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ROLE OF CIRCULATING (PRO)RENIN IN TISSUES: IN VIVO STUDIES ON TRANSGENIC MOUSE MODELS.

by

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Summary

The renin-angiotensin system (RAS) is involved in the regulation of blood pressure and electrolyte balance as well as in the pathogenesis of several diseases, including hypertension. The RAS has been described as an endocrine system in which all of the constituents are present in the circulation. Renin catalyses the cleavage of liver-derived angiotensinogen thereby initiating the first and rate-limiting step in the generation of the potent vasoactive peptide angiotensin II (Ang II) in circulation. Renin is secreted primarily from the kidneys along with it biosynthetic precursor, prorenin. Observation of presence of these constituents in tissues has led the proposal of the existence of local or tissue RAS. In cardiovascular tissues, the origin of renin is still a controversial issue. Previous studies have provided evidence *in vitro* for the uptake and local activity of circulating renin in such tissues. However, direct evidence from *in vivo* studies is still lacking.

We have created an *in vivo* model to directly test whether circulating renin and prorenin are taken up by tissue to contribute to local generation of angiotensin peptides. In this model, transgenic mice which release human active renin and/or prorenin into the circulation exclusively from the liver were mated to mice expressing human angiotensinogen exclusively in the heart. The measurement of the products of the reaction, Ang I and II, in the hearts of double-transgenic mice serves as a direct measure of the ability of circulating renin and prorenin to promote the activity of a local cardiac RAS. Our results provide the first direct evidence that circulating renin contributes by a pressure-independent mechanism to the production of angiotensin peptides in the heart of intact animals. Moreover, we provide the first direct evidence that circulating prorenin contributes to the production of angiotensin peptides in the tissues of intact animals supporting a role for circulating prorenin.

To directly test whether circulating prorenin plays a physiological role in mice by competing for the uptake and tissue action of circulating renin, we have created a second *in vivo* model. In this model, transgenic mice harbor a chronic elevation of circulating mouse prorenin. Our results fail to support a model in which circulating prorenin could act as a

natural antagonist of renin binding in the vasculature and be responsible for the circulatory modifications and pathologies associated with its chronic elevations.

In conclusion, our studies permitted us to confirm that uptake and local activity of both circulating renin and prorenin take place *in vivo*. However, no significant blood pressure-lowering role can be attributed to circulating prorenin in mice.

Résumé

Le système rénine-angiotensine (SRA) est reconnu comme un facteur prépondérant dans le maintien de la pression sanguine et de l'équilibre électrolytique ainsi que dans la pathogénèse de diverses maladies tel que l'hypertension. Ce système est décrit comme endocrinien et implique la rencontre de ces composantes présentent en circulation. La rénine catalyse le clivage de l'angiotensinogène produite du foie. Cette première réaction représente l'étape limitante menant à la génération de l'angiotensine II, un peptide vasoactif. La rénine est sécrétée principalement du rein tout comme la prorénine, son précurseur biologique. L'observation de la présence de ces composantes dans les tissus a mené à proposer l'existence de SRA locaux (tissulaires). L'origine de la rénine dans les tissus du système cardiovasculaire fait encore l'état d'une controverse. Des études antérieurs tendent à démontrer que la rénine peut être capter de la circulation par différent tissus et qu'elle possède une activité enzymatique locale (tissulaire). Toutefois, aucune étude n'a permis de confirmer de telle observation *in vivo*.

De ce fait, nous avons créé un modèle expérimental nous permettant de tester directement l'hypothèse voulant que la rénine et que la prorénine soient captées par diverses tissus pour contribuer à la génération locale de peptides angiotensine *in vivo*. Ce modèle consiste au croisement de souris transgéniques exprimant le gène codant pour la rénine ou la prorénine humaine dans le foie avec des souris exprimant le gène codant pour l'angiotensinogène humain dans le coeur. Le dosage de l'angiotensine I et II, les produits de la réaction, dans le coeur des souris transgéniques porteur des deux gènes servira à déterminer la capacité de la rénine et de la prorénine humaine circulante à promouvoir l'activité d'un SRA cardiaque local. Nos résultats ont permit de fournir l'évidence directe que la rénine en circulation contribue à la production de peptides angiotensine dans le coeur d'animaux intacts, et ce indépendamment de la pression sanguine. De plus, nous avons fournis l'évidence directe de la contribution de la prorénine circulante à la production de peptides angiotensine dans les tissus d'animaux intacts ce qui appuie un rôle pour la prorénine.

De façon à tester directement si la prorénine en circulation joue une rôle physiologique chez la souris, nous avons développé un second modèle expérimental in vivo. Ce modèle consiste à des souris transgéniques surexprimant le gène codant pour la prorénine de souris. Nos résultats ne supportent pas le modèle voulant que la prorénine circulante agit comme un antagoniste naturel de la liaison de la rénine aux récepteurs présent dans la parois des vaisseaux et qu'elle soit responsable des modifications et pathologies des vaisseaux sanguins associées normalement à son taux plasmatique élevé de façon chronique.

En conclusion, nos études nous ont permis de confirmer que la rénine en circulation était bien captée par divers tissus *in vivo* et qu'elle était active localement. De plus, nous avons démontré que la prorénine en circulation avait la capacité d'être active localement lui permettant de fournir une activité rénine au niveau tissulaire. Toutefois, il semble que celle-ci ne joue aucune rôle physiologique important chez la souris.

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List of Abbreviations

ACE	angiotensin-converting enzyme
ANF	atrial natriuretic factor
APTES	3-aminopropyltriethylsilane
Ang	angiotensin
AT	angiotensin receptor
bFGF	basic FGF
bp	base pair; kb, kilobase pair
BSA	bovine serum albumin
°C	degree Celcius
cDNA	complementary deoxyribonucleic acid
Ci	curie(s); μCi, microcurie(s)
Da	dalton; kDa, kilodalton
DAB	diaminobenzidine
DAG	diacylglycerol
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetracetic acid
ELISA	enzyme-link immunosorbant assay
ET	endothelin
FACS	fluorescence-activated cell sorting
FGF	fibroblast growth factor
g	gram(s); mg, milligram(s); pg, picogram(s); μg, microgram(s)

g	velocity
h	hour(s)
HRP	horseradish peroxidase
Ig	immunoglobulin
IGF	insulin growth factor
IP_3	inositol 1,4,5-triphosphate
IR	immunoreactive
JG	juxtaglomerular gland
K_{cat}	catalytic constant
K_d	dissociation constant
K_m	Michaelis-Menten constant
L	liter(s); mL, milliliter(s)
m	meter(s); mm, millimeter(s); nm, nanometer(s); μm, micrometer(s)
M	molar; mM, millimolar; nM, nanomolar; μM, micromolar
MHC	myosin heavy chain
min	minute(s)
mol wt	molecular weight
mRNA	messenger RNA
NGS	normal goat serum
PAGE	polyacrylamide gel electrophoresis
PDGF	platelet derived growth factor
PRA	plasma renin activity
PCR	polymerase chain reaction

PRC	plasma renin concentration
RAS	renin-angiotensin system
RIA	radioimmunoassay
RNA	ribonucleic acid
RnBP	renin-binding protein
SBP	systolic blood pressure
SD	standard deviation
SDS	sodium-dodecyl sulfate
SEM	standard error measurements
SMG	submandibular gland
TBS	tris-buffered saline
TGF	transforming growth factor
TRC	total renin concentration
TTR	transthyretin
Tris	tris(hydroxymethyl)aminomathane

If we knew what we were doing,
it wouldn't be called research, would it?
- Albert Einstein (1879-1955)

To Natalie, Maryanne
and my mother

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Preface

The present thesis, consisting of 5 chapters, has as a theme the functional role of circulating (pro)renin in tissues. Chapter 1 is a literature review covering our present knowledge of the roles of circulating RAS and of its constituents. Emphasis is made on the evidence for the existence and function of local RAS, especially in the heart. Subsequently, the question of the origin of renin in tissues is presented introducing the concept of renin uptake. Finally, hypotheses concerning prorenin function *in vivo* are presented as well as the experimental approach used for the present studies (e.g. transgenic mouse model) to test these hypotheses.

Chapter 2 to 4, inclusively, are comprised of scientific articles, in their original form, in accordance with the McGill University "Guidelines for Thesis Preparation" which reads as follows:

> Candidates have the option of including, as part of the thesis, the text of one or more papers submitted or to be submitted for publication, or the clearly-duplicated text of one or more published papers. These texts must bound as an integral part of the thesis.
>
> If this option is chosen, connecting texts that provide logical bridges between the different papers are mandatory. The thesis must be written in such a way that it is more than a mere collection of manuscripts; in other works, results of a series of papers must be integrated.
>
> The thesis must conform to the other requirements of the "Guidelines for Thesis Preparation". The thesis must include: a Table of Contents, an abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a review of the literature, a final conclusion and summary, and a thorough bibliography or reference list.
>
> Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgment to be made of the importance and originality of the research reported in the thesis.

> In the case of manuscripts co-authored by the candidate and others, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. Supervisors must attest to the accuracy of such statements at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the authors of the co-authored papers.

Thus, in accordance with these regulations, small connecting texts will be presented before each of the chapters 2 to 4 describing the logical progression of the work and the contribution of each co-authors.

Finally, chapter 5 includes a detailed discussion of the relevance of the findings in the thesis and attempts to describe the future prospects of the studies on uptake and local activity of circulating (pro)renin in tissues. This is followed by a claim to originality, the Appendices in which additional studies are reported and, finally, a general bibliography follows and includes the references for chapters 1 and 5. The references for chapters 2 to 4 inclusively are found at the end of the respective chapter.

CHAPTER 1

General introduction

More than 100 years ago, a pressor activity was discovered in kidney extract and the name renin was coined in 1898 (1). Renin remained a mysterious entity until it was purified in stable form in the 1970s. During this 70-year period, the pathophysiological importance of renin in renovascular hypertension was established. Identification of renin as an enzyme that produces the pressor peptide angiotensin (a hybrid of angiotonin and hypertensin) laid a foundation for the future development of research on the renin-angiotensin system (RAS), as we know it today (2). This led to extensive studies on angiotensins and their formation from angiotensinogen by renin and angiotensin-converting enzyme.

1 The circulating renin-angiotensin system

The RAS has traditionally been viewed as a hormonal (endocrine) system. However, unlike many other hormones, angiotensin II (Ang II), the effector component of the system, is not released into the circulation by a group of specialized cells in one single organ, but it is produced in the circulation and the components required for angiotensin synthesis are derived from many different organs (Figure 1.1). The precursor protein, angiotensinogen, is produced in the liver. It serves as the substrate for renin, an aspartyl protease originating from the kidney to form the decapeptide angiotensin I (Ang I). Ang I is subsequently converted into the octapeptide angiotensin II (Ang II) by angiotensin-converting enzyme (ACE), a membrane-bound metallopeptidase, located at the luminal side of the vascular endothelium. ACE also circulates in an active soluble form in blood plasma. Ang II is a potent hormone and its synthesis and degradation are tightly controlled. Angiotensin-degrading enzymes, so-called angiotensinases, present at the luminal surface of the vascular endothelium and in the circulating blood, degrade Ang I and II into smaller, mostly inactive, fragments.

