.6.2. Human renin perfusion and fixation in whole cotyledons for immunohistochemistry

Two cotyledons dissected from the same placenta were perfused as described above with heparin and Krebs-Hanseleit solution, followed by 50 ml of culture supernatant containing human active renin (activity approximately 5000 ng AI/ml/hr) in complete DMEM at a flow rate of 2ml/min. This was followed by a 60 ml wash with DMEM

devoid of human renin and a final perfusion with 15 ml of Bouin's fixative solution (75% picric acid, 25% formaldehyde, 5% glacial acetic acid) at the same flow rate. The tissues were dissected into small pieces (0.5 cm x 0.5 cm) and post-fixed in the Bouin's fixative solution at 4°C overnight.

2.6.3. Treatment of tissues for immunohistochemistry

Tissues were removed from the Bouin's solution and rinsed in 70% ethanol and dehydrated by placing them in graded alcohol baths (70% to 100% ethanol) followed by 3 separate baths of 100% xylene with stirring for 30 minutes. The tissues were then embedded in paraffin and cut into 5 micron thick slices and mounted on gelatin-coated slides according to standard protocols.

2.6.4. Immunohistochemistry on cotyledon tissues perfused with human renin

Slides of cotyledon tissues were returned to the aqueous phase by immersion in 100% xylene baths followed by solutions ranging from 100% ethanol to 50% ethanol for 5 minutes each. Slides were then rinsed for 10 minutes in running water and treated for elimination of endogenous peroxidase by immersion in a solution of 3% H_2O_2 in methanol for 30 minutes at room temperature with stirring. The slides were then rinsed with TBS and treated in a manner identical to the method used for immunohistochemistry on GH_4Cl transfected cells beginning with the blocking step with 1% donkey serum in TBS. Tissues were once again dehydrated, a drop of Permount (Fisher) added and a coverslip placed over the slides.

2.6.5. Immunofluorescence studies

Slides were treated in a manner identical to that described in section 2.6.4. except that the slides were incubated with a higher concentration of anti-HA1 primary antibody (5 µg/ml instead of 1 µg/ml) in TBS containing 5% Carnation instant skim milk powder

at 4°C overnight. The following day, slides were rinsed with TBS and incubated with an anti-mouse IgG-FITC (Fluorescein isothiocyanate) from goat (Boehringer Mannheim) at a 1:100 dilution for 1 hour at room temperature. The slides were then rinsed with water, a drop of PED (phenylenediamine) /glycerol placed on the tissues, and mounted with a coverslip.

2.7. Membrane binding experiments

2.7.1. Cotyledon perfusion for membrane preparations

Human placentas were dissected into individual cotyledons and perfused with heparin and Krebs-Hanseleit solution as described above. The cotyledon was then removed from the apparatus and cut into small pieces (1cm x 1cm) and frozen in a solution of isopentane and dry ice that was maintained at -30°C to-40°C. After 1 minute, the frozen tissue was stored at -80°C until required for binding experiments (see below).

2.7.1.1. Cotyledon membrane preparations

Approximately 6 grams of placental tissue was allowed to thaw in 50 ml Falcon tubes and then minced with a razor blade. To eliminate most of the blood, 40 ml of buffer 4 (10mM Tris pH 7.5, 10mM $MgCl_2$, 1mM KCl, 0.1% EDTA and 0.3 M sucrose) was added to the tissue, the precipitate allowed to settle, and the supernatant discarded. This step was repeated several times until the supernatant was clear (approximately 3-4 times). The precipitate was then resuspended in 10 ml of buffer 4 and homogenized for 30 seconds at medium speed in the Polytron tissue homogenizer. The homogenate was then transferred to Oakridge tubes and completed to 3/4 full with buffer 4 and centrifuged at 5000 rpm for 10 minutes at 4°C. The supernatant was filtered through 3-4 layers of cheesecloth and kept on ice. Buffer 4 (10 ml) was added and the pellet rehomogenized and repelleted. The supernatant was filtered and combined to the supernatant from the previous step and the pellet discarded. The pooled supernatants

were centrifuged at 18 000 rpm for 30 minutes at 4°C. The supernatant was discarded and the resulting pellet resuspended in 20 ml of buffer 3 (same as buffer 4 but without sucrose) and rehomogenized, topped to 3/4 full with buffer 3 and repelleted. The pellet was then resuspended in 10 ml of 4M $MgCl_2$ and allowed to incubate for 1 hour on ice to remove endogenous renin. Buffer 3 (20 ml) was then added and the solution recentrifuged at 18 000 rpm for 30 minutes at 4°C. The supernatant was discarded and the pellet rehomogenized with 10 ml of buffer 3 and repelleted. The final pellet was resuspended in 1 ml of buffer 3 and the protein concentration determined using the Bradford protein assay.

2.7.2. Bradford protein assay to determine cotyledon membrane concentration

Placental membrane preparation (20 µl) was incubated with 30 µl H_2O and 50 µl of 1M NaOH for 5 minutes at room temperature. PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) pH 7.4 (700 µl) and 200 µl of Bio-Rad protein assay dye was added, the solution vortexed and allowed to sit for 10 minutes at room temperature. The absorbance was measured using a UV-Visible Recording Spectrophotometer (UV160U-Shimadzu) and compared against a protein standard curve of bovine thyroglobulin (Sigma) ranging from 0-30 µg.

2.7.3. Iodination of renin

Purified, recombinant human renin (Bio-mega) was iodinated as follows. 0.3 M $NaPO_4$ (pH 7.6) (15 µl), 1 mCi (40 µl) of I^{125}, and 5 µl of chloramine T (0.2 mg/ml in 300mM sodium orthophosphate pH 7.4) was allowed to incubate with 5 µg of renin (25 µl) for 10 seconds at room temperature. The reaction was stopped with 50 µl of tyrosine (1 mg/ml in TBS pH 7.4) and 200 µl of sodium iodide (2 mg/ml in TBS containing 1 mg/ml lysozym). Free iodine was separated from I^{125}renin by passing the mixture through a Sephadex G50 medium size exclusion resin, particle size 50-150µ (Pharmacia) . Fractions were collected every minute for 50 minutes on 100 µl of 1%

BSA in H_2O and 10 µl were counted on an LKB-Wallac CliniGamma 1272 automatic gamma counter. Fractions in the void volume containing the highest counts were pooled and used for binding experiments.

2.7.4. Binding experiments using radioactive renin

A saturation binding curve was constructed for ^{125}I-renin binding to placental membrane preparations. Briefly, 40 µg of membrane was incubated with increasing amounts of ^{125}I-renin ranging from 5000 to 2 million cpm either with (nonspecific binding) or without (total binding) saturating concentrations of cold renin for 1 hour at room temperature. Samples were then filtered onto a Schleicher & Schuell glass fiber filter paper #34 (pre-treated by incubation with 5% Carnation skim milk powder in buffer III, 30 minutes in a shaker at 65 rpm) using a 30 tube harvester (Brandel). Filters were then placed in clean tubes and counted on a LKB-Wallac CliniGamma 1272 automatic gamma counter. Specific binding was determined by subtracting nonspecific binding from total binding. The experiment was repeated 3 times.

2.7.5. Saturation binding curve experiments using renin collected from GH$_4$Cl stable cell lines

Membrane preparations (15 µg) were incubated at 4°C overnight (14-18 hours) with increasing concentrations of renin (2.3×10^{-11}M to 4.65×10^{-10}M) in a total volume of 150 µl. Nonspecific binding was determined using prorenin at a concentration of 8.85 nM. As discussed below, preliminary findings indicated that renin and prorenin bound with equal affinity to membrane preparations. Since prorenin cannot cleave Aogen into AI unless trypsin activated, membrane-bound prorenin is not detected by our RIA for AI. Any renin still bound to the membrane in the presence of excess prorenin would thus represent nonspecific binding. The following day, the samples were briefly centrifuged for 5 seconds, 13 000 rpm and 100 µl samples were loaded onto a Schleicher & Schuell glass fiber filter paper #34 (pre-treated as described above)

placed in a Bio-rad 96-well BioDot apparatus. The wells were washed 5 times with 250 µl of buffer III and once with 500 µl of buffer III. The filters were then cut with a razor blade and allowed to incubate at 37°C with 36 µl 5X TAB, 144 µl buffer III, 0.49µl PMSF, 49 µl Maleate buffer, 0.49 µl dimercaprol, 0.49 µl 8-Hydroxyquinoline solution and 5 µl of semipurified sheep angiotensinogen (total volume of 235 µl) for 1 hour. After the incubation period, 105 µl samples were placed into 2 separate tubes and the RIA for AI was carried out as usual (see above). These experiments were repeated on three different days on the same batch of cotyledon membrane preparations.

2.7.6. Competition binding experiments

Membrane preparations (15 µg) were incubated at 4°C for 14-18 hours with 3.5 nM renin and either deglycosylated prorenin or renin active site mutant (Asp +226/Asn) at concentrations ranging from $1x10^{-12}$M to $1x10^{-08}$M. The samples were treated in a manner identical to the saturation binding curve experiments for renin except that these samples were filtered using the Schleicher & Schuell Minifold II Slot-Blot system.

2.8. Preliminary studies for future transgenic mouse work

It is important to test whether the uptake of renin occurs *in vivo*. Transgenic mice expressing the HA1 tagged renin constructs under the control of a liver-specific promoter will be used to address this question. Different tissues can be collected from these animals and use of immunohistochemistry will allow us to see whether renin uptake has occurred.

2.8.1. Plasmid construction for expressing HA1-tagged renin and prorenin in transgenic mice

The cDNA for HA1-tagged renin and prorenin was cloned into a Bluescript plasmid under the control of the liver-specific promoter, transthyreitin (TTR). Briefly, the - 3 Kb to +20 sequences of the promoter for the TTR gene (a generous gift from Robert H. Costa, The University of Illinois at Chicago) was excised from the plasmid using Eco RI and Hind III enzyme restriction digestion and recloned in the Bluescript (BS) (S/K +) plasmid. The resulting plasmid was then cut with Hind III and the 5' phosphates removed using calf intestinal phosphatase (CIP). The inserts (HA1-tagged proteins) were excised from the RSV-LTR/ß-globin-vector using Hind III and Nde I (already blunt-ended). The vector and inserts were added together in approximately equal ratios, the ends repaired with DNA Polymerase I Klenow fragment (Life Sciences) and blunt-end ligated at 16°C overnight. Electrocompetent DH5α cells were electroporated as in standard protocols and positive clones selected by restriction enzyme digestion with Bgl II and Stu I and Kpn I to verify the correct DNA migration pattern.