Ang II is the active mediator of the cardiovascular actions of the RAS. It is involved in the regulation of blood pressure in several ways. Not only is Ang II a potent vasoconstrictor, it also regulates blood volume and sodium balance through stimulation of water- and sodium retention by the kidneys (Figure 1.2). The action of Ang II in renal water and sodium handling are mediated by the peptide's direct effect on renal tubule cells (3) and the renal vasculature, as well as by its indirect effects on the kidney via the

production and release of aldosterone from the adrenals (4,5). Ang II also acts within the heart, the central and autonomous nervous systems to amplify its volume-retaining and vasoconstrictive effects on peripheral vascular systems. In addition to cardiovascular homeostasis, Ang II effects on tissue involve biological responses such as angiogenesis (eye), gluconeogenesis (liver), fibrosis (heart and kidney), and altered exploratory behavior (brain) (6-16).

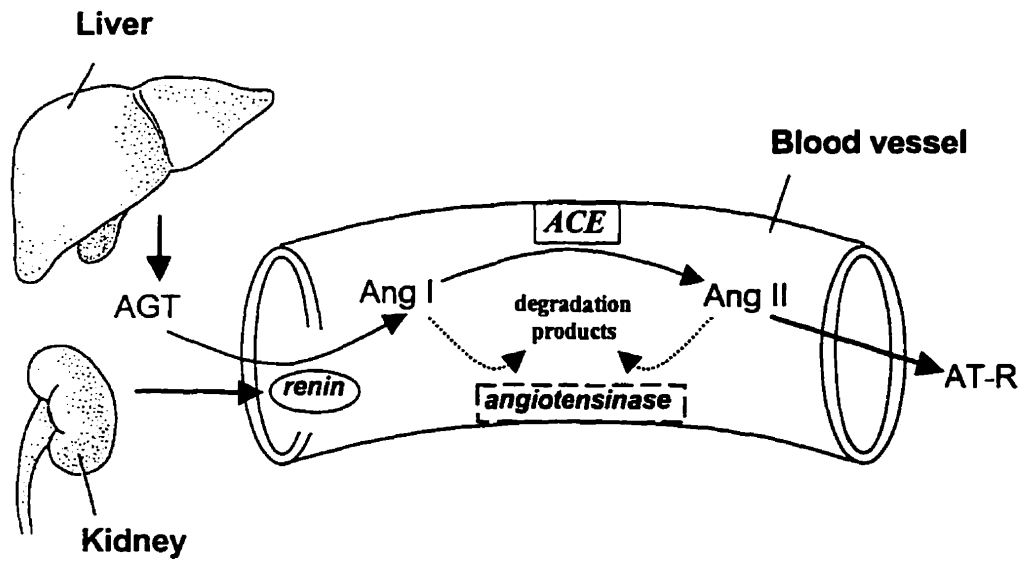

Figure 1.1 The classical concept of the renin-angiotensin system. Components: angiotensinogen (AGT), angiotensin (Ang) I and II, and angiotensin II receptors (AT-R).

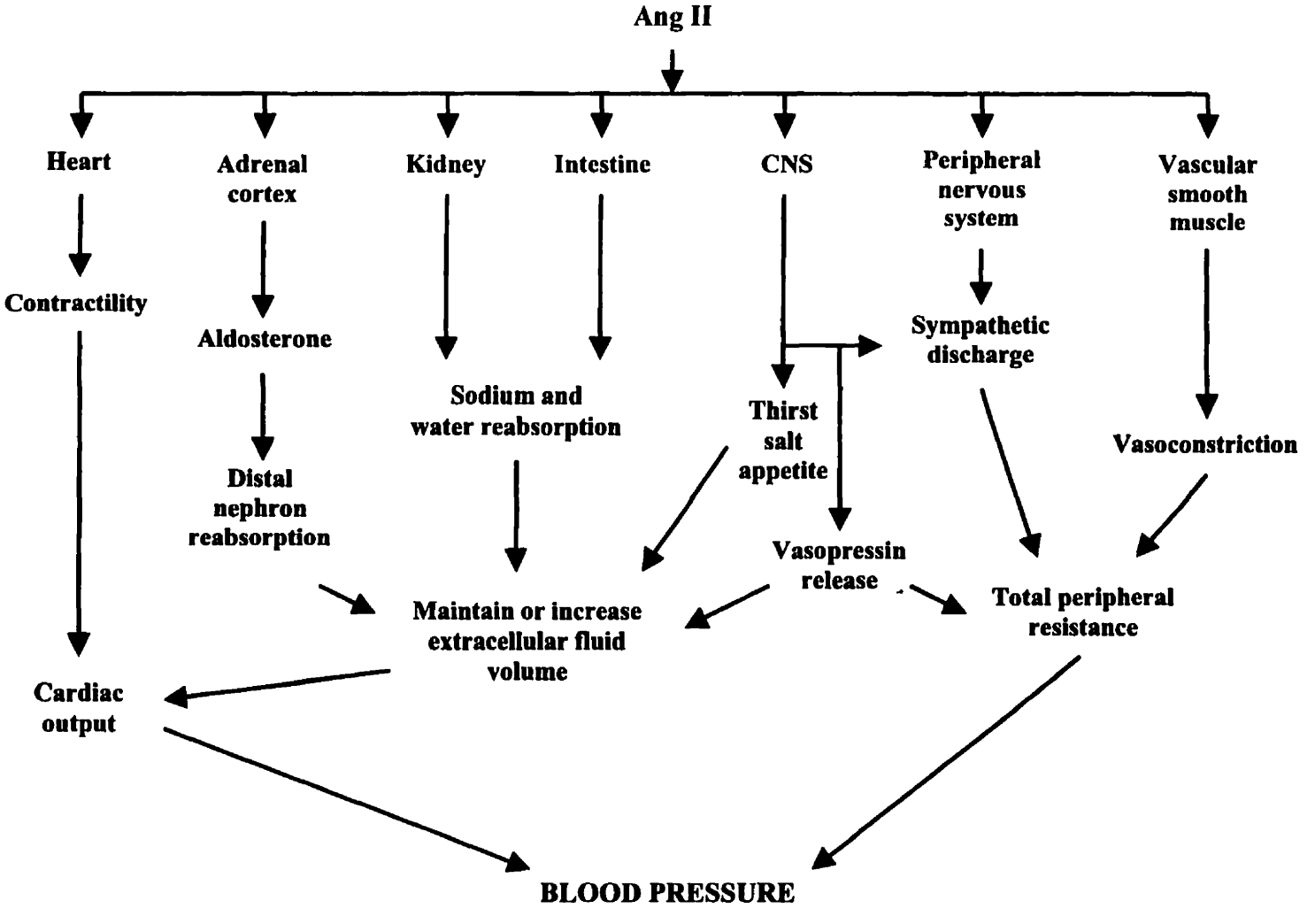

Figure 1.2 Schematic representation of the major effects of angiotensin (Ang) II on blood pressure. CNS, central nervous system

All of the biological actions of Ang II are mediated through binding to specific membrane receptors, followed by an intracellular cascade of second messenger reactions, leading eventually to the cellular response. For Ang II, at least two different subtypes of receptors, AT_1 and AT_2, have been identified, based upon differential binding of non-peptide Ang II receptor antagonists (17). Both subtypes belong to the seven transmembrane domain receptor superfamily. Most of the known physiological responses of Ang II are mediated by the AT_1 receptor. In mouse and rat two isoforms of the AT_1 subtype receptor can be distinguished based on their amino acid sequences, AT_{1a} and AT_{1b} (18-22). Because pharmaceutical inhibition of the AT_1 receptor does not discriminate between these isoforms, the relative physiological roles of AT_{1a} and AT_{1b} and their relationship to human AT_1 receptor functions have been difficult to identify. Recent gene-targeting experiments have clarified the relative role of the AT_{1a} and AT_{1b} receptors in the periphery, demonstrating a predominant role for AT_{1a} receptors in regulation of vascular tone (23,24). Stimulation of the AT_1 receptor is associated with a hypertrophic or hyperplastic growth response in fibroblasts and myocytes (25-28), whereas stimulation of the AT_2 receptor seems to be anti-proliferative and pro-apoptotic (29-32).

The signal transduction events stimulated by Ang II binding to AT_1 receptor lead, via interaction with a G protein (e.g., mainly $G_{q\,\alpha}$), to the activation of phospholipase C, which hydrolyses phosphatidylinositol 4,5-biphosphate to inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 mobilizes Ca^{2+} from the endoplasmic reticulum, while DAG activates protein kinase C (33,34). Additional signal transduction pathways reported to be triggered by AT_1 receptor stimulation include the JaK/STAT pathway and the Ras/MAP kinase pathways (35-39). The signal transduction events of the AT_2 receptor are not as well defined, but they have been shown to include inhibition of MAP kinase activation (30,40,41). Coupling of the AT_2 receptor to a G protein (e.g., G_i) has been demonstrated (30,32,42,43). Moreover, apoptosis attributed to AT_2 receptor stimulation seems to be mediated through the second messenger ceramide (44,45).

In addition to the AT_1 and AT_2 plasma membrane receptors, Ang II binding sites have been detected in the cytosol and nucleus of cells (46-52). Some of these binding proteins have AT_1 receptor-like characteristics (46,48,52). The function of these intracellular

receptors is presently unknown. However, a small but growing body of evidence has developed to indicate that intracellular receptors could have effects on the transcription regulation of renin and angiotensinogen, on cell-to-cell communication and impulse propagation (53-56).

Although the primary and most important effector product of the RAS is Ang II, recent evidence has demonstrated that some amino and carboxy terminal derived fragments of Ang II possess some biological activity. These angiotensin metabolites include angiotensin III (Ang III), angiotensin IV (Ang IV), and angiotensin [1-7] (Ang [1-7]) (Figure 1.3, Table 1.1).