2.8.2. Testing the specificity and activity of the transthyreitin promoter in HepG2 cells

HepG2 (derived from a human hepatocellular carcinoma, ATCC HB 8065) and GH_4Cl cells were transiently transfected with HA1-tagged renin and prorenin under transcriptional control of the TTR promoter using the CellPhect transfection kit (Pharmacia Biotech). Renin and prorenin activities were then compared between HepG2 and GH_4Cl cells to see if expression was limited to the liver cells.

2.8.3. Purification of inserts for transgenic mice

DNA plasmid (30 µg) was cut with the appropriate restriction enzymes to release the insert from the plasmid vector and electrophoresed on a 0.8% agarose gel at 25 volts overnight. Bands were isolated by NA-45 DEAE cellulose membranes (Schleicher & Schuell). Briefly, cellulose membranes were placed in front and behind the fragment of interest and electrophoresed for 10 minutes at 100 volts. The membrane containing the fragment was placed in an eppendorf and washed with a low salt solution (0.15 M NaCl, 0.1 mM EDTA, 20 mM Tris-HCl, ph 8.0). The wash was then discarded and the fragment eluded with 50µl of 3M high salt solution (3 M NaCl, 0.1 mM EDTA, 20 mM Tris-HCl, ph 8.0) at 55°C for 10 minutes. The solution was then filtered using 0.45 µm HV filters (Millipore), diluted to 1M salt with TE (10 mM Tris-HCl, 1 mM EDTA) pH 8.0, extracted once with phenol-chloroform and precipitated at 4°C overnight. The following day, the tube was centrifuged at 13 000 rpm for 15 minutes, the supernatant discarded and the pellet washed with 70% ethanol. The DNA was then lyophilized in a speed vac for 5 minutes and resuspended in 2.4 ml of TE containing 3.0 g of ultrapure CsCl (density of 1.70 ± 0.01 g/ml). The DNA was then centrifuged at 20°C for 48 hours at 40 000 rpm. Fractions (200 µl) were collected and 3 µl samples were electrophoresed on a 0.8% agarose gel. Those containing DNA were pooled and dialyzed in TE pH 8.0 at 4°C. The buffer was changed four times over 48 hours. Solutions were then filtered through a 0.2 µm sterile acrodisc (Gelman Sciences) and the DNA concentration determined.

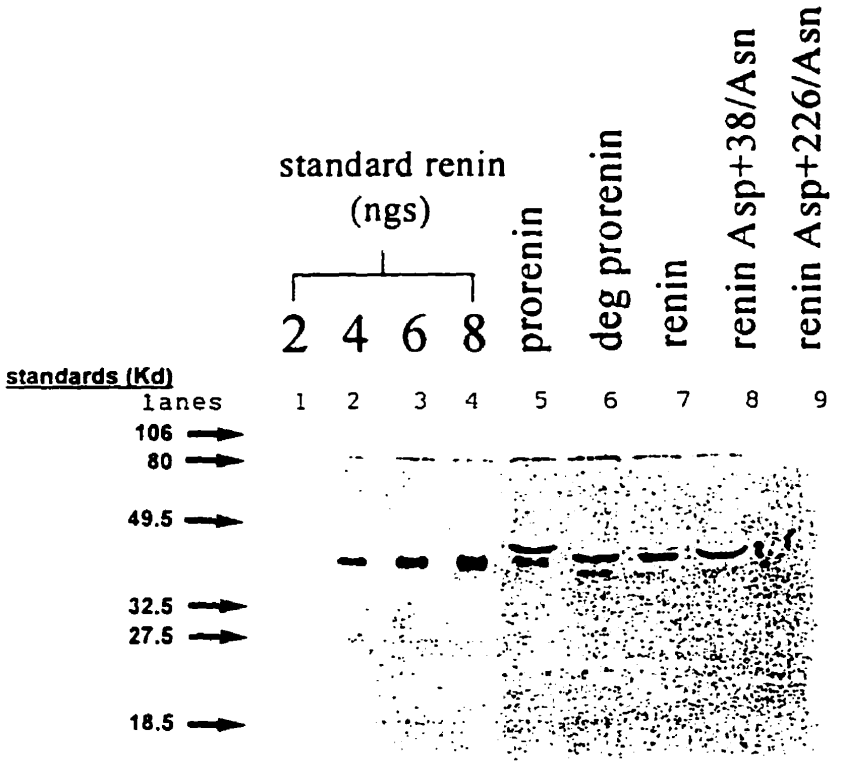
3. Results

3.1. Quantitation and assessment of renin activity in stably transfected GH_4Cl cells

The concentration of active renin present in the supernatants of stably transfected GH_4Cl cells was estimated by western blotting and densitometry of exposed X-ray films using purified human renin as a standard. As shown in Figure 10, lane 7, there are two bands with molecular weights of approximately 43 kDa and 48 kDa, corresponding to active renin and prorenin respectively in supernatants of furin-cleaved renin transfected cells. The presence of a small amount of the higher molecular weight band (corresponding to prorenin) suggests that cleavage by furin in the endoplasmic reticulum or the golgi apparatus is not complete, in agreement with earlier findings (T.L.Reudelhuber, personal communication). The concentration of renin averaged approximately $9.3x10^{-9}$M (Figure 10). The enzymatic activity of the protein was assessed by incubation with excess sheep angiotensinogen and production of AI determined by RIA. The activity of renin averaged a production of 98 000 ng AI/ml/hr (Table 1).

3.2. Enzymatic measurement of renin uptake by whole, perfused cotyledon

To determine whether renin could be taken up from the circulation in human tissues, placentas obtained at full term were dissected into individual cotyledons and perfused with renin through the cannulated artery as illustrated in Figure 9. After extensive washing with DMEM, angiotensinogen was perfused and eluates from the vein were collected and assayed for AI and AII. AI levels initially rose dramatically to 6.4 ng/ml and rapidly decreased to 0.2 ng/ml (Figure 11 and Table 2). Levels of AII reached a maximum of 0.28 ng/ml and dropped to 0.02 ng/ml (Figure 11 and Table 2). Concomitant with this increase in AII was a rise in arteriolar blood pressure of 6 mm



Spot denso results from a standard curve fitting program (IS-1000 version 2.0, Alpha Innotech Corporation)

		IDV	ng (input)	ng (calculated)	calculated concentration*
standards	renin standards	15287	2	2	
	(Bio-mega)	33191	4	4	
		52417	6	6	
		65075	8	8	
unknowns	prorenin	44501		5	1.0E-08 M
	deg prorenin	47442		5	1.2E-08 M
	renin	41578		4	9.3E-09 M
	renin Asp+38/Asn	37868		4	9.3E-09 M
	renin Asp+226/Asn	19279		2	4.7E-09 M

Figure 10: Quantitation of prorenin, deglycosylated prorenin, renin and renin active site mutants (Asp+38/Asn and Asp+226/Asn) concentrations in the supernatants of stably transfected GH_4Cl cells. IDV, integrated intensity value; deg prorenin, deglycosylated prorenin.

* The calculated protein concentrations convert the ng obtained from the western blot to Molarity (M) by taking into the account the molecular weight of the protein (43 000 for renin and 48 000 for prorenin).

Sample	Molarity	Active Renin (ngAI/ml/hr)	Total Renin (active+inactive) (ngAI/ml/hr)	Equivalency of renin activity for 1E-12 M protein (ngAI/ml/hr)
renin	9.3E-09	9.8E+04	n/a	10.5
prorenin	1.0E-09	6.3E+02	1.0E+05	10
deg prorenin	1.2E-08	25	1.1E+04	1
renin Asp+38/Asn	9.3E-09	0	0	0
renin Asp+226/Asn	4.7E-09	0.6	0	0

Table 1: Typical activities of supernatants collected from GH_4Cl cells stably transfected with renin, prorenin, deg prorenin (deglycosylated prorenin), and renin active site mutants (Asp+38/Asn and Asp+226/Asn) plasmid constructions as determined by RIA for AI generation. n/a, not applicable.

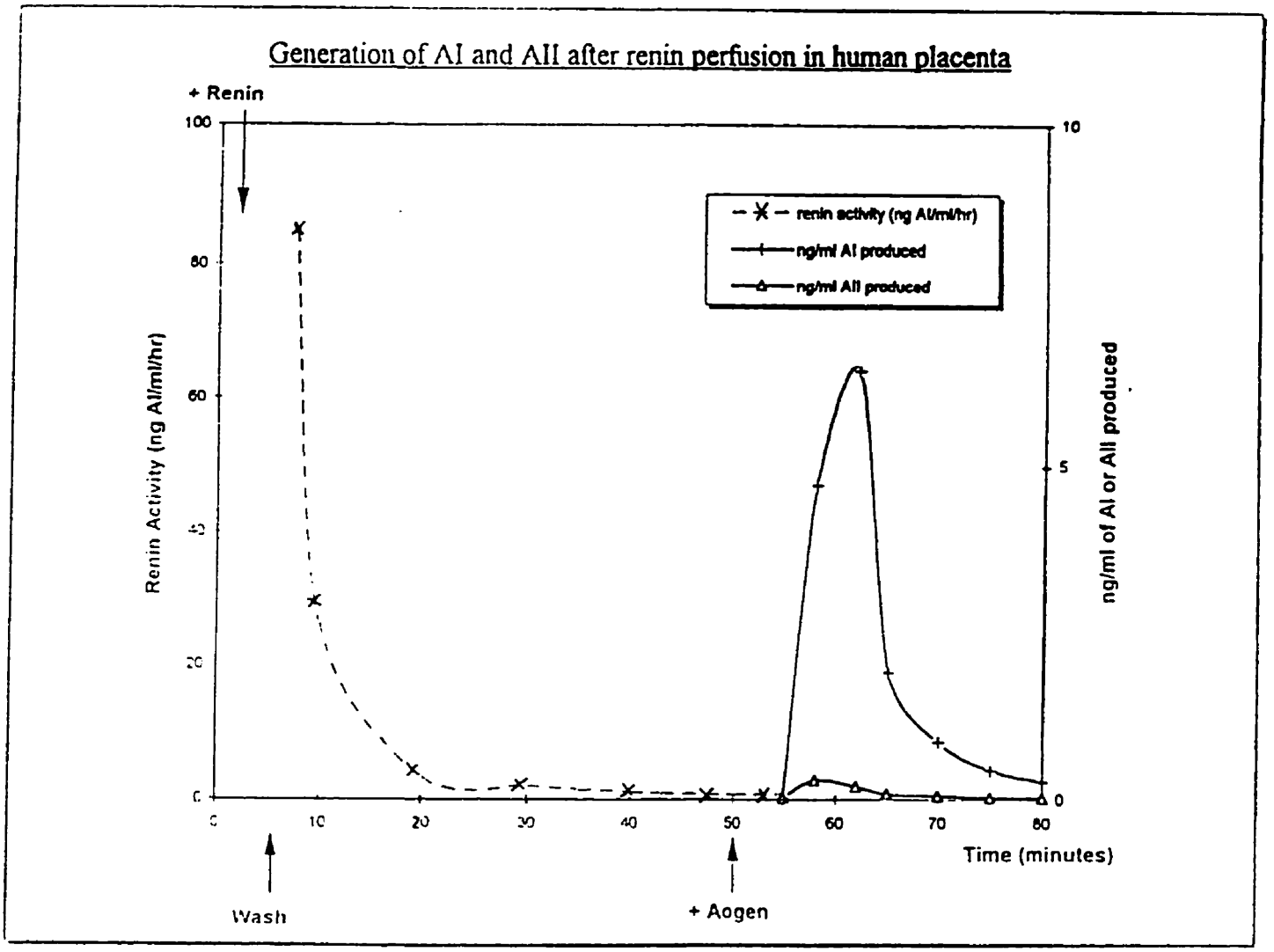


Figure 11: Evidence for renin uptake in the whole, perfused human cotyledon. 50 ml of active human renin (activity approximately 5000 ng AI/ml/hr) in DMEM was perfused through the artery followed by a 100 ml wash with DMEM. Next, a 50 ml solution of semi-purified sheep Aogen (approximately 1μM) was infused. All solutions were perfused at a flow rate of 2 ml/min. The venous returns were collected for analysis of renin activity and any angiotensins produced. As indicated by the dashed line, the activity of renin decreases during the washing phase suggesting that any unbound renin is expelled and detected in the venous return. When Aogen is perfused, a rapid increase in AI is observed along with a smaller increase in AII (indicating the presence of ACE) implying that renin had been taken up by the tissues and was able to cleave its substrate. Because the increase in AI and AII is not sustained but decreases after several minutes, this suggests that once renin cleaves its substrate, it is released and washed out into the venous return. In the control cotyledon, DMEM devoid of human renin was used and all others perfusions were the same. Here, no increase in AI or AII was observed. This figure is representative of two experiments performed on separate days and the cotyledons were obtained from different placentas.

		renin-perfused placenta			*control-perfused placenta*		
Time sequence	**Samples collected from venous return**	**Renin Activity of venous return during DMEM wash**	**AI (ng/ml) in venous return during Aogen infusion**	**AII (ng/ml) in venous return during Aogen infusion**	**Renin Activity of venous return during DMEM wash**	**AI (ng/ml) in venous return during Aogen infusion**	**AII (ng/ml) in venous return during Aogen infusion**
1. 50 ml infusion of renin or DMEM in control							
2. start of DMEM wash	1st 5 ml aliquot	85,0	ND	ND	0	ND	ND
	2nd 5 ml aliquot	26,4	ND	ND	0	ND	ND
	3rd 5 ml aliquot	3,3	ND	ND	0	ND	ND
	4th 5 ml aliquot	1,9	ND	ND	0	ND	ND
	5th 5 ml aliquot	1,3	ND	ND	0	ND	ND
	6th 5 ml aliquot	0,95	ND	ND	0	ND	ND
	7th 5 ml aliquot	0,75	ND	ND	0	ND	ND
	8th 5 ml aliquot	0,55	ND	ND	0	ND	ND
3. start of Aogen infusion	9th 5 ml aliquot	ND	0	0,02	ND	0	0
	10th 5 ml aliquot	ND	4,7	0,28	ND	0,01	0,01
	11th 5 ml aliquot	ND	6,4	0,19	ND	0,2	0,02
	12th 5 ml aliquot	ND	1,9	0,08	ND	0,1	0,02
	13th 5 ml aliquot	ND	0,8	0,05	ND	0,01	0,01
	14th 5 ml aliquot	ND	0,4	0,03	ND	0,1	0,01
	15th 5 ml aliquot	ND	0,3	0,02	ND	0,1	0,06
	16th 5 ml aliquot	ND	ND	ND	ND	0,8	0,03

Table 2: Evidence of renin uptake in the whole, perfused cotyledon. In step 1, 50 ml of active human renin (activity approximately 5000 ng AI/ml/hr) in DMEM was perfused through the artery followed by a 100 ml wash with DMEM (step 2). Next, a 50 ml solution of semi-purified sheep Aogen (concentration approximately 1 μM) was infused (step 3). The venous returns were collected for analysis of renin activity and AI and AII. In the control cotyledon, DMEM devoid of renin was perfused and all other steps were the same. The numbers are representative of 2 experiments performed on separate days and on cotyledons obtained from different placentas. Please refer to Figure 11 for graphical representation and the text for details. ND, not determined.

Hg corresponding to the known vasoconstrictive activity of this peptide (Figure 12). In control cotyledons perfused with medium lacking renin, no increase in AI or AII was evident after angiotensinogen perfusion. AI and AII levels remained at 0.07 ng/ml and 0.06 ng/ml respectively (Table 2). Identical results were obtained in two separate experiments (not shown).

3.3. Immunohistochemical measurement of renin uptake by whole, perfused cotyledon

3.3.1. Preliminary studies on the efficiency of the HA1 antibody in recognizing the HA1 epitope on GH_4Cl cells transfected with HA1-tagged renin

Because the placenta itself produces large amounts of prorenin and our polyclonal renin antibody recognizes both prorenin and renin, it was impossible to distinguish between endogenous and exogenous renin in the perfused cotyledons. To address this problem, we added a DNA cassette encoding 9 amino acids of the hemagglutinin (HA1) protein of the influenza virus to the carboxy terminus of all our renin constructs. GH_4Cl cells were transiently transfected with these peptide-tagged proteins and the efficiency of the commercial anti-HA1 antibody in recognizing the HA1 epitope was assessed by immunohistochemistry. As seen in Figure 13, the anti-HA1 antibody was effective in recognizing only cells that had been successfully transfected with the HA1-tagged proteins as visualized by the brown staining inside the cells. Untransfected cells show no staining. These tagged proteins were therefore used in subsequent cotyledon perfusion experiments.

3.3.2. Immunohistochemistry on cotyledons perfused with HA1-tagged renin

In efforts to visualize the cells responsible for renin uptake in the human placenta, cotyledons were perfused with HA1-tagged renin and uptake examined using the anti-HA1 antibody in immunohistochemistry and electron microscopy studies. The typical

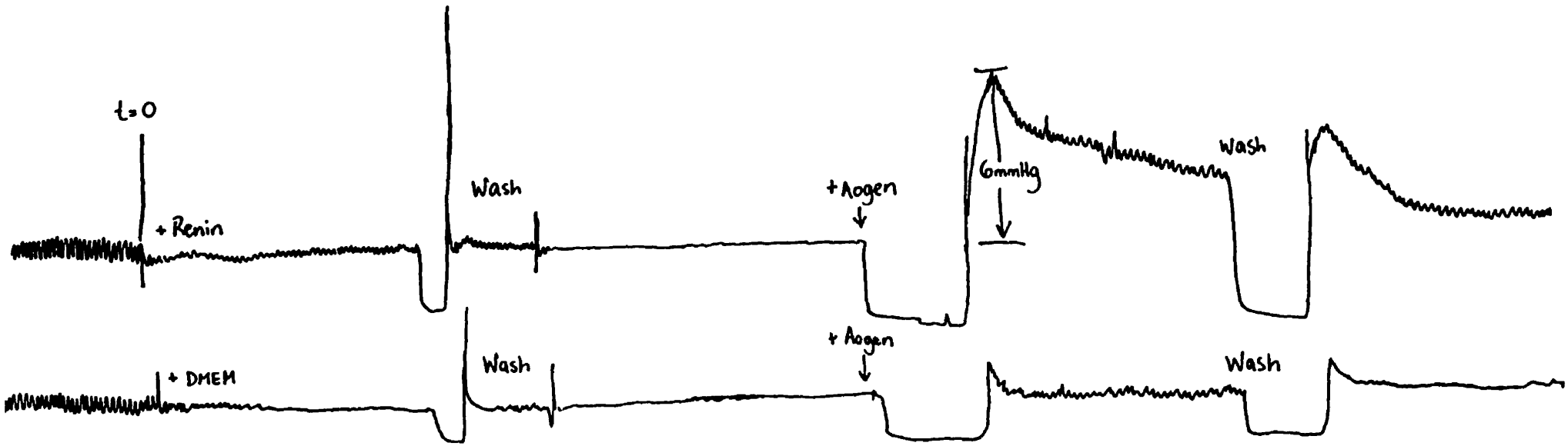


Figure 12: In one experiment, following renin and angiotensinogen perfusion of the human cotyledon, an increase in pressure of 6 mm Hg was observed, corresponding to the vasoconstrictive activity of AII generated.