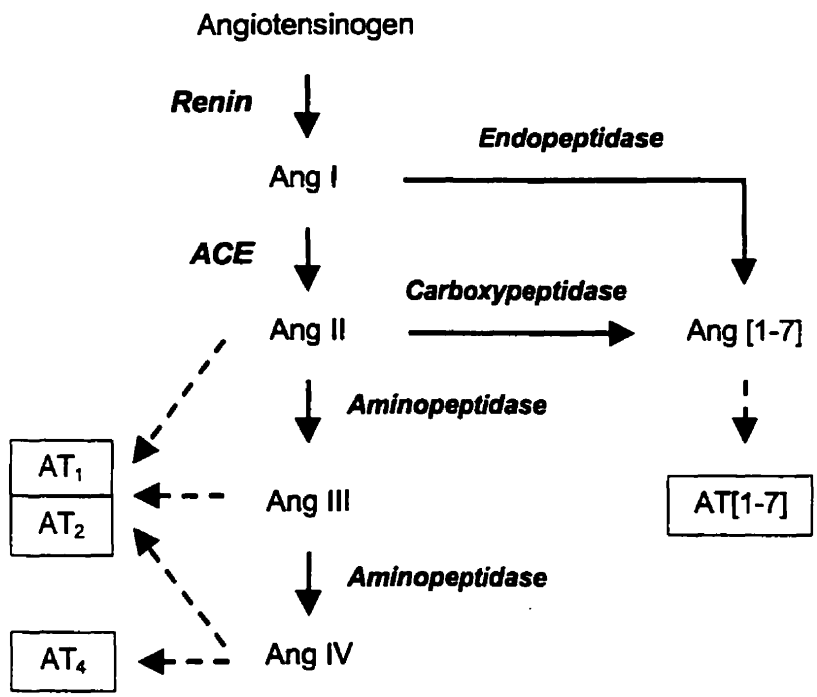

Figure 1.3 Generation of angiotensin preptides (Ang) from angiotensinogen and activation of various angiotensin receptor (AT) by angiotensin II and its metabolites.

Table 1.1 Angiotensin peptide sequences

Angiotensin peptide	*Amino acid sequence (position)* 1 2 3 4 5 6 7 8 9 10 11 12 13 14
AGT	NH_2-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Asn-**R**-COOH
Ang I	NH_2-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-COOH
Ang II	NH_2-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-COOH
Ang III	NH_2-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-COOH
Ang IV	NH_2-Val-Tyr-Ile-His-Pro-Phe-His-Leu-COOH
Ang [1-7]	NH_2-Asp-Arg-Val-Tyr-Ile-His-Pro-COOH

* Sequence derived from human angiotensinogen. **R** in angiotensinogen consists of 438 amino acids. AGT, angiotensinogen. Ang, angiotensin.

Ang III is a direct metabolite of Ang II, produced by aminopeptidases (mainly aminopeptidase A: APA) (Figure 1.3). In the brain, Ang III effects are similar to Ang II causing pressor and dipsogenic effects as well as appetite when injected into cerebral ventricles (57). Recent evidences suggest that Ang III is the major effector peptide in the brain, accounting for some physiological effects thought to be mediated by Ang II (58). Ang III biological activity is mediated through binding AT_1 and AT_2 receptors (59,60).

Recently the existence of a new angiotensin receptor type, AT_4, has been proposed that preferentially binds Ang IV, a product from further metabolism of Ang II or Ang III peptide (61-63) (Figure 1.3). Its natural ligand may however be Leu-Val-Val-hemorphin 7 rather than Ang IV (64). This receptor is prominent in the brain (65-67). Peripheral tissues that reveal a high content of AT_4 are kidney, bladder, heart, spleen, prostate, adrenals, and colon (68). The primary functions thus far associated with this Ang IV/AT_4 system include memory acquisition and retrieval, the regulation of blood flow, inhibition of renal tubular sodium reabsorption, and cardiac hypertrophy (69). There is a preliminary indication that

this system may also be involved in neurite outgrowth (57,70), angiogenesis, and stimulation of endothelial cell expression of PAI-1 (71), and repair of blood-brain barrier (72).

Ang [1-7] is primarily a product of Ang I metabolism by endopeptidases (e.g., neprilysin, metallo- and prolyl endopeptidases) (73), although it may also be cleaved from Ang II by a carboxypeptidase (e.g., post-proline carboxypeptidase) (74) (Figure 1.3). Ang [1-7] biological effects include vasodilatation, stimulating the synthesis and release of vasopressin, prostaglandins, and nitric oxide, and potentiating the hypotensive effects of bradykinin (75). These effects seem to be mediated via a non-AT_1/AT_2 receptor subtype as suggested by it sensitivity to non-selective angiotensin antagonist, [Sar^1-Thr^8] Ang II (76). Furthermore, a relatively high affinity-binding site (10-20 nM) for Ang [1-7] has been described (77-79). However, characterization of these sites has not yet been completed (75). Recent reports indicate that Ang [1-7] may bind to ACE to be further metabolized (80-83).

2 The RAS components: renin, ACE and angiotensinogen

2.1 Renin

Renin belongs to the class of aspartyl proteinases which consist of two similar domains each containing a catalytically essential aspartic acid residue with the active form produced from the proenzyme by a proteolytic cleavage of the N-terminal propeptide. However, renin differs from the others members of this class in several respects. It has a pH optimum of 5.5-7.5 instead of 2.0-3.4 (84). This neutral pH optimum is essential for it to be functional in the plasma. Renin also differs to other aspartyl proteinases by having a highly restricted substrate specificity. Unlike other aspartyl proteinases, renin activation is not autocatalytic. Renin has no known biochemical role other than the proteolysis of renin substrate, angiotensinogen. Renin has a very high degree of selectivity for the amino acid sequence on either side of the unique scissile peptide bond of angiotensinogen. Human renin cleaves the bond between the Leu^{10} and Val^{11} residues in human angiotensinogen (Table 1.1). The minimum size of a substrate cleaved by renin is the octapeptide Pro^7-Phe^8-His^9-Leu^{10}#Val^{11}-Ile^{12}-His^{13}-Asn^{14}; this reflects the size of the active site and gives clues to

the nature of the residues involved in substrate binding. Additionally, renin from almost all species including human cleaves the Leu^{10}-Leu^{11} bond in pig, horse and sheep angiotensinogen. It is well known that non-primate renins react very poorly with primate angiotensinogen, whereas human renin reacts relatively well with non-primate angiotensinogen. The presence of an asparaginyl *N*-glycosylation site near the scissile bond may very well account for the selectivity. The enzyme activity of renin is determined by measuring the rate of production of Ang I from angiotensinogen by radioimmunoassay (85) or enzyme immunoassay (86).

Mature native renin is a single-chain polypeptide of about 40-50 kDa, pI 5.7, which contains two disulfide bonds and two potential *N*-glycosylation sites (87). Renin is encoded by a single gene locus (*Ren-1*) located on chromosome 1. The renin gene is ~12.5 kb in size and contains 10 exons and 9 introns. The structures of renins (derived from the *ren-1* gene) were deduced from the nucleotide sequence of cDNA (human and rat) which revealed the presence of preprorenin and prorenin consisting of about 400 amino acid residues (88,89). A single-chain form of renin of 42 kDa was produced by recombinant methods.

Renin is produced as prorenin with a 43 residue prosegment attached at the N-terminus of mature renin (90-94). Prorenin is an inactive proenzyme that is activated *in vitro* by trypsin, cathepsin B and other proteinases (95). Prorenin has to be sorted to the dense core secretory granules in the juxtaglomerular cells to be processed into mature renin. Prorenin, as well as renin, are released and present in the circulation and prorenin accounts for approximately 90% of total renin (prorenin + renin) in circulation.

The prosegment of prorenin is responsible for the inhibition of renin. Prorenin has less than 10% of the full activity of renin, but its activity is non proteolytically increased by lowering the pH or temperature, or by lipids (96-99). The first two phenomena have been called acid-activation and cryoactivation, respectively. Recombinant prorenin has also been reversibly activated by exposure to low temperature or acidic pH (100) and a conformational change can be induced in the active cleft by adding nonpeptide renin inhibitors (101).

2.2 Angiotensin converting enzyme (ACE)

ACE is a zinc metallopeptidase which cleaves the C-terminal dipeptide from Ang I to produce Ang II and inactives bradykinin by the sequential removal of two C-terminal dipeptides (102). In addition to these two main physiological substrates, ACE hydrolyzes a wide range of substrates including neurotensin, [Met^5]-enkephalin-Arg^6-Gly^7-Leu^8, β-neoendorphin, donorphins, the insulin B chain, substance P and luteinizing hormone-releasing hormone (LH-RH) *in vitro*. Clearly, ACE displays a wide substrate specificity and is implicated in a range of physiological processes unrelated to blood pressure regulation such as immunity, reproduction and neuropeptide regulation.

ACE is an ectoenzyme anchored to the plasma membrane with the bulk of its mass exposed at the extracellular surface of the cell. There are two ACE isoforms: a somatic form of around 150-180 kDa found in endothelial, epithelial and neuronal cells and a smaller isoform (mol wt 90-110 kDa) present in germinal cells. Somatic ACE is found in the plasma of membrane vascular endothelial cells, particularly in the lung, and where it is well placed to metabolize circulating substrates (103,104). A soluble form of ACE is present in many biological fluids, such as serum, seminal, amniotic and cerebrospinal fluid. It appears to derive from proteolysis of the membrane-bound form of the enzyme in endothelial cells. The smaller isoenzyme of ACE (germinal ACE) is found exclusively in the testis (105,106).

Molecular cloning of the somatic form of ACE demonstrated that the enzyme is composed of two homologous domains, called hereafter N- and C-domains. The germinal form contains only one of the C-terminal domain. Both domains contain a putative catalytic site characterized the zinc-binding motif HEXXH widely found in metalloproteases (107).

2.3 Angiotensinogen

Angiotensinogen is a globular glycoprotein with a molecular mass between 55 and 65 kDa, depending on its state of glycosylation. It contains four putative N-linked glycosylation sites. Angiotensinogen belongs to the serpin (serine protease inhibitor) superfamily, which includes α1-antitrypsin, α1-antichymotrypsin, and antithrombin III (108,109). Based on its structural similarity with these proteins (~20 % amino acid sequence identity),

angiotensinogen represents one of the most distant members of the serpin superfamily and has probably lost its serine protease-inhibitory activity. Angiotensinogen has no known physiological role other than to be the precursor of angiotensin peptides. Angiotensinogen is usually measured by an enzymatic assay for Ang I after its complete hydrolysis by excess renin. However, a direct immunoassay has also been developed (110).

3 Regulation of circulating RAS activity

As an endocrine system, the circulating RAS is subject to feedback inhibition. It is activated under conditions of reduced blood pressure, sympathetic stress, or volume contraction and is inactivated when these conditions are normalized. The compensatory effect of this system is generally thought to be mediated through changes in the rate of secretion of renin from the kidney. This come from the facts that the Michaelis-Menten constant (K_m) of renin-angiotensinogen reaction (1.25 μM) is similar to the angiotensinogen concentration in plasma (1 μM), that angiotensinogen is secreted constitutively from liver and its half-life is quite long: 10 h (111) compared to the renin half-life of 20-30 min (112-114). Renin is stored in granules and released upon stimulation. The plasma concentration of renin is in the nanomolar range (1000 times less than angiotensinogen) in normal conditions.