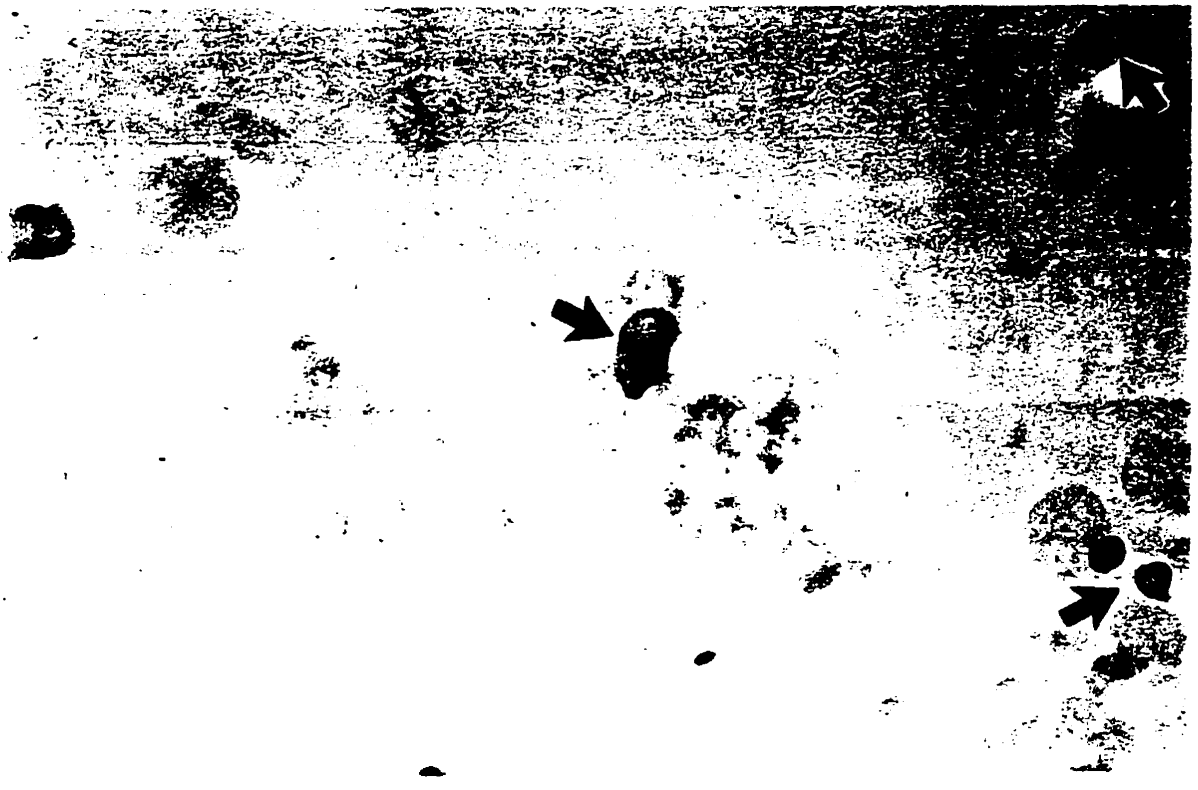


Figure 13: GH_4Cl cells were stained with HA1 specific antibody after transient transfection with the expression vector for the peptide-tagged human renin. Heavy arrows indicate cytoplasmic staining in HA1 expressing cells.

structure of the human cotyledon is shown in the electron micrograph picture seen in Figure 14. It was not possible to see a difference between renin-perfused cotyledons (Figure 15) and controls (Figure 16) in either immunohistochemistry or immuno-electron microscopy studies (not shown).

3.4. Examining renin binding to cotyledon membranes

3.4.1. Radioligand binding experiments

Initially, the binding experiments had been conducted using ^{125}I labeled renin for classical radioligand binding experiments. The nonspecific binding as well as the background binding (when only ^{125}I renin was filtered through the glass fiber filter paper) turned out to be very high and hence no conclusions could be made from these experiments and this approach was abandoned (results not shown). Iodination of rat renin and classical binding experiments with rat tissues gave similar results (D.F.Cantanzaro, personal communication).

3.4.2. Detection of renin and prorenin binding by enzymatic activity

To solve this problem, placental membrane preparations were incubated with renin and uptake assessed by enzymatic activity. Prorenin from supernatants collected from GH_4Cl stably transfected cells was quantified by western blotting as described above (Figure 10, lane 5). Prorenin, having a slightly higher molecular weight than renin (48 kDa versus 43 kDa due to the prosegment) migrated at a slightly higher level than renin as expected. A slightly lower band, corresponding to renin, shows that a small amount of prorenin was activated to renin, possibly due to proteases present in the culture supernatant. Prorenin concentration was roughly $1X10^{-08}M$ (Figure 10) and activity, determined by trypsin activation of the protein, averaged 100 000 ng AI/ml/hr (Table 1); renin activity (due to prorenin activation in the culture supernatant) was roughly 600 ng AI/ml/hr (Table 1). Initial experiments had shown that renin and

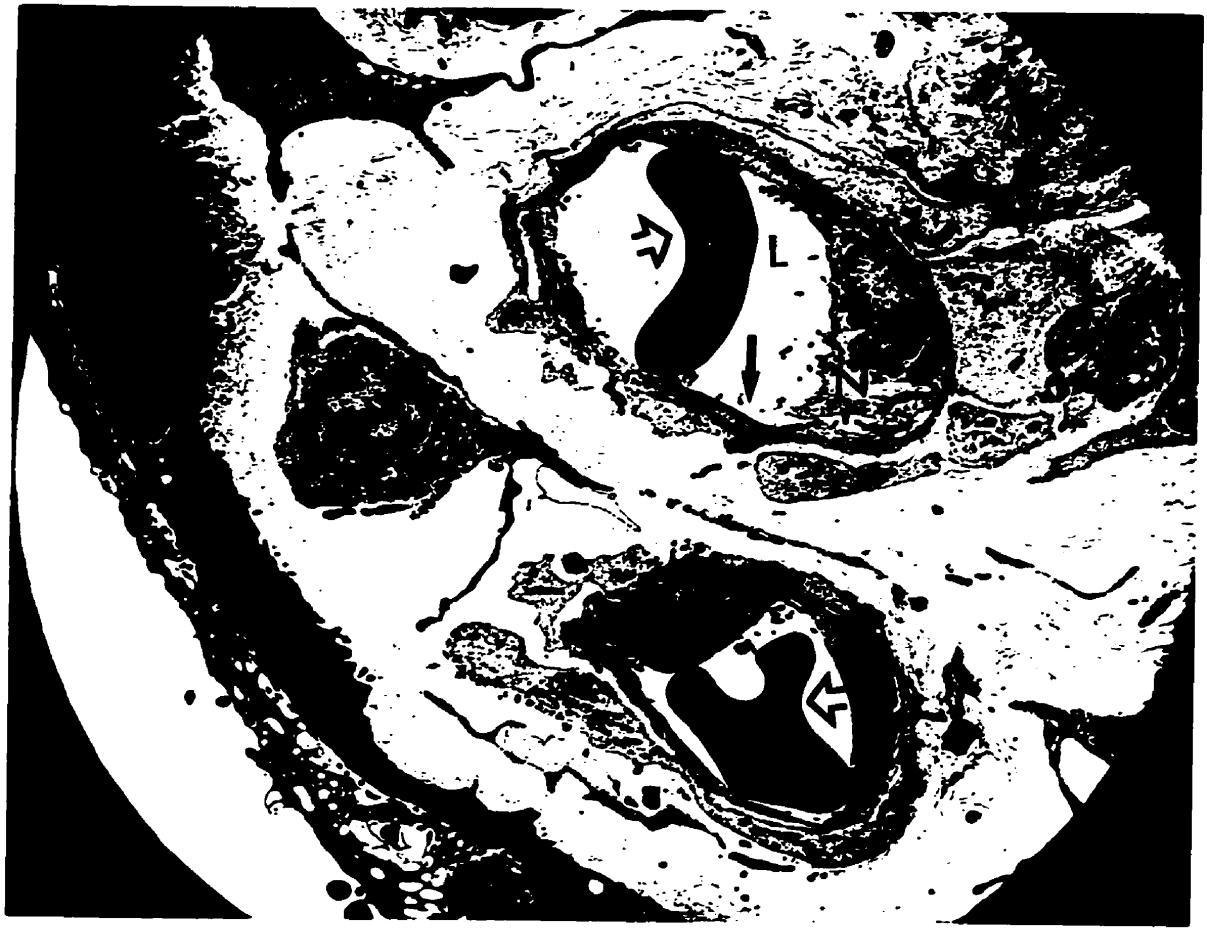


Figure 14: Electron micrograph of a tissue section from a human cotyledon. Open arrows show red blood cells trapped in the lumen of blood vessels, closed arrows indicate vascular endothelial cells. L, lumen; N, nucleus. Magnification X2.5K.

Figure 15: A tissue section from a human cotyledon was stained with HA1 specific antibody after human HA1-tagged renin perfusion and fixation of the whole cotyledon. Open arrows indicate endothelial cells surrounding blood vessels and where we would expect to see staining. No staining was evident. L, lumen. Magnification X25.