Activation of the circulating RAS is marked by the stimulation of renin secretion from the kidney and is accompanied primarily by an increase in renin mRNA in these cells (115). Furthermore, under maximal stimulatory conditions, some of the renin secretion stimulators also elicit a transdifferentiation mechanism in neighboring smooth muscle cells which are "recruited" for renin expression (116,117). Under these conditions, smooth muscle cells along the entire afferent arteriole begin to synthesize renin. Different mechanisms have been shown to control the release of renin. Adrenergic stimulation was found to suppress renin release, whereas high salt diet (the macula densa mechanism), high blood pressure (the baroreceptor mechanism), and the direct action of Ang II (short-loop negative feedback) were shown to regulate renin release in response to minute-to-minute changes in these physiological conditions to maintain blood pressure homeostasis.

In the liver, angiotensinogen synthesis and release is stimulated by estrogens, glucocorticoids, thyroid hormones and Ang II (118-120).

ACE has been detected on many cell types, but the conversion of circulating Ang I is mainly mediated by endothelial ACE, plasma ACE being of minor importance (121). Plasma ACE levels are partly determined by the so-called insertion/deletion ACE gene polymorphism (122). This may also apply to tissue (i.e. endothelial) ACE (123). Moreover, ACE expression is subject to negative feedback by Ang II (124).

4 Measurement of circulating RAS activity

In view of the fact that some forms of hypertension are directly associated with RAS activity, much interest has been directed toward the measurements of plasma renin levels as an indication of RAS activity in circulation. Plasma renin activity (PRA) is the most commonly used index for it activity and represents the rate of formation of Ang II *in vivo*. PRA is estimated from the amount of Ang I generated from endogenous angiotensinogen during incubation of the plasma sample with inhibitors of Ang I degradation. However, in view of the substrate-dependence of PRA (e.g. reaction follows the classic Michaelis-Menten kinetics), changes in renin activity are not exclusively related to changes in renin concentration (125-127). Therefore, measurement of plasma renin concentration (PRC) and/or the concentration of angiotensinogen is required for quantitative interpretation of changes in plasma renin activity. PRC is measured under the identical conditions with the exception that sufficient exogenous angiotensinogen is added to the incubation mixture to saturate the renin present in the sample. Assuming substrate saturation, the reaction process becomes zero-order with respect to angiotensinogen and thus the velocity is dependent solely upon the amount of enzyme present.

5 Pharmacological blockade of RAS

Pharmacological blockade of RAS is an effective tool for the treatment of hypertension and its complications. Blockade is possible at the level of renin, ACE or the AT_1 receptor. ACE inhibitors, the class of RAS blockers that was developed first, have the disadvantage that ACE is not a specific enzyme. It hydrolyses several substances other than Ang I, such as

bradykinin, substance P and enkephalins (128). Some side effects observed with ACE inhibitor therapy may be related to this non-specificity.

Angiotensinogen is the only known substrate for renin, and renin inhibition may therefore be devoid of the side effects observed during ACE inhibition. The non-peptide renin inhibitors that have been developed so far are effective blood pressure lowering agents (129-131). However, because of their poor oral bioavailability, renin inhibitors are not widely used in the clinic.

The most recently developed class of blockers of the RAS is the non-peptide AT_1 receptor antagonists (132,133). Somewhat surprisingly, these drugs do not completely eliminate the side effects of ACE inhibitors, however, they may overcome the problems arising from the fact that long-term treatment with ACE inhibitors does not fully suppress the levels of Ang II, especially those at tissue sites (134). This incomplete suppression of Ang II production may be due to enhanced Ang I formation (as a consequence of stimulated renin release) or generation of Ang II by enzymes other than ACE.

5.1 Effect of RAS inhibitors on circulating RAS components

Inhibition of the RAS always results in the onset of feedback processes (Table 1.2). Remikiren (a non-peptide renin inhibitor) inhibits plasma renin activity, thereby leading to reduced plasma Ang I and Ang II concentrations (129). As a consequence of removing Ang II-mediated feedback, renin release from the kidney will increase, resulting in elevated circulating renin levels (129,131). However, due to the presence of the renin inhibitor, this renin is enzymatically inactive. ACE inhibitors also cause a rise in plasma renin, and as a result of that, a rise in plasma Ang I. Plasma Ang II is reduced initially, but it may rise to levels above normal during chronic treatment, as a result of the increased renin and Ang I concentrations (135). AT_1-receptor antagonists will not only increase plasma renin and Ang I, but plasma Ang II as well (133,136).

Table 1.2 Effect of selective RAS inhibition on RAS component levels in blood plasma and heart*

	Immuno-reactive renin	*Enzymatically active renin*	*Ang I*	*Ang II*	*Ang II/I ratio*
Plasma					
Renin inhibition	↑	↓	↓	↓	n.d.
ACE inhibition	↑	↑	↑	↓	↓
AT_1 receptor blockade	↑	↑	↑	↑	↔
Heart					
Renin inhibition	n.d.	n.d.	n.d.	n.d.	n.d.
ACE inhibition	↑	↑	↑	↔↓	↓
AT_1 receptor blockade	n.d.	n.d.	↑	↑	↓

* Data are taken from references (129,131,133,134,136-138). ↑, increase. ↓, decrease. ↔, no change. n.d., not determined.

5.2 Effect of RAS inhibitors on tissue RAS components (heart)

At present, not much is known about changes in cardiac RAS component levels during treatment with RAS inhibitors (Table 1.2). Renin is elevated in cardiac tissue of both humans (137) and pigs (138) treated with ACE inhibitors, whereas cardiac angiotensinogen is decreased under these conditions (137,138). ACE inhibition with perindopril leads to a 2-4 fold increase in cardiac Ang I (134) whereas cardiac Ang II did not change unless very high doses of the ACE inhibitor quinalapril are applied. The AT_1 receptor antagonist losartan increases cardiac Ang I and II approximatively 7- and 2-fold, respectively (139). As a consequence of these non-parallel changes in cardiac Ang I and II, the cardiac Ang II/I ratio decreased both with quinalapril and losartan. The decrease in cardiac Ang II/I ratio during quinalapril treatment most likely illustrates the degree of ACE inhibition obtained in cardiac tissue. The decrease in cardiac Ang II/I ratio during losartan treatment is more

difficult to explain, especially since cardiac ACE activity is unchanged following losartan treatment (139). The authors speculate that a proportion of the measured tissue level of Ang II may have been receptor-bound and protected from metabolism and that the displacement of receptor-bound Ang II by losartan may have accelerated local tissue metabolism of Ang II, with a consequent decrease in the Ang II/I ratio.

6 Local (or tissue) renin-angiotensin systems

Based upon discrepancies observed more than twenty years ago between acute changes in circulating levels of RAS components and the changes in blood pressure, it was proposed that so-called local RAS exists in addition to the circulating RAS (140). Subsequent studies showing the presence of RAS components (i.e. angiotensinogen, renin, ACE, angiotensin peptides) at tissue sites appear to confirm this theory (141). However, one has to keep in mind that the mere presence of RAS components in tissue cannot be taken as direct evidence for their local production and participation in a local RAS. Only during the last decade or so have the tools of molecular biology enabled us to prove beyond doubt that the components of the RAS are in fact present locally in many organs and tissues and that they have the capacity to participate in a local RAS. It is now acknowledged that such local RAS may existence in kidney, adrenal, brain, heart, blood vessel wall, ovary, testis, adipose tissue and eyes (142-151).

There is no full agreement on how a local RAS should be defined. Some have defined it as a system that functions completely independently of the circulating RAS (i.e. in situ synthesis of all RAS components required for Ang II production), whereas others support the idea that local Ang II generation depends, at least in part, on blood-derived components (i.e. uptake from circulation of RAS components required for local Ang II production) (141,152). However, irrespective of this debate, the significance of local Ang II formation is widely acknowledged. Produced in the immediate vicinity of their sites of action, local Ang II is implicated in pathophysiological conditions such as hypertension, myocardial infarction, heart failure, restenosis, and atherosclerosis (153-157). Rather than restricting the action of the RAS to a circulating endocrine type, investigations of the tissue RASs have expanded the role of the RAS to actions at the local level. Indeed, locally synthesized

Ang II can exert autocrine, paracrine, or even intracrine effects (158), as opposed to the endocrine effects of circulating Ang II (Figure 1.4).

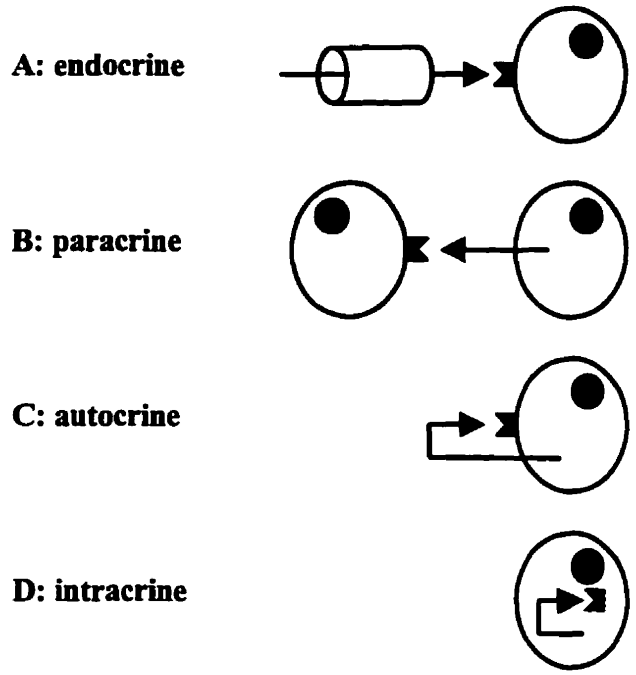

Figure 1.4 Alternative mechanisms of action of angiotensin II (Ang II) in addition to its traditional function as an endocrine factor.

A: Blood-borne Ang II reaches its distant target organ via the circulation, where the peptide produces its effect.

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

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

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

7 Cardiovascular RAS

Among the various tissues in which local RAS has been postulated, the heart has been the focus of intensive investigations, mainly due to more convincing evidence for local Ang II production coming from the recently discovered beneficial effects of the ACE inhibitors and AT_1 receptor antagonists in heart failure and post-infarction remodeling,

which are independent, at least in part, of their systemic effect on blood pressure (132,133,136,159-164). A reduction of Ang II production locally in the heart has been proposed to explain the blood pressure-independent effects of ACE inhibition in cardiac patients. Indeed, substantial evidence for the existence of a functional RAS in cardiovascular tissues has been provided (165-167). Evidence from *in vitro* studies suggests that the Ang II produced by tissue can elicit functional responses in the heart. For example, isolated, perfused hearts from rabbits pretreated with ACE inhibitor demonstrate significant reduction in contractility on pacing (168), suggesting that locally produced Ang II potentiates mechanical activity by altering catecholamine levels at sympathetic nerve terminals. Further evidence for a functional vascular and cardiac RAS has been provided by studies demonstrating that Ang II has direct effects on cardiovascular cells (i.e. fibroblasts, myocytes and endothelial cells), which are associated with growth (i.e. hypertrophy and/or hyperplasia) (25-28), as well as anti-proliferative and pro-apoptotic responses (29-32). Moreover, Ang II also stimulates collagen production by cardiac fibroblasts (169). These lines of evidence support the view that a local RAS is involved in the development and/or progression of cardiovascular disease.