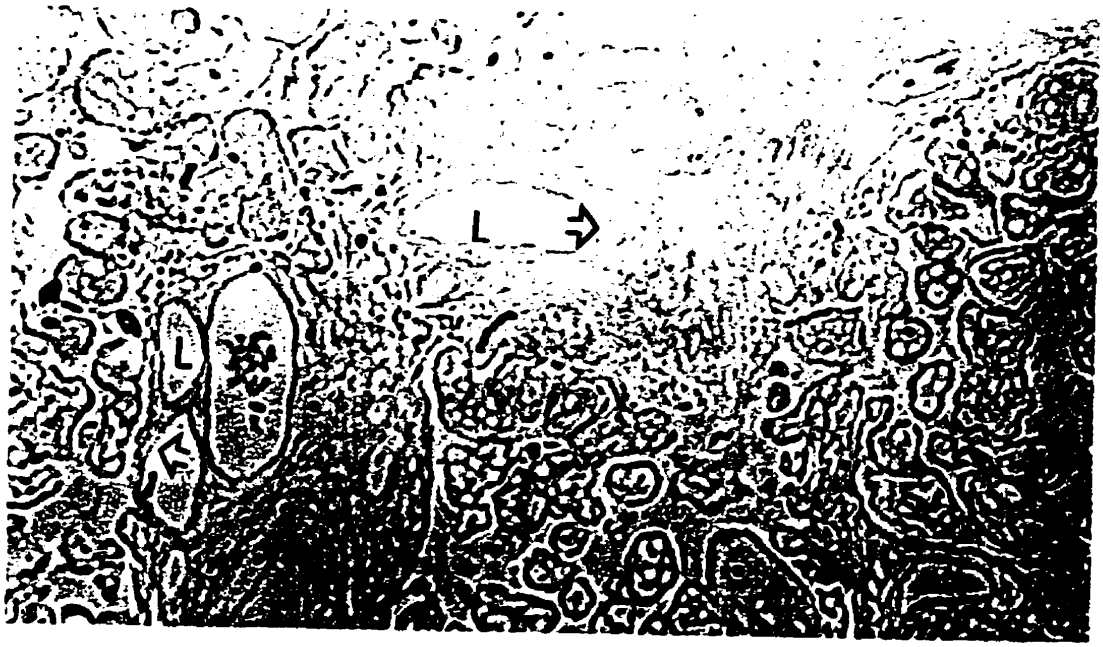


Figure 16: Control. A tissue section from a human cotyledon was stained with HA1 specific antibody after DMEM perfusion and fixation of the whole cotyledon. Open arrows indicate endothelial cells surrounding blood vessels. No staining was evident. L, lumen. Magnification X25.

prorenin bind with approximately equal affinities to the placental membranes, with prorenin perhaps having a slightly higher binding affinity (Figure 17 and Table 3). For example, at $4.65X10^{-10}M$ of renin added, the protein remaining bound to the membrane had a renin activity of 139.5 ng AI produced/mg cotyledon membrane protein. At $5.2X10^{-10}M$ of prorenin added, prorenin activity was 221.2 ng AI produced/mg cotyledon membrane protein. Since n = 1 for the prorenin binding experiment, this increase in activity may not be statistically significant, but is still useful in showing that both bind with similar affinities to the membranes.

3.4.3. Saturation binding curve experiments

Since prorenin bound with a comparable affinity to renin in placental membrane preparations, but prorenin activity is masked unless trypsin activated, prorenin was used as a competitor to determine nonspecific binding of renin in saturation binding experiments. Increasing concentrations of renin ranging from $2.3X10^{-11}$ M to $4.65X10^{-10}$ M were incubated with placental membrane preparations in the presence (nonspecific binding) or absence (total binding) of $8.85X10^{-09}$ M prorenin. Specific binding was determined by subtracting the nonspecific binding from the total binding (Figure 18 and Table 4). The K_d for human renin binding to the cotyledon membrane preparations was estimated by plotting the specific binding renin activity against the log of renin concentration and was calculated to be approximately 125pM (Figure 19).

3.4.4. Competition binding experiments to examine regions of prorenin/renin important for binding to cotyledon membrane preparations

In order to identify the regions of renin and prorenin important for binding, competition binding experiments were performed by displacing renin with prorenin, deglycosylated prorenin and a renin active site mutant. The concentrations of the renin variants present in the culture supernatants was determined by western blotting as

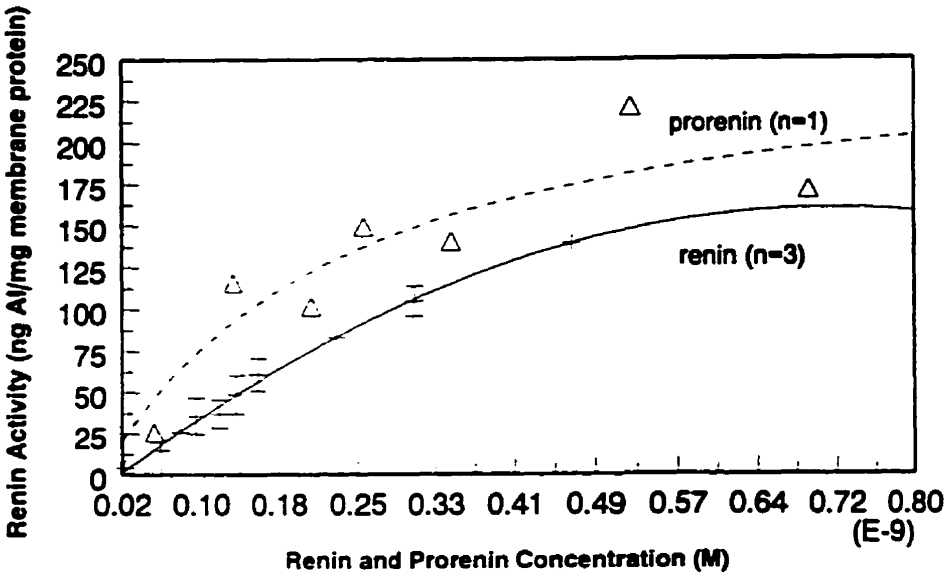


Figure 17: Renin and prorenin binding to cotyledon membranes. Polynomial 2 curve fit was used for renin and prorenin binding curves.

Renin Activity Added (M)	Renin Activity retained (ng AI/ml/hr)	Prorenin Activity Added (M)	Prorenin Activity retained (ng A1/ml/hr)
4,65E-10	139,45	6,94E-10	171,2
3,10E-10	104,9	5,21E-10	221,2
2,33E-10	83,2	3,47E-10	140,5
1,55E-10	60,8	2,60E-10	149,3
1,33E-10	48,6	2,08E-10	101,8
1,16E-10	37	1,30E-10	115,7
9,30E-11	35,8	5,21E-11	26
7,75E-11	25,9		
5,81E-11	15		
2,33E-11	4,3		

Table 3: Comparing prorenin versus renin binding to cotyledon membrane preparations. Prorenin and renin remaining bound to membranes after the binding experiments were measured by enzymatic activity and generation of AI detected by RIA. The experiment was performed once for prorenin binding studies and three separate times for renin binding studies.

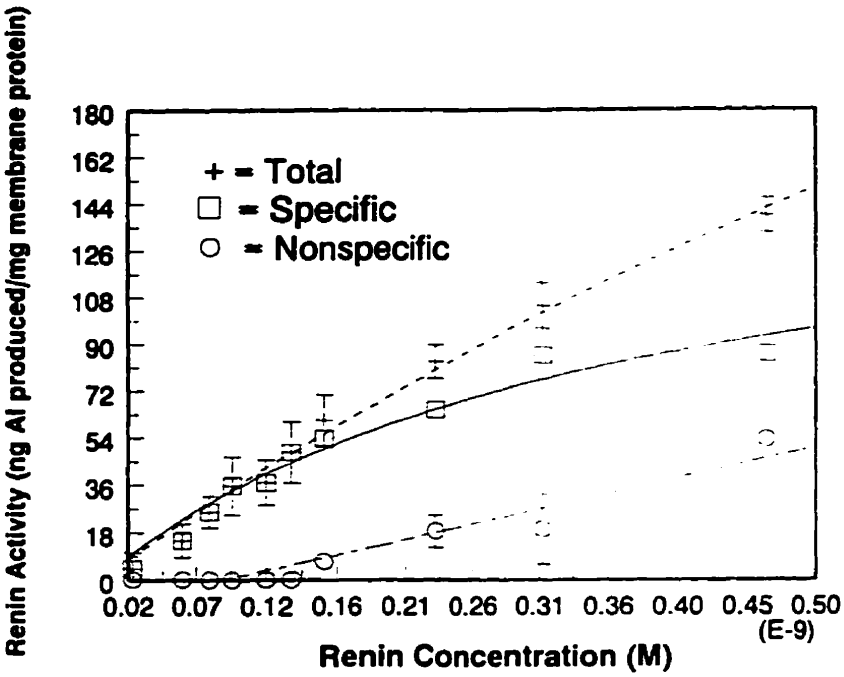


Figure 18: Saturation binding curve for renin. Nonspecific binding was determined using $8.85X10^{-09M}$ prorenin. Polynomial 2 curve fit was used for total and specific binding curves. Linear curve fit was used for the nonspecific binding curve.

Renin Activity Added (M)	log of Renin Activity Added (M)	Total Binding (ng AI/ml/hr)	Nonspecific Binding (ng AI/ml/hr)	Specific Binding (ng AI/ml/hr)
4.65E-10	-9.33	139.5	53.4	86.1
3.10E-10	-9.51	104.9	19.3	85.6
2.33E-10	-9.63	83.2	18.5	64.7
1.55E-10	-9.81	60.8	7.1	53.7
1.33E-10	-9.88	48.6	0.14	48.5
1.17E-10	-9.94	37	0.1	36.9
9.30E-11	-10.03	35.8	0.01	35.8
7.75E-11	-10.11	25.9	0.02	25.9
5.81E-11	-10.24	15	0.07	14.9
2.33E-11	-10.63	4.3	0.07	4.2

Table 4: Saturation binding curve for human renin binding to cotyledon membrane preparations. Increasing amounts of renin were added to a fixed concentration of membranes (15µg). Nonspecific binding was defined using 8.85 nmol/L of human prorenin. Specific binding was determined by subtracting nonspecific binding from the total binding. These experiments were repeated on three separate occasions.