8 Enigma of the tissue RAS (emphasis on the cardiac RAS)

Although a substantial body of evidence documenting the existence, local integration, and physiological function of a local RAS in cardiovascular tissues has been provided, investigators can only speculate on the precise origin of renin in those tissues, the functional significance of local angiotensin and of renin precursor, prorenin. This enigma comes from the fact that, so far, it is still difficult to clearly separate the circulating and local RAS. Although local synthesis of RAS components has been demonstrated in cardiovascular tissues, early investigations have pointed out the possibility that the circulating RAS may serve to deliver renin and possibly angiotensinogen to tissue sites (i.e., in blood vessel wall and heart), where local angiotensin production may then occur. A better understanding of the origin of the RAS components participating in tissue RAS, and physiological function of prorenin may shed further light on the mechanism(s) by which local RASs exert their (patho)physiological effects.

9 Origin of renin in cardiovascular tissues: local synthesis versus uptake from circulation

Unlike ACE and angiotensinogen, it is still debated whether local synthesis of renin occurs in the heart. Renin has been detected in heart and other cardiovascular tissue based on its enzyme activity and immunoreactivity (165). However, the presence of renin mRNA is still controversial (170,171). Although the angiotensinogen and ACE genes are expressed in considerable abundance in the heart (172-174), renin mRNA is barely detectable, even with a quantitative polymerase chain reaction determination (175-177). The bulk of the evidence to date goes against the local synthesis of renin in cardiovascular tissues, and in support of the uptake of renin from circulation.

9.1 Local synthesis of renin

Renin mRNA levels in normal hearts are low or undetectable (178-181), suggesting that under normal circumstances cardiac renin synthesis is unlikely to occur. It is possible, however, that during fetal development (182) or under pathophysiological conditions (179), renin gene expression is increased in the heart. Considering that the kidney is the only tissue known to be capable of converting prorenin to active renin, local expression of the renin gene should lead to generation of the inactive precursor, prorenin. To date, we still have no clear evidence that prorenin is activated in non-renal tissue, either by proteolytic or non-proteolytic process.

Based on the assumption that all molecules produced in a tissue will be eventually released in the interstitial space and then into circulation, studies performed on cardiac and vascular cultured cells failed to demonstrate release and detection of renin in the cultured medium (183,184). Accordingly, studies performed on isolated heart perfused with physiological buffer failed to demonstrated release and detection of renin in the perfusate indicating that renin is not synthesized locally in cardiovascular tissues (185,186).

Therefore, it appears that the renin responsible for cardiac angiotensin generation is not synthesized locally. Renin may then reach the heart via the circulation and thus be of renal origin.

9.2 Kidney as a source of cardiac renin

The kidneys are the primary and apparently the unique source of active renin in the circulation. At least 48 hours of bilateral nephrectomy is needed for renal renin to completely disappear from the circulation. In studies in which bilateral nephrectomy was performed in pig, renin was no longer detectable in cardiac tissue after 20 hours (187). In the rat, aortic renin also disappeared after 24 to 48 hours nephrectomy, with a longer half life than plasma renin (178,187-190) and the cardiac level of renin decreased in parallel with the plasma level of renin to level close or below the limit of detection after a bilateral nephrectomy (178,187).

These data suggest that most, if not all, renin present in the normal heart originates from the kidney. Thus, the heart is capable of sequestrating renin form the circulation.

9.3 Uptake (or sequestration) of renin from circulation

Evidence of the uptake of renin from circulation has been provided by several lines of evidences:

Measurement of renin in cultured cells, tissue or plasma samples

The renin levels in cardiac tissue *in vivo* are too high to be explained by simple diffusion into the interstitial fluid, and renin is enriched in membrane fractions prepared from freshly obtained cardiac tissue (187). Prorenin has been reported to be extracted by the vascular wall (191), the heart (192,193) and kidney (194). Loudon *et al.* (191,195) injected partially purified renin into rats and observed a steep increase in aortic renin-like activity. Swales *et al.* (190) injected mouse renin into rats and found by immunofluorescent studies of the aorta and intrasplenic arteries that this exogenous renin was taken up predominantly by the media. Skeggs (196) infused hog renin for several days into hypertensive rabbits and found a transformed form of renin in the aorta, the carotid arteries (both times mainly at the regions of the smooth muscle cells), the heart, and in the kidney.

The amount of angiotensin release from the isolated perfused Langerdorff heart or hindquarter (well know models to study cardiac and vascular angiotensin production, respectively) depends on the addition of renin to the perfusion fluid (185,186,197-199).

The release of Ang I from the isolated heart during perfusion with renin is too high to be attributed to Ang I generation in either intravascular or interstitial fluid, indicating a role for vascular surface-bound renin (186). Interestingly, angiotensin release continued after discontinuation of renin perfusion, i.e., at a time when renin was no longer present in the perfusate (198,199) suggesting that renin is retained in the vascular wall.

Renin clearance studies

Physiological data supporting the compartmentalization (i.e. sequestration) of tissue renin have been provided by bilateral nephrectomy studies. For example, the rate of disappearance of vascular renin was determined to be much slower than that of circulating renin after bilateral nephrectomy (200). When renin was injected into binephrectomized rat, this renin taken up subsequently in the vasculature was cleared at a much slower rate than that of the rat endogenous plasma renin (191). The data of Skeggs *et al.* (201) would also support this conclusion. These observations taken together support the concept that tissue renin (regardless of its origins) may have concentrations or clearance rates different from that of circulating renin and thereby may act locally in the tissue compartment with enzyme-substrate concentration, kinetics, and clearance that differ from the circulating compartment.

Identification of renin binding protein

In view of the absence of significant renin synthesis at cardiac tissue sites, one may speculate that the heart possesses specific mechanisms to sequester (pro)renin from the circulation. Several groups have reported on the existence of (pro)renin binding proteins and/or receptors (137,202-209). An intracellular renin-binding protein (RnBP, mol wt 40 kDa) was discovered in the early eighties in humans, rats and pigs (207-209). Binding to this RnBP reduces the Ang I generating activity of renin by more than 80%. Recently, this RnBP was found to correspond to the enzyme N-acyl-D-glucosamine 2-epimerase (210), and targeted inactivation of it gene revealed that it might be involved in the intracellular processing of renin rather than in renin uptake and/or regulation (211).

Subsequently, using chemical cross-linking, two vascular RnBPs (mol wt 40 and 70 kDa, respectively) were identified by Campbell and colleagues in membranes isolated from rat mesenteric arteries or cultured rat aortic smooth muscle cells. Interestingly, binding to these RnBPs was inhibited by a specific, active site-directed renin inhibitor, suggesting that the active site of the renin molecule might be involved in the binding process (205).

More recently, with the use of radiolabeled (pro)renin, high affinity renin binding sites/receptors ($K_d \approx 1$ nM) were demonstrated in human mesangial cells and in membranes prepared from rat tissues (204,206). In the rat, these binding sites bound prorenin and renin equally well, which suggest that neither the prosegment nor the active site is involved in the binding process (204). This contrasts with Campbell's findings. In human mesangial cells, renin binding led to the induction of DNA synthesis, most likely without Ang II playing a role as an intermediary between renin binding and the hypertrophic response (206). Thus, a renin receptor may have been identified that directly transduces an intracellular signal.

Schalekamp *et al.* have also reported that cardiac (i.e., cultured neonatal rat cardiomyocytes and fibroblasts) and endothelial cells (i.e. cultured human umbilical vein endothelial cells) were capable of binding and internalizing prorenin and renin, and of activating prorenin after its internalization (202,203). The process has been shown to involve the mannose 6-phosphate receptor, known for it function in the process of intracellular lysosomal enzyme sorting (212). Failure to detect any active renin in the cell culture media suggests that active renin generated from prorenin is not secreted from the cells which take it up (202,203). Whether this phenomenon represents true activation or intermediary processing of prorenin leading to its degradation is not known.

Clinical studies

The relative importance of circulating renin and its possible participation in a cardiac RAS has been highlighted by data provided by both prospective and retrospective clinical studies of hypertensive patients. These results demonstrate a strong correlation between elevated circulating renin levels in hypertensive patients and the risk of myocardial infarction independent of blood pressure (213,214).

Taken together, these findings suggest that renin is not synthesized in cardiac and vascular tissues but rather sequestered from circulation. Moreover, tissue-bound renin may contribute to local angiotensin production in those tissues. However, as we may see from these findings, direct evidence from *in vivo* studies is still lacking.

10 Hypothesis concerning prorenin function *in vivo*

Various hypotheses have been put forward to assess a role for prorenin and, hence, to justify its relative abundance in the circulation (215,216). Most of these hypotheses remain within the framework of the aforementioned enigma. They are summarized in Table 1.3. Some of them are quite obvious, others are highly speculative.

Table 1.3 Hypotheses concerning prorenin

	Hypotheses	*Reference*
1	Only the kidney processes prorenin to renin.	(216)
2	Prorenin is a source of renin, by conversion to renin in the circulation.	(215)
3	Prorenin could play a direct role, not mediated by renin and not necessarily related to fluid, electrolyte and blood pressure homeostasis.	(215)
4	Prorenin can mimic the function of renin *in vivo* without cleavage of its prosegment.	(187)
5	Prorenin could be the transporter of potential renin activity to various target organs, where it acts directly.	(215)
6	Circulating prorenin is a "spill-over" from renal and (or) extrarenal sources, where renin or prorenin functions within those tissues or organs and not in the circulation.	(215)
7	The tissue renin system functions via prorenin.	(187)
8	Circulating renin and prorenin may bind to tissue by specific binding sites.	(216)
9	Prorenin may displace renin from binding sites and thus decrease local renin activity.	(219)
10	Prorenin is a by-product of renin synthesis and thus, possess no functions.	

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

Let us first consider observational data. Anephric subjects have very low, although not zero (217), circulating levels of renin, Ang I and Ang II in the presence of near-normal levels of prorenin and high levels of angiotensinogen (218). This indicates that prorenin is not activated in the circulation and, hence, hypothesis 2 in Table 1.3 is unlikely. Hypothesis 1 may be restated as: circulating renin derived from the kidney.

Experimental evidence against hypothesis 2 has also been obtained from infusion studies of prorenin and from observations in transgenic animals. Short-term infusion of recombinant human prorenin in monkey did not lead to higher renin, Ang I or Ang II argues against hypothesis 2. Binding of prorenin to tissues or cells has been demonstrated (202,204,206), although not necessarily to cells derived from kidney or liver. Activation of prorenin to renin by extrarenal cells has also been confirmed (202,203). The physiological significance of renin binding was investigated in an experiment by Hu *et al.* (219) who infused either renin or Ang II in rats to extent that both regimens yielded identical plasma levels of Ang II. If renin activity after renin binding would be important, renin infusion would be expected to have additional hemodynamic and hormonal effects. This appeared not to be the case. This argues against hypothesis 9. This latter hypothesis was also challenged in a study by Muller *et al.* who infused human prorenin and renin in a rat transgenic for human angiotensinogen (220). Infusion of prorenin alone or in combination with renin did not affect blood pressure or Ang II levels.