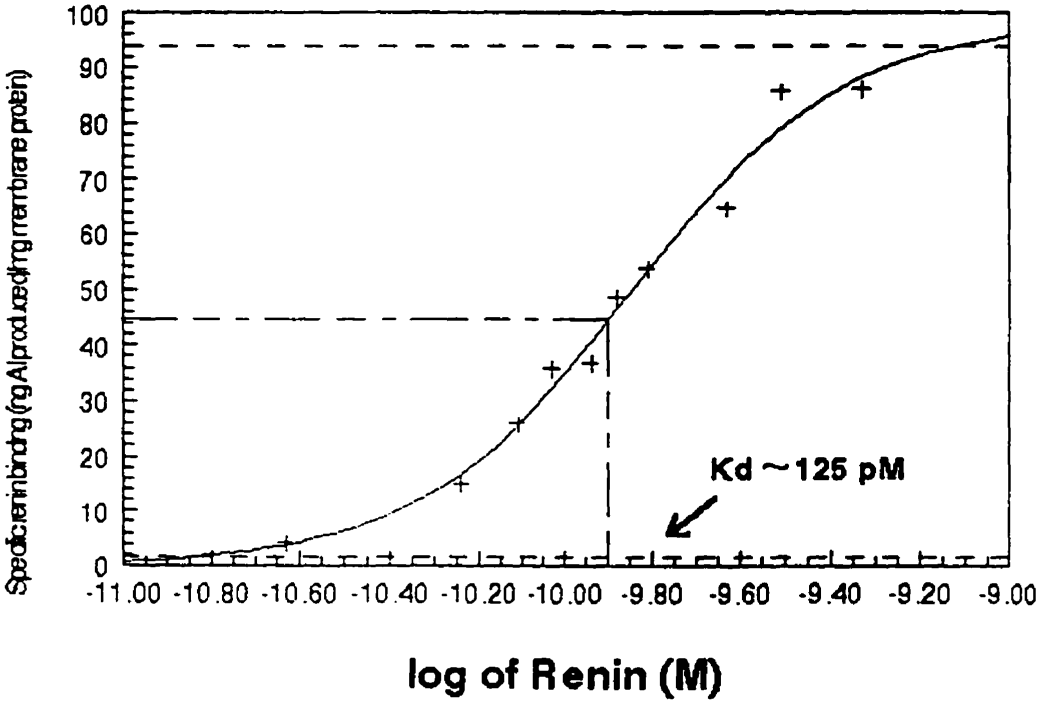


Figure 19: Calculating the K_d for human renin binding to placental membranes. A nonlinear sigmoidal curve fit was used.

described above (Figure 10). The competition binding curve experiments were performed in duplicate.

3.4.4.1. Competition binding experiments between renin and prorenin

Renin ($9.3X10^{-10}$M) was incubated with increasing concentrations of prorenin ranging from $1X10^{-12}$ M to $1X10^{-08}$ M. Prorenin was able to displace renin as shown in the competition binding curve in Figure 20 (A) and Table 5. The IC50 was calculated to be 100 pM.

3.4.4.2. Competition binding experiments between renin and deglycosylated prorenin

Deglycosylated prorenin also migrated at the same position as the renin standards (roughly 43 kDa) (Figure 10, lane 6). The absence of glycosylation due to mutation of the asparagine residues to serine at positions 5 and 75 of the protein resulted in a lower molecular weight than native prorenin. Again, the presence of a lower molecular weight band suggests that a small amount of deglycosylated prorenin was activated to deglycosylated renin in the supernatant. The average concentration of the deglycosylated prorenin in the undiluted culture supernatant was roughly $1.2X10^{-08}$ M (Figure 10). The effect of deglycosylation seemed to lower the activity since equivalent amounts of protein resulted in a higher generation of AI content (roughly a 10 fold increase) for native prorenin over deglycosylated prorenin (Table 1, 10 ng AI/ml/hr for prorenin versus 1 ng AI/ml/hr for deglycosylated prorenin at a concentration of $1X10^{-12}$ M protein). Renin ($9.3X10^{-10}$M) was incubated with increasing concentration of competitor ranging from $1X10^{-12}$ M to $1X10^{-08}$ M. Deglycosylated prorenin was able to displace renin from the membranes as illustrated in the competition binding curve in Figure 20 (C) and Table 5. The IC50 was calculated to be 100 pM.

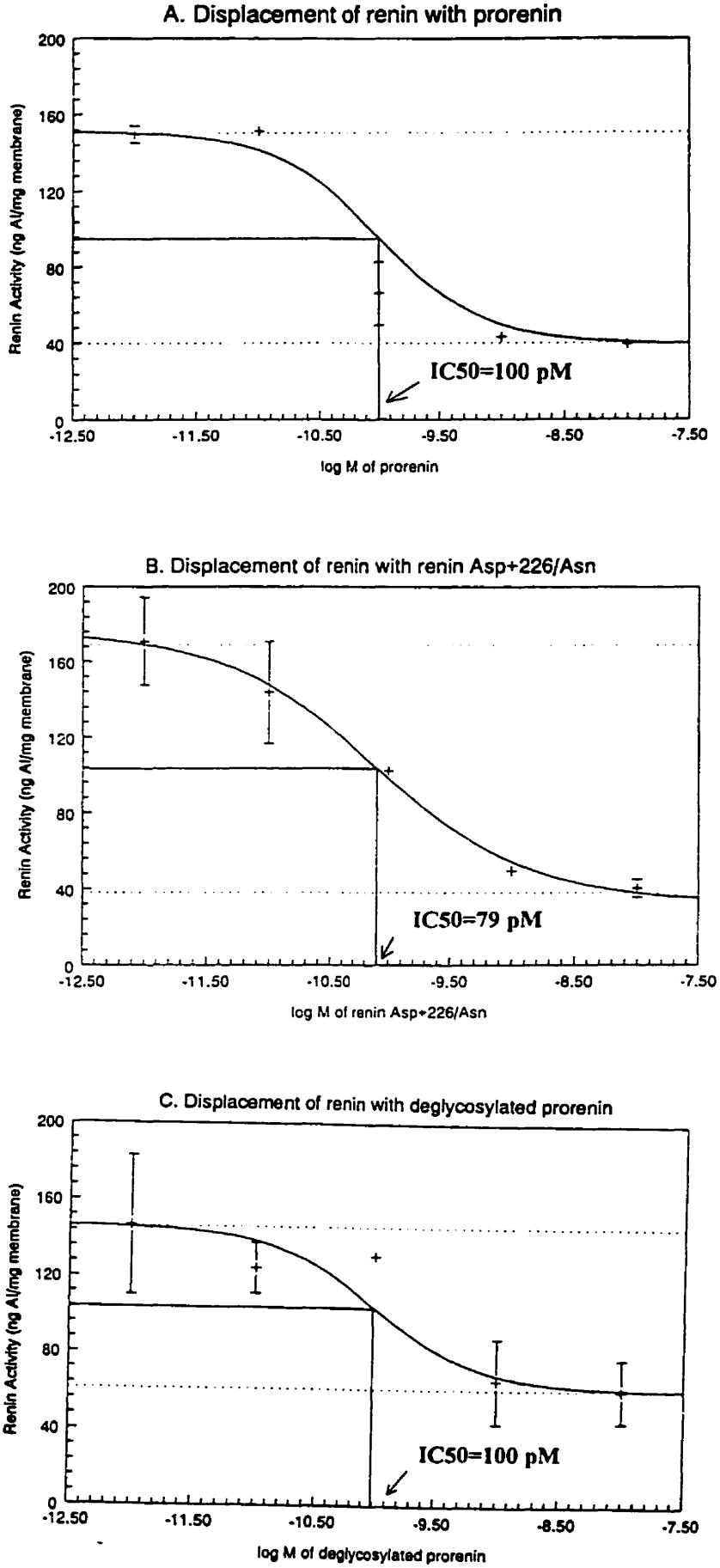


Figure 20: Displacement of renin with prorenin (A), renin active site mutant, Asp +226/Asn (B), and deglycosylated prorenin (C). The IC50s were all quite similar and calculated to be 100 pM, 79 pM, and 100 pM for prorenin, renin Asp+226/Asn and deglycosylated prorenin respectively.

Concentration of competitor	log of competitor	competitor		
		prorenin	renin Asp+226/Asn	deg prorenin
		Renin Activity remaining (ng AI/ml/hr)		
1E-08	-8	39,1	40,5	60,3
1E-09	-9	42,9	49,6	65,5
1E-10	-10	65,8	102,1	131,1
1E-11	-11	151,3	143,8	124,4
1E-12	-12	149,7	171	146,5

Table 5: Competition binding curve between renin and prorenin, deglycosylated prorenin (deg prorenin) and renin active site mutant, Asp+226/Asn. $1X10^{-12}$ M to $1X10^{-08}$M of competitor were used to displace $9.3X10^{-10}$M renin. This experiment was repeated two times on the same day.

3.4.4.3. Competition binding experiments between renin and a renin active site mutant

The renin active site mutant (Asp+226/Asn) was used in competition binding experiments to displace active renin. This mutant was arbitrarily chosen over the Asp+38/Asn active site mutant; both mutants were inactive as assessed by RIA (Table 1). As expected, the mutation of the active site of renin did not change its molecular weight and hence the protein migrated at the same position as the renin standards (Figure 10, lanes 8 and 9). The concentration of the mutant protein (Asp+226/Asn) in the undiluted culture supernatant was roughly $4.7X10^{-9}$ M (Figure 10), although in some culture supernatants, concentrations were as high as $1.4X10^{-08}$ M (not shown). Renin ($9.3X10^{-10}$ M) was incubated with increasing concentrations of the mutant ranging from $1X10^{-12}$ M to $1X10^{-08}$ M. As shown in the competition binding curve in Figure 20 (B) and Table 5, the construct was able to effectively displace renin. The IC50 was calculated to be 79 pM.

3.5. Preliminary studies for future transgenic mouse work

3.5.1. Expression of transthyreitin transgenes in transfected cells

For transgenic mice studies, a promoter for transthyretin was used to direct renin expression to the liver. To test whether expression was indeed specific to the liver, human hepatoma-derived HepG2 cells were transiently transfected with a vector encoding HA1-tagged human renin and prorenin under the transcriptional control of the liver specific promoter. Renin and prorenin activity was measured to be 53.8 ng Al/ml/hr and 29.6 ng AI/ml/hr respectively by RIA. In control experiments, the same constructs were transfected in GH_4Cl cells, however, only negligent amounts of renin could be detected, indicating that expression was specific to liver cells (Table 6).

Sample	cells	Renin Activity (ng AI/ml/hr)	Prorenin Activity (ng AI/ml/hr)
TTR3kb/Prorenin	GH_4Cl	n/a	0
TTR3kb/Prorenin	Hep G2	n/a	29.6
TTR3kb/Renin	GH_4Cl	1	n/a
TTR3kb/Renin	Hep G2	53.8	n/a

Table 6: Expression of prorenin and renin constructs under transcriptional control of a liver specific promoter (TTR) in a transfected liver cell line (HepG2) and a non-liver cell line (GH_4Cl). n/a, not applicable.