An interesting transgenic rat model has been developed by Mullins *et al.* (221). They inserted the murine Ren-2 renin gene in the genome of a rat strain. Transgenic rats developed Ang II-dependent hypertension with grossly elevated plasma prorenin levels. The renin gene was over-expressed in adrenals, but not in kidney. Although the mechanism by which hypertension is caused in these rats is still elusive, the model raises the possibility that renin or perhaps prorenin may cause hypertension independently of the plasma RAS. On the other hand, transgenic rats, over-expressing rat prorenin in the liver, showed normal blood pressure. In spite of the absence of hypertension, however, cardiomegaly and severe renal lesions were present (222). Plasma prorenin levels in these transgenic rats are

increased 400-fold. This suggests that circulating prorenin has a role in the development of cardiac and renal pathology and refutes hypothesis 10.

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

Studies that appear to refute definitively one hypothesis or the other are scarce. The main obstacle is probably the fact that it is nearly impossible to study prorenin in animals in the absence of renin. Any change in the level of prorenin is usually accompanied by a change in the level of renin. Infusion studies as mentioned above were performed with heterologous prorenin and renin, and this leads to suppression of endogenous prorenin and renin. Results may therefore not be extrapolated to the physiology of homologous, endogenous prorenin. Furthermore, in humans, prorenin and renin may only be studied in blood, whereas it may well be that tissue concentrations are more important.

11 The transgenic mouse as a tool to study the RAS and its components

To overcome these limitations, the transgenic mouse system can be used. This powerful system, first described by Gordon *et al.* (223), is amenable to the study of complex biological alterations such as hypertension and oncologic transformation and this approach entails microinjection of DNA into one of pronuclei of fertilized eggs, followed by the transfer of the microinjected eggs into the uteri of foster mothers. This can result in the stable integration of the foreign DNA into the genome of the offspring and some of these animals will express the introduced gene (224). This approach offers the great advantage that the foreign gene product is likely to be exposed to many physiological influences, some of which may be expressed constitutively or only transiently during development.

So far, transgenic mice have been used as a model in which the action of the RAS can be assessed in a living host capable to mounting a physiological response to the development of hypertension (225). All human RAS components (i.e. renin and angiotensinogen) have been successfully introduced in mice (226). Most of those mice carry the human RAS gene under the control of its endogenous (natural) promoter. Studies have been performed to characterize the mechanisms or regulation of individual RAS

component and its relative importance in the normal and pathological and physiological responses of the RAS. All individual RAS components have been investigated using the lost-of-function approach (i.e. gene knockout) and/or the gain-of-function approaches (target expression of specific gene) (227-231), but no one has ever reported the use of the transgenic mouse model to access either the tissue uptake of circulating (pro)renin or the contribution of the latter to local RAS activity.

11.1 RAS in mouse and it relevance to the human RAS

The RAS has been found to be a very conserved system between all species studied so far. The mammalian RAS consists of the same constituents, regulation mechanisms and major functions (i.e. homeostasis). However, there are minor interesting differences related mainly to the renin gene and the Ang II receptors type 1:

Renin

Unlike man and mammals in which renin is encoded by a single gene locus, mice have either one gene (*ren-1^c*) or two gene loci (*ren-1^d*, *ren-2^d*) which are contiguous (232). The sequence homologies and relative chromosomal localization of the two renin genes suggests that the two copies originate from a recent gene duplication. In the mice harboring two renin genes, both copies are functional, and have the ability to compensate for one another (233,234). Moreover, both mouse renins harbor the same enzyme activity characteristics (i.e. affinity to angiotensinogen). The tissue distribution of renin in mice differs from human but reflects the presence of the second gene copy. Mouse renin is primarily found in kidney, the adrenal and the submandibular gland (SMG).

In the mouse, plasma active renin originates from the kidney, adrenal and SMG, the latter two tissues releasing both Ren-1 and Ren-2. Prorenin/renin plasma ratio differs: in human and rats 90% of total renin is prorenin (114,235) while in mice the majority of renin is the active form: prorenin accounts for only 30-50% of total renin (236-238). The plasma renin concentration in mice is normally extraordinarily high (>200 ng Ang I/mL per hour when measured using homologous mouse substrate) compare to human, but angiotensinogen levels are low and limit Ang I production by mouse renin (observation

provided by Yan *et al.* (238)). Thus, in mice, angiotensinogen is the rate limiting RAS component, not renin.

AT_1 receptor subtypes

Unlike men, mice possess two receptor subtypes for AT_1: AT_{1a} and AT_{1b}. The respective role is described above (refer to Section 1).

11.2 Experimental design

Our system is based on the strict species specificity of renin kinetics with respect to angiotensinogen. It has been shown that the action of renin on its substrate is dependent on the species from which these substances are derived (239,240). For example, mouse renin is capable of cleaving angiotensinogen derived from mouse, rat, rabbit, porcine, canine, bovine, sheep and goat sources, whereas renin derived from human, rat, hamster, rabbit, and porcine species does not have the ability to cleave mouse angiotensinogen. This property has been exploited in the development of a human plasma renin assay using sheep and human substrate where zero-order kinetics is achieved.

Our system consists of two transgenic mouse lines (illustrated in Figure 2.1 and 3.1, panel A). One transgenic mouse line, the enzyme producer, expresses either human active renin or prorenin. The second transgenic mouse line, the substrate supplier, produces human angiotensinogen in a specific tissue (e.g. the heart). Crossing the two mouse lines, mice harboring both the enzyme and substrate are produced, and used to assess the ability of the enzyme to enter and act in tissue expressing the substrate. Immunological identification of the enzyme *in situ* should prove it presence while measurement of tissue Ang I should determine it local activity. Moreover, since the amino acid sequence of human Ang I is identical to the mouse Ang I, this system has the great advantage that once Ang I is released from human angiotensinogen, this peptide can be further processed to Ang II by mouse ACE. Therefore, this approach permits to assess the local physiological effects of increased tissue Ang II.

12 Aim of the thesis

The uptake and local activity of circulating (pro)renin in tissues of intact animal has not yet been investigated. Based on the hypothesis that circulating (pro)renin is taken up by tissues to participate in local RAS *in vivo*, we performed studies in transgenic mice with the following aims:

1. To assess the tissue uptake of circulating renin and prorenin in heart and other tissues, and thereby determine its respective tissue distribution and cellular localization.
2. To determine if plasma-derived renin and/or prorenin are enzymatically active within tissues.
3. To investigate the role of locally generated Ang II in the heart relative to development of cardiac hypertrophy and fibrosis.
4. To assess the function of circulating prorenin relative to it implication in the systemic control of blood pressure and development of vascular pathophysiology.

Three transgenic mouse models have been developed in this study and each model constitute the core of each following chapter. In chapter 2, we describes the results of the distribution and localization of circulating renin and prorenin in tissues, and the contribution of the latter to local generation of angiotensin peptides. Implication of increased local synthesis of those peptides in the heart is discussed. In chapter 3, we describe the results of the distribution and localization of plasma-derived prorenin in tissues, and the local activity of the latter within tissues. In chapter 4, we describes the results of the function of prorenin in circulation relative to it implication in the systemic control of blood pressure in mice. A general discussion is presented in chapter 5 followed by a summary and conclusion. Finally, we present in appendices results not shown or published from each study.

CHAPTER 2

Contribution of circulating renin to local synthesis of angiotensin peptides in the heart

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Renin-angiotensin system (RAS) has been described as an endocrine system in which all RAS constituents are present in circulation. Observation of presence of these constituents in tissues led to the proposal of the existence of local RAS. In cardiovascular tissues, the origin of renin is still a controversial issue. Previous studies have provided evidence for the uptake and local activity of circulating renin in such tissues. However, direct evidence from *in vivo* studies is still lacking. This work presents evidence for the uptake and local activity of circulating (pro)renin in the heart.

This is a multi-authored work. Dr. David W. Silversides from the Centre de Recherche en Reproduction Animal (CRRA) at St-Hyacinthe generated all transgenic mouse lines. Sui Mei Linda Chui, Master's student, and Daniel Methot, Ph.D. student in our laboratory provided the expression vectors used to generate the TTRhRen and MHChAgt mice lines, respectivively. Chantal Mercure, a laboratory technician, performed the electron microscopy study presented in Figure 2.6. All the remaining of the work was carried out by myself under the supervision of Dr. Timothy L. Reudelhuber.

ABSTRACT

The activity of a local cardiac renin-angiotensin system has long been suspected in the promotion of cardiac pathologies including hypertrophy, ischemia and infarction. All of the components of the renin-angiotensin system cascade have been demonstrated to be synthesized within the heart with the possible exception of the first enzyme in the cascade, renin. In the current study, we provide direct evidence that circulating renin can contribute to cardiac-specific synthesis of angiotensin peptides. Furthermore, we demonstrate this effect is independent of blood pressure and that in animals of comparable blood pressure, elevated circulating renin significantly enhances cardiac fibrosis. These results may serve to explain some of cardiac pathologies associated with the renin-angiotensin system.

INTRODUCTION

In mammals, the cleavage of the decapeptide angiotensin I (Ang I) from the circulating hepatic glycoprotein angiotensinogen is the rate-limiting step in the renin-angiotensin system (RAS) and is carried out by the kidney-derived aspartyl protease renin (Figure 2.1A). Ang I is subsequently processed by endothelial-derived angiotensin converting enzyme to the octapeptide angiotensin II (Ang II) which exerts its effects on vasoconstriction, aldosterone release and cell growth/apoptosis through its interaction with specific receptors (AT_1-R and AT_2-R). The components of the RAS as well as their corresponding mRNAs have also been reported to be expressed within certain tissues, leading to the suggestion that tissue RAS (tRAS) could influence long term hemodynamic changes through local generation of Ang II which can in turn affect surrounding tissues or cells. Ang II is capable of stimulating the expression of nuclear proto-oncogenes such as c-*fos*, *c-jun, jun-B, egr-1* and c-*myc* as well as growth factors such as PDGF, FGF, and TGF-β1, and late markers of cardiac hypertrophy such as skeletal α-actin and ANF (1-4), raising the possibility that locally generated angiotensin peptides act as growth factors in the heart. The resulting structural changes may be linked to decreased cardiac function.