4. Discussion

In this study we present evidence for the existence of a renin and prorenin binding site in membrane preparations from the human placenta. While other investigators have reported vascular and renal uptake of renin in the rat, uptake of renin by human tissues has not been addressed. As discussed in greater detail below, we show that uptake of renin and prorenin occurs via a specific and saturable binding mechanism in the human placenta and that the active site and carbohydrate moieties are not implicated in this recognition and binding process.

4.1. Production of AI and AII in whole, perfused placenta; preliminary evidence of renin uptake

In efforts to study renin uptake in human tissues, our studies began by examining this phenomenon through enzymatic measurement of renin uptake in the whole, perfused placenta. The observation that renin and angiotensinogen perfusion in the human placenta resulted in a rapid rise in AI and AII followed by their progressive decrease suggests a mechanism for renin uptake and release. A possible explanation is that the perfused renin is taken up by the cells via an existing renin binding site or a specific receptor present on the surface of the cell membranes. Once its substrate is presented to the enzyme, renin is released into the circulation where it cleaves Aogen to AI. The presence of ACE on the endothelial cell membranes causes the resulting AI to be converted to AII, hence accounting for the small increase in arteriolar blood pressure due to the vasoconstrictive activity of the peptide (Figure 12).

4.2. The placenta is a major prorenin factory

The placenta itself produces and secretes large amounts of prorenin.. During the first few weeks of pregnancy, the levels of plasma prorenin increase approximately 10 fold

and then decrease slightly, but remain elevated throughout the duration of pregnancy [54]. It seems that the initial rise in prorenin levels is due to ovarian prorenin gene expression induced by the increased levels of human chorionic gonadotrophin [55] and the sustained high levels are maintained by the uteroplacental unit. Renin mRNA as well as the corresponding proteins (mainly the immature form, prorenin) have been detected in the placenta [56], myometrium [57] and decidua [57]. The exact role of the remarkably high levels of prorenin present during pregnancy is not known, however, it is possible that it could be involved in controlling the local RAS by antagonizing the binding of renin to tissues.

4.3. Use of HA1-tagged renin in immunohistochemistry

In an effort to identify the cell type responsible for renin and prorenin uptake, we used immunohistochemistry to detect the retained renin in perfused whole cotyledons. Initial pilot experiments failed to see any difference between renin perfused placenta and controls due to high levels of staining in both sets of tissues. This was due to the large amounts of endogenous prorenin present in the placenta and the fact that our polyclonal antibody recognized both renin and prorenin. To solve the problem of distinguishing between endogenous prorenin and exogeous perfused renin, we constructed a renin tagged with the hemagglutinin HA1 epitope such that we could directly detect the perfused renin by immunohistochemistry.

4.3.1. Use of HA1-tagged renin in immunohistochemical studies of GH_4Cl transfected cells

The antibody against the HA1 tag was effective in immunohistochemical studies on GH_4Cl cells transfected with HA1-tagged renin (Figure 13), indicating that indeed the anti-HA1 antibody could specifically and effectively recognize the HA1 tag in the folded protein.

4.3.2. Use of HA1-tagged renin in immunohistochemical studies in the whole, perfused placenta

However, studies on the perfused cotyledon tissues were not as encouraging. As shown in the electron microscopy photograph of a representative cotyledon section (Figure 14), the structure is very complex, comprised of many different cells and small arteries and veins that contribute to a very dense structure. The photograph depicts the endothelial cells (and its nuclei) wrapped around two small vessels with a red blood cell trapped in one of the lumen of the vessels. Because the endothelial cells line the surface of the arteries and veins, it is in these cells that the binding and uptake of renin is postulated, and the place where we would expect to see staining in our immunohistochemical studies. However, in our studies of the cotyledon tissue sections, it was not possible to see a difference in tissues obtained from placental perfusion studies between renin-perfused cotyledons (Figure 15) and controls (Figure 16). There are several plausible explanations for these results. First of all, it is likely that that the amount of renin uptake in the placenta is extremely small and therefore uptake could not be visualized. It is also possible that once the protein is bound to the tissue, a conformational change is induced in the protein whereby the HA1 tag is not accessible for recognition by the antibody and hence no staining would be evident. Alternatively, no conformational change may be involved but simple binding of the protein into the pocket of the site may hide the HA1 tag. Another possible explanation for the failure to detect exogenous renin is that perfusion is not homogeneous and that the pieces of tissue that were dissected out from the cotyledons for the studies had not been perfused with the tagged protein. This is idea is supported by the picture of the cotyledon (Figure 14) which shows that a red blood cell is trapped in the lumen of the artery. The red blood cell would have been washed away during the perfusion process had the cotyledon been properly perfused. For these reasons or a combination of these reasons, visual specific uptake of renin in the perfused human cotyledon was not possible.

4.4 Binding experiments with human cotyledon membranes

4.4.1. Initial radioligand binding experiments

Binding experiments were not conducted using the conventional radioligand binding studies because initial studies using iodinated human renin resulted in high backgrounds. Often, non-specific binding measured by incubation of membranes with labeled renin and a large excess of cold renin resulted in counts similar to membranes incubated with labeled renin alone (total binding). These experiences were repeated 5 times with three different batches of iodinated renin. It is likely that iodination of renin causes conformational changes in the protein detrimental to binding and hence may be responsible for these high non-specific backgrounds. For this reason, we had to pursue our binding studies using nonradioactive renin and assess binding by determining residual renin activity on the membranes after binding experiments.

4.4.2. Prorenin and renin can bind to the same site

Examination of prorenin and renin binding to placental membrane preparations by enzymatic assay show that both proteins bind with approximately equal affinities, as shown in the saturation binding curve in Figure 17 and the competition binding curve in Figure 20 (A). The IC50 for prorenin competing with renin for binding sites was calculated to be 100 pM, which is very close to the K_d measured for renin (125 pM), suggesting that indeed, these two proteins bind to the same site. These results are consistent with a recent report conducted by Sealey et al. who reported affinities of 200 and 900 pmol/L for binding of rat prorenin and renin respectively to renal cortical membranes [52].

4.4.3. Is prorenin a natural antagonist to the tRAS?

The fact that prorenin binds to the same site as renin but is unable to generate AI when angiotensinogen is present *in vitro* suggests that prorenin may be a natural antagonist to the tRAS *in vivo*. For example, as mentioned earlier, prorenin is present in very high amounts during pregnancy. In addition, all components of the RAS, including angiotensinogen, ACE and AII receptors are present in the organs of the reproductive system. It is possible that a local uteroplacental RAS is functional whereby locally generated AII may act in a paracrine or autocrine manner to control local circulation separately from the circulating system. Prorenin may act as an antagonist to the tRAS by competing with renin for binding sites without eliciting generation of AI when angiotensinogen is present and thus eliminating the normal biological response associated with renin. In support of the postulated vasodilatory function of prorenin is the correlation of high levels of this protein present in areas which maintain high blood flows including the reproductive organs, eyes [41] and kidneys [58]. Further indication for the vasodilatory action of prorenin comes from observations that patients suffering from diabetes mellitus often have a 10 fold increase in plasma prorenin levels over normal levels and 100 fold increase over their circulating renin levels [59]. While in the early stages of the disease the renal blood flow and glomerular filtration are increased, this often ends up in renal failure, possibly due to abnormal renal vasodilation that results in hyperperfusion injury [60].

4.4.4. Alternatively, can prorenin contribute to local AII levels?

An alternative hypothesis would be that prorenin can bind to the same site as renin and elicit the same biological response (catalyze the production of AI upon substrate presentation). The demonstration that prorenin can have reversible catalytic activity *in vitro* at room temperature and 4°C [61] and at low pH [62] without removal of the prosegment may suggest a mechanism whereby prorenin binding to tissues can mimic renin to generate AI (which is then cleaved to AII by ACE) from angiotensinogen

which may then act as an angiogenic or growth factor or control local circulation. However, the results obtained in this study show that prorenin is not active when bound to membrane preparations and that activity could be induced only upon removal of the prosegment through trypsin activation. Nevertheless, the possibility of reversible catalytic activity *in vivo* cannot be ignored.

4.4.5. The K_d for human renin binding to cotyledon membrane preparations

The K_d for human renin binding (125 pmol/L) measured from our saturation binding curve experiments was found to be slightly lower than that reported by Sealey et al. This K_d was only a rough estimate since we were unable to concentrate prorenin (without some activation of prorenin to renin (Table 1)) to levels high enough to be in 100 fold excess of renin at the higher concentrations of the saturation binding curve experiments. Hence it is possible that we did not reach the saturation point and that our measured K_d is an underestimate of the true value. In other words, the affinity of renin to the binding site is actually lower than we report and would thus be in better agreement with the value of 900 pmol/L measured by Sealey's group. Furthermore the K_d could not be calculated by the usual Scatchard plot (due to the fact that the free renin concentration was not known) but was determined by plotting the specific renin binding against the log of renin concentration to give a sigmoidal curve where the K_d was calculated as the concentration of renin where we observed half of the maximal renin binding response.

4.4.6. Competition binding experiments to determine regions important for renin binding

4.4.6.1. The carbohydrate residues of prorenin are not essential in the uptake process

It is also of great importance to determine which parts of the protein are responsible for recognition and binding to the tissue preparations. Human renin contains two consensus sequences for N-linked glycosylation (Asn-X-Thr) at positions 5 and 75 relative to the mature polypeptide sequence (Figure 4 and 7). Prior experiments had shown that the glycosylation residues were not essential for the processing, sorting or secretion of the protein in AtT-20 cells [63], however, they seem to be involved in protein clearance from the circulation [64]. It was therefore of interest to see if they were important for renin binding to membrane preparations. Competition binding experiments (Figure 20, C) show that deglycosylated prorenin could still effectively displace native renin (IC50 approximately 100 pM). This is similar to the K_d calculated for renin (125 pM) suggesting that these two proteins bind to the same site and that the carbohydrate residues are not essential in the binding process. These observations are in agreement with earlier findings reported by Campbell et al. [51] who showed that mouse SMG renin, void of potential glycosylation sites, bound to rat tissues with equal affinity as compared to rat renin which contains 3 consensus sequences for N-linked glycosylation. It thus appears that the degree of protein deglycosylation has no effect on binding.

4.4.6.2. The active site of renin is not essential in the uptake process

Furthermore, our findings that a renin active site mutant could effectively compete with native renin for binding (Figure 20, B) suggests that the active site is not essential for binding. The IC50 (79 pM) was similar to the K_d calculated for renin (125 pM) indicating that both proteins bind to the same site with comparable affinities. These

results agree with a hypothesis put forth by both Campbell and Sealey's group, who similar to this study, show renin activity in membrane bound renin, implying that binding of renin to tissues does not involve the renin active site. Campbell's group explains their finding that renin inhibitors and angiotensinogen are able to inhibit renin binding by inducing a conformational change in the protein once they are bound to active site and not the fact that the active site is implicated in the recognition and uptake process. One possible mechanism in which these inhibitors may function *in vivo* may be to prevent binding of renin to tissues, therefore limiting the local levels of AII. It will be important in future experiments to determine what protein regions are responsible for recognition and uptake.

4.7. Renin-binding protein (RnBP) is not responsible for the uptake described in this study

Previous studies have reported the cloning of a renin binding protein (RnBP) [65] present in kidney lysates, with a molecular weight of approximately 40 000. RnBP inactivates renin by binding to the protein with high affinity (0.2 nM for porcine RnBP and renin) and species specificity [66]. However it is improbable that that is the same protein responsible for the observed renin binding in our studies. First of all, there is no obvious signal peptide in the RnBP gene and thus it is found mostly in the cytosol and not on the surface of cell membranes. Moreover, the renin taken up in our binding studies was still active whereas RnBP is known to inactivate renin. In addition, this protein does not bind prorenin, and furthermore, RnBP mRNA has not yet been reported in the placenta and therefore it is unlikely to be implicated in the binding observed in our placental tissues.

4.8. Future Directions

4.8.1. Identifying peptide regions in renin responsible for binding

It is important to identify the regions of renin that are implicated in the uptake process. Since renin is bilobal, there may be 2 binding sites in the protein that are important for binding. To test this, chimera proteins can be constructed using a combination of native human renin and Cathepsin D or pepsin (related aspartic proteinases that are structurally similar to renin), or rat renin to test if binding still occurs. Alternatively, fusion proteins made from various portions of HA1-tagged prorenin or renin fused to immunoglobulin can be constructed to see if those regions alone can direct uptake in the tissues.

4.8.2. Future experiments with transgenic mice

Transgenic mice will be generated expressing the HA1-tagged protein constructs under the control of the transthyreitin promoter and direct specific expression to the liver. Immunohistochemistry on different tissues obtained from these animals will help to provide visual evidence for renin uptake and to confirm that important binding regions identified in future *in vitro* studies are also fundamental *in vivo*. This approach will allow us to test for uptake of human renin *in vivo* without disrupting the endogenous mouse RAS. This is due to the fact that human renin does not cleave mouse angiotensinogen [67]. This is also confirmed in transgenic mice studies where mice carrying human renin and human angiotensinogen genes caused sustained increases in blood pressure, but single gene carriers had no change in blood pressure levels [68]. Because of the high degree of sequence homology that exists between mouse and human renin genes, it is likely that the mouse renin-binding protein will be able to recognize and bind human renin. Preliminary studies on the efficiency and specificity of the transthyreitin promoter show that indeed expression of the renin-expressing plasmid is restricted to HepG2 liver cells and that no expression was seen in a non-

liver cell line (GH_4Cl). Renin and renin variants will thus be expressed in the liver and other organs such as the heart, brain, kidneys and reproductive organs can be examined to see if uptake occurs. Furthermore, the specificity of this renin-binding protein will be tested by generating mice transgenic for HA1-tagged Cathepsin D (an aspartyl protease similar to renin) to see if uptake still occurs.

4.9. Implications of a renin binding site in tissues

The identification of a renin binding site would lend support to the existence of tRAS that would control local levels of AII in a manner distinct from that of the circulating RAS. The physiological importance of these binding sites will have to be assessed by further studies on the exact nature, location and structure of the sites. If these sites are in reality binding proteins, then their purification and characterization are an essential key to the puzzle involving the tRAS. Development of specific inhibitors to the binding sites may provide a new and effective way to blocking the local RAS and provide a new avenue for the treatment of hypertension.

5. CONCLUSIONS

In this study we were able to demonstrate for the first time in human tissue, the existence of a high affinity, saturable binding site for renin and prorenin. The K_d of human renin binding to cotyledon membrane preparations was estimated to be 125 pM. In light of all the evidence underlying the importance of the tissue renin-angiotensin system, this finding is great. The presence of a renin binding site allows us to formulate a mechanistic explanation on how local angiotensin peptides can be generated. Renin in the circulation could be taken up by tissues via this specific binding site and would be able to act upon locally generated or circulation-derived angiotensinogen to generate angiotensin I. Angiotensin converting enzyme, which is abundant in the membranes of endothelial cells, would be able to cleave the decapeptide to angiotensin II, which could then act in an autocrine or paracrine manner to control local circulation or to contribute to the maintenance of long term hemodynamics.

The interesting finding that prorenin binds to the same site as renin but without the ability to generate angiotensin peptides following substrate presentation suggests that prorenin may act as a natural antagonist to the tRAS. By competing with renin for the same binding sites, prorenin could thus limit the amounts of locally generated angiotensin II.

We have shown in these studies that neither the carbohydrate residues nor the active site of renin is essential in this uptake process. It will be interesting in future experiments to determine what regions of the protein are necessary for recognition and binding to tissues. Furthermore, the purification and characterization of this binding site will provide invaluable information to the whole question of the functional presence of a tRAS. In addition, the long term possibility of developing specific

inhibitors to renin uptake may eventually lead to novel treatments for controlling hypertension.

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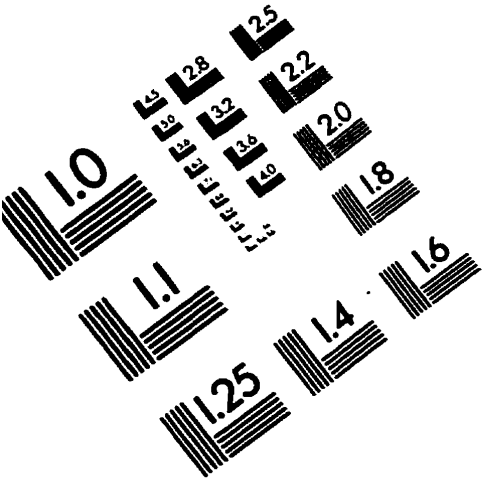
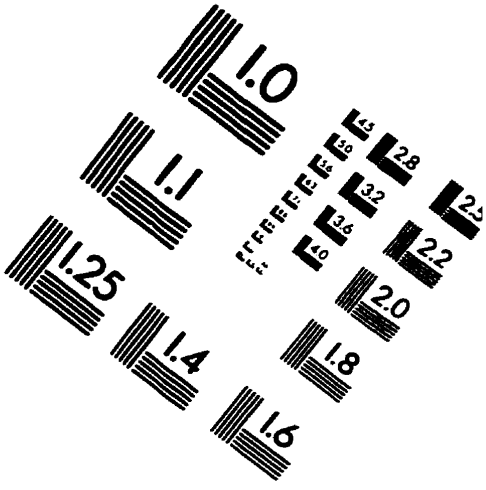
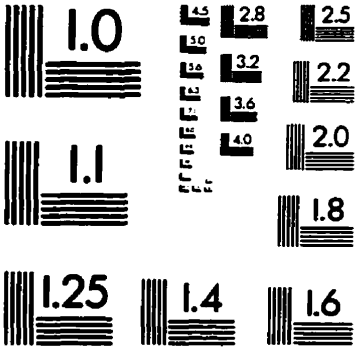
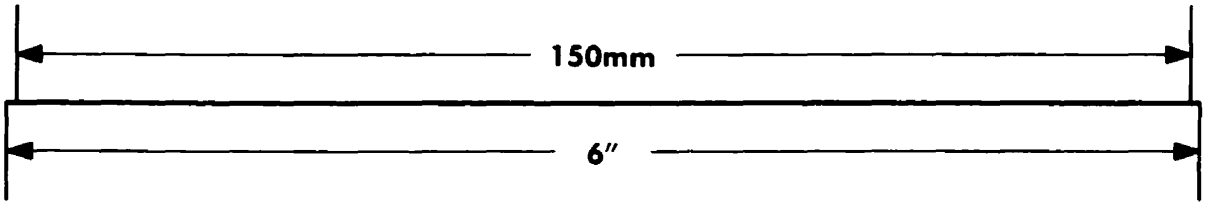
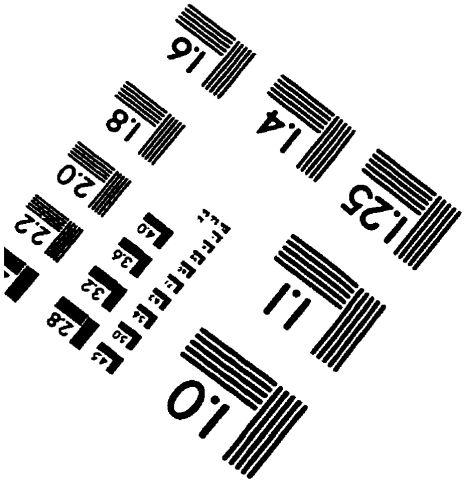
IMAGE EVALUATION TEST TARGET (QA-3)

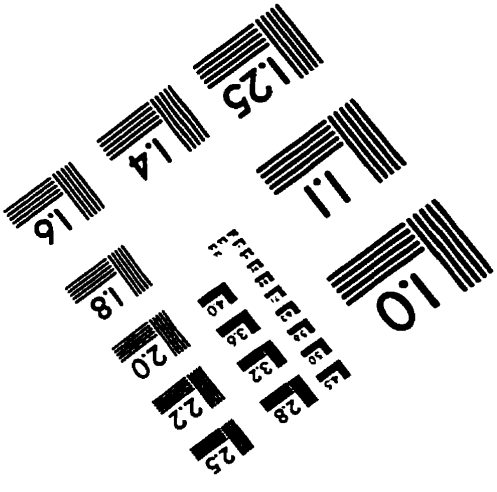












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