The existence of a localized RAS in the heart has been supported by several lines of clinical evidence. For example, RAS inhibitors are beneficial in the treatment of chronic heart failure, acute myocardial ischemia and in regression of cardiac hypertrophy (5-8). Both prospective and retrospective clinical studies of hypertensive patients have shown a strong correlation between elevated circulating renin levels in hypertensive patients and the risk of myocardial infarction independent of blood pressure (9,10). Although the mRNA for all of the components of the RAS have been detected in the heart, the mRNA for renin has variously been reported as being absent or expressed at very low levels and there is some debate as to whether there is sufficient active renin generated within the heart to catalyze an intra-cardiac RAS (11,12). Some studies have suggested that renin might be taken up by vascular tissues, including the heart: For example, the enzymatic activity of intra-cardiac and plasma renin in the whole animal parallel one another and both virtually disappear with removal of the kidneys, the primary source of circulating active renin (13). *In situ* perfusion studies also support the notion that the heart and

vasculature can retain circulating renin (14-16) and two groups have reported the existence of high affinity renin receptor proteins in rat membrane preparations (17,18). Nevertheless, the physiological relevance of the renin binding seen in these *in situ* and *in vitro* approaches is still unclear. In the current study, we have created an *in vivo* model to directly test whether circulating renin can contribute to local generation of angiotensin peptides in the heart. In this model (Figure 2.1B), transgenic mice which release human active renin into the circulation exclusively from the liver were mated to mice expressing human angiotensinogen exclusively in the heart. The measurement of the products of the reaction, Ang I and II, in the hearts of double-transgenic mice serves as a direct measure of the ability of circulating renin to promote the activity of a local cardiac RAS. Our results provide the first direct evidence that circulating renin contributes by a pressure-independent mechanism to the production of angiotensin peptides in the heart of intact animals and may point to new avenues in the treatment of certain forms of heart disease.

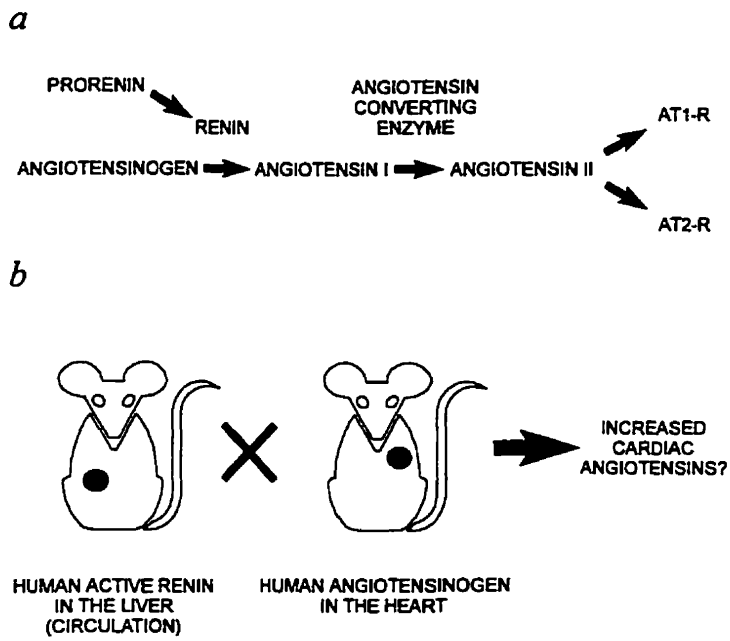

Figure 2.1 (A) Schematic diagram of the renin-angiotensin system. AT1-R and AT2-R; angiotensin II type 1 and type 2 receptors. (B) Strategy used in this study to test for the contribution of circulating renin to cardiac angiotensin peptide production.

MATERIALS AND METHODS

All animal protocols were approved by the institutional Animal Protection Committee of the Clinical Research Institute of Montreal.

Expression of human renin and angiotensinogen in transgenic mice

To express human renin in mouse liver, a 3 kb region of the transthyreitin gene promoter (a generous gift from Robert H. Costa, The University of Illinois at Chicago) was cloned upstream of the human prorenin cDNA. To generate active human renin, a cleavage site for the ubiquitous protease furin was inserted at the juncture of the prosegment and the active renin molecule, resulting in prosegment removal by endogenous proteases in the secretory pathway of expressing cells (19). Expression of human angiotensinogen in the mouse heart was achieved by cloning the cDNA downstream of a 6 kb fragment of the alpha-myosin heavy chain gene promoter (a generous gift from Jeffrey Robbins, University of Cincinnati).

FVB/N mouse embryos were microinjected according to standard protocols (20) and all subsequent breeding was carried out in the FVB/N line. Tissue-specific expression of the human transgenes was verified by an RNAse protection assay from total tissue RNA as previously described (21).

All animal tested were male at 10 weeks of age unless otherwise stated.

Biochemical and physiological characterization of transgenic mice

Renin and prorenin assays (Table 2.1) were performed as follows: Blood samples obtained by orbital puncture of mice lightly anesthetized with ether were collected into ice-cold microcentrifuge tubes containing EDTA and immediately centrifuged to isolate plasma. Plasma was stored at -20°C until assayed. Human plasma renin concentration (PRC) was determined by the rate of Ang I generation from an excess of human angiotensinogen to take advantage of the species specificity of the reaction between renin and angiotensinogen (21). Under the assay conditions, mouse renin generated barely detectable levels of Ang I from human angiotensinogen. Briefly, 0.25 μL (transgenic) or 5 μL (non transgenic) of plasma was incubated with 100 ng of purified human substrate (>95%; Sigma Chemical

Co., St-Louis, Mo, USA) at 37°C for 0, 10, 20, and 30 minutes in a total volume of 150 μL of buffer, pH7.5. Reactions were stopped on ice and subsequent steps performed at 4°C. The Ang I generated was measured by radioimmunoassay (RIA). Total renin concentration (TRC) was determined after incubation with trypsin (0.3 mg/mL; Boehringer Mannheim, Germany) at room temperature for 10 minutes in a total volume of 50 μL of buffer, pH8.0. Prorenin was calculated as the difference between total and active renin content.

Blood pressures of transgenic mice were measured by tail cuff plesmithography using a *BP-2000* system (Visitech Systems, Apex, NC, USA) according to previously published procedures (22). Briefly, mice were trained to the apparatus for a total of 8-9 uninterrupted days and measurements were recorded only for the last 2 days.

The degree of cardiac hypertrophy was estimated by calculating the ratio of cardiac ventricle wet weight by total body weight.

Ang I and II were measured by RIA of acid-soluble extracts of either plasma or heart tissue with a modification of the method of van Kats *et al.* (23). Briefly, mice were anesthetized by intraperitoneal injection with 3 mg sodium pentobarbital (MTC Pharmaceuticals, Cambridge, Ontario) and 250 μL whole blood was collected by cardiac puncture in presence of inhibitor solution (1 μM remikiren, 1 μM captopril, and 10 mM EDTA final concentration) and cleared immediately by centrifugation. Plasma samples (150 μL) were adjusted to 2 mL by addition of acid extraction buffer (80% ethanol, 0.1 M HCl) and again cleared by centrifugation at 13,000 X *g* for 30 minutes. Ethanol was evaporated and 2 mL of 1% ortho-phosphoric acid was added to each sample. Samples were again cleared by centrifugation, 2 mL of 1% ortho-phosphoric acid was again added. The samples were loaded onto Sep-Pak hydrophobic c18 cartridges (Waters Corp, Milford, MA, USA) which was subsequently washed with 2x 5 mL H_20. Angiotensin peptides were eluted with 3 ml of absolute methanol (Anachemia Canada Inc, Montreal, Quebec, Canada). Samples were then split in two equal half for the separate measurement of Ang I and II. Lyophilized peptides were quantitated by RIA. The Ang I antibody used is specific for Ang I peptide with no detectable cross reactivity with Ang II or metabolites whereas the Ang II antibody used (CD3) shows 100% cross reactivity with both Ang III and IV, but none with Ang I (data not shown). For measurement of Ang I and II contained in heart

tissue, animals were euthanized and excised hearts were pressed repeatedly onto blotting paper to remove excess blood before being flash frozen in liquid nitrogen. Frozen hearts were pulverized with a mortar and pestle, and the powder was immediately homogenized in 2 ml of the acid extraction buffer. After clearing by centrifugation, the samples were treated as described above for the determination of Ang I and II content.

Antihypertensive treatment

Three to four animals of each group (non transgenic, single-transgenic for human renin and human angiotensinogen, and double-transgenic expressing both transgenes) received either tap water (vehicle) or losartan (a gift from Merck; 30 mg/kg/day in drinking water). Individually housed mice were treated for 8 consecutive days in which drinking volume was measured every day and dosage was adjusted daily for fluid intake. Blood pressure measurements were performed as described above.

Histochemistry

Mice were anesthetized by intraperitoneal injection with 3 mg sodium pentobarbital (MTC Pharmaceuticals, Cambridge, Ontario). Blood was chased from major vessels by whole body perfusion of saline solution (20 mL) through the heart, followed by *in situ* organ fixation using 40 ml of either 1) Bouin's fixative solution (0.9% picric acid, 10% formaldehyde, 5% glacial acetic acid) for light microscopy, or 2) 0.5% glutaraldehyde/4% paraformaldehyde, pH7.2 for electron microscopy. Organs were then quickly removed and post-fixed in respective solution for 5 and 16 hours. All fixed tissues were stored in 70% ethanol at 4°C until analyzed.

For light microscopy histochemistry, tissues were dehydrated, embedded in paraffin blocks, cut into 5 μm sections, and mounted on 3-aminopropyltriethoxysilane (APTES)-coated slices (Sigma Chemical Co., St-Louis, Mo, USA) . The sections were deparafinized, rehydrated and washed with H_20. Staining with Sirius Red was performed as follow: rehydrated slides were stained with 0.5x hematoxylin (BDH, Toronto, Ontario) for 1 min, rinsed for 5 min with H_2O and counter-stained for 30 min with Sirius Red solution (saturated Bouin's solution containing 0.1% Sirius Red dye). Stained slides were again rinsed in H_2O, dehydrated and mounted for observation. For immunohistochemistry,

non-specific antibody binding was blocked by incubation with 1% donkey serum in Tris-buffered saline (TBS; 50 mM Tris-HCl, 154 mM NaCl, pH7.4) for 1 hour at 25°C. Tissue sections were incubated with rabbit polyclonal antibody to human renin (BRI-6, 1:600; a generous gift from Daniel F. Catanzaro, Weill Medical College of Cornell University, NY) in TBS containing 5% Carnation milk powder. The sections were then incubated with a biotinylated donkey anti-rabbit IgG (1:200 dilution; Amersham, Oakville, Ontario), followed by streptavidin-horseradish peroxidase (HRP, 1:300 dilution; Amersham, Oakville, Ontario). Positive staining was detected using 0.025% diaminobenzidine (DAB) and 0.03% H_2O_2 for 8 minutes. The sections were dehydrated, mounted with Permount (Fisher Scientific Ltd, Nepean, Ontario), and photographed using Nomarski optics. For cell-type identification, we used M3/38, a monoclonal rat anti-mouse macrophage specific antigen MAC2 (ATCC, Manassas, VA), EPOS-anti-α-smooth muscle actin and EPOS-anti-vimentin (DAKO, Mississauga, Ontario) conjugated with HRP. Antigen retrieval treatment with 0.1% trypsin/Tris (pH7.6) was applied for vimentin immunostaining.

For electron microscopy immunohistochemistry, tissues were embedded in LR White hard resin (London Resin Company Ltd, England) according to the manufacturer's protocol. Sections (90 nm) were mounted on copper grids and incubated by floating on Tris-BSA buffer (20 mM Tris-HCl, 500 mM NaCl, 0.1% BSA, 0.13% NaN_3, 0.05% Tween 20, pH8.0) containing 2% normal goat serum (NGS) for 15 min. The grids were then transferred to a drop of human prorenin antibody BRI-6 diluted 1:200 in Tris-BSA containing 2% NGS for an overnight incubation at 4°C. After rinsing in Tris-BSA, sections were incubated on a drop of goat anti-rabbit IgG immunogold conjugate: 15 nm (British BioCell International, Cardiff, UK) at a dilution of 1:30 in Tris-BSA for 1 hour at 25°C. After rinsing, sections were stained using uranyl acetate and lead citrate before examination with the electron microscope (JEOL JEM 1200 EX).

RESULTS

Expression of transgenes

Mice transgenic for the human renin cDNA under the control of the transthyretin promoter revealed expression of the transgene in the liver as shown by RNAse protection assays (Figure 2.2A). A small amount of expression was also detectable in the brain in agreement with previous studies documenting promoter activity in the choroid plexus (24). Expression was not detected in the other organs tested, including the heart and kidneys. *In situ* hybridization of sections from mouse heart and liver further confirmed that the human renin transgene was not expressed in the heart of transgenic animals while being generally expressed in hepatocytes (data not shown). Expression of the human angiotensinogen transgene under control of the myosin heavy chain promoter was detectable in the heart of transgenic mice (Figure 2.2B). Some expression was also detected in the kidney and the lungs of the founder line used in this study, but this expression should have no bearing on generation of angiotensin peptides in the heart.

Physiological and biochemical characterization of single- and double-transgenic mice

Expression of human active renin in the liver of transgenic mice (TTRhRen-A3) leads to release and detection of human renin in the circulation of which ~86% is active renin (Table 2.1). These mice also exhibit a significant elevation of blood pressure and cardiac hypertrophy as compared to non-transgenic littermates. In contrast, the human angiotensinogen-expressing mouse lines (MHChAgt-2) showed no increase in blood pressure or heart weight as compared to non-transgenic animals. The blood pressure and degree of cardiac hypertrophy seen in the double transgenic mice was identical to that seen in the mice expressing only active human renin in the liver.

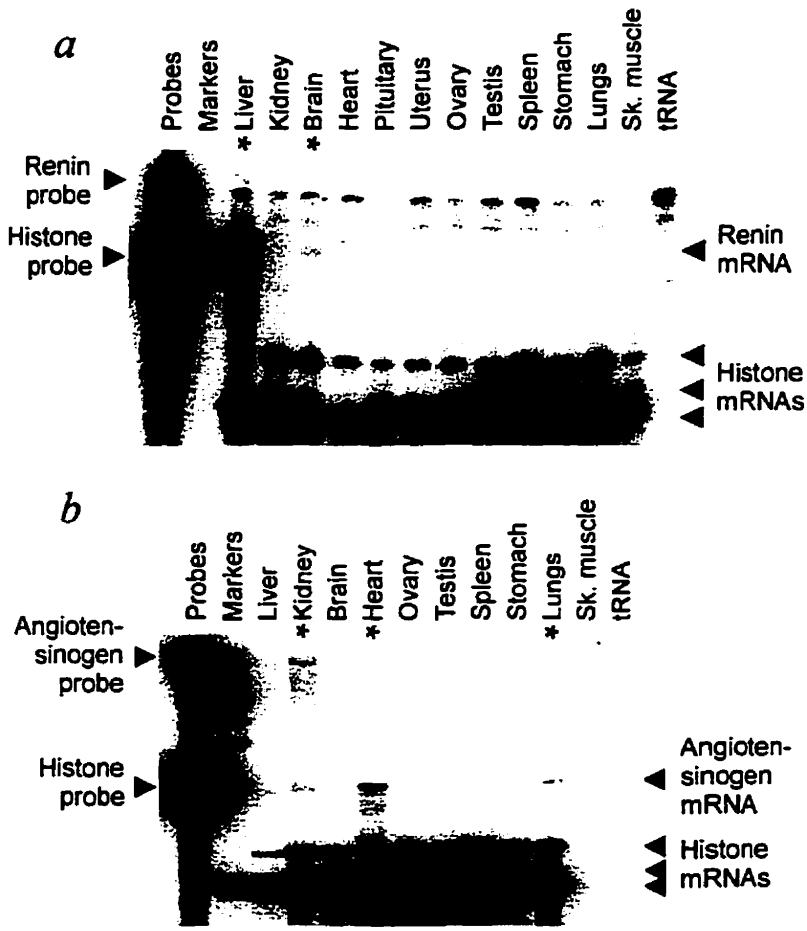

Figure 2.2 Tissue distribution of transgene expression as measured by RNase protection assay. (A) Expression of human renin cDNA in various tissues of TTRhRen-A3 mice. (B) Expression of human angiotensinogen cDNA in tissues of MHCAgt-2 mice. Asterisks (*) denote sites of expression. Histone H4 mRNA is included as a normalization control.

Table 2.1 Physiological characterization of transgenic mice*

Mouse line	*Human protein expressed (tissue)*	*PRC (ng Ang I/mL/h)*	*TRC (ng Ang I/mL/h)*	*SBP (mmHg)*	*HW/BW (mg/g)*
non-transgenic	none	1±1	2±1	126±3	3.70±0.16
TTRhRen-A3	active renin (liver)	3307±508	3854±574	163±3†	4.51±0.05†
MHChAgt-2	angiotensinogen (heart)	nd	nd	124±4	3.69±0.12
TTRhRen-A3 X MHChAgt-2	active renin (liver) + angiotensinogen (heart)	nd	nd	164±4†	4.70±0.06†

* Results represent the mean (± SD) values of single determinations on 5-12 individual animals. PRC, plasma renin concentration. TRC, total renin concentration. SBP, systolic blood pressure. nd, not determined. † $P<0.001$ as compared to non-transgenic mice by ANOVA using Student's t-test.

Function of circulating renin in the heart

To test whether circulating renin can contribute to cardiac RAS activity, transgenic mice expressing human renin in the liver (TTRhRen-A3) were mated to mice expressing human angiotensinogen exclusively in the heart (MHChAgt-2). Double-transgenic mice were tested for circulating and cardiac content of angiotensin peptides (Figure 2.3). The results demonstrate that while the single-transgenic animals showed either low or undetectable angiotensin peptides in the heart, double-transgenic mice exhibited a dramatic increase in cardiac content of both Ang I and II. Notably, the circulating levels of the angiotensin peptides did not increase in double-transgenic mice as compared to single- and non-transgenic controls, suggesting that enhanced production of angiotensins in the double-transgenic mice was restricted to the heart. These results were reproduced in matings between additional founder lines of transgenic mice (not shown) and are consistent with the ability of circulating renin to act on its substrate within the heart.

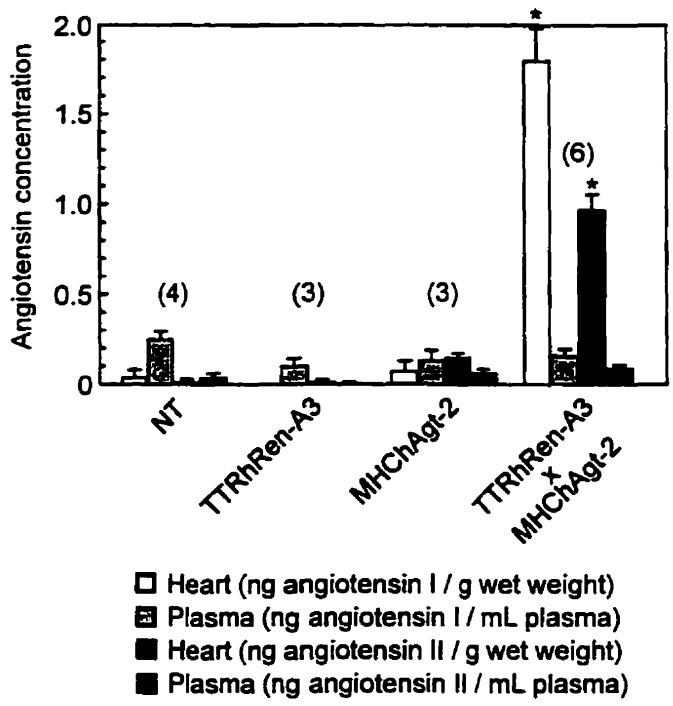

Figure 2.3 Concentration of Ang I and II in plasma and hearts of non-, single- and double-transgenic animals. Number in parentheses represent the number of animals analyzed in each group. * $P<0.001$ as compared to non-transgenic mice by ANOVA using Student's t-test.

Effects of blood pressure on cardiac activity of circulating renin

The entry of circulating renin into the heart could be mediated by either a specific capture mechanism (e.g., receptor/acceptor protein) or by passive diffusion. In the latter case, diffusion might be enhanced by a pressure gradient from the circulation. Indeed, mice expressing active human renin in the liver are hypertensive as compared to their non-transgenic littermates (see above). To test whether renin was taken up from the circulation by a pressure-dependent mechanism, double-transgenic animals were treated with an anti-hypertensive agent for a period of 1 week to normalize their blood pressure and their cardiac and circulating levels of Ang I were compared to that of vehicle treated littermates. Results (Table 2.2) demonstrate that even though anti-hypertensive treatment led to a significant reduction in the blood pressure of the double-transgenic animals, there was no decrease in the ratio of cardiac to circulating Ang I in the treated animals as compared to untreated littermates. These data suggest that the contribution of circulating renin to the cardiac RAS is not mediated by a pressure-dependent mechanism.

Table 2.2 Treatment of transgenic mice with losartan*.

Mouse line	*Human protein expressed (tissue)*	*Treatment*	*SBP (mmHg)*	*Ang I conc. in heart (ng Ang I/g wet weight)*	*Ang I conc. in plasma (ng Ang I/mL plasma)*
non-transgenic	none	vehicle	125±9	0.045±0.036	0.318±0.056
TTRhRen-A3 X MHChAgt-2	active renin (liver) + angiotensinogen (heart)	vehicle	153±6†	1.654±0.109‡	0.381±0.012
TTRhRen-A3 X MHChAgt-2	active renin (liver) + angiotensinogen (heart)	losartan	129±3	1.970±0.100‡	0.327±0.082

* Results represent the mean (± SD) values of single determinations on 3-4 individual animals. SBP, systolic blood pressure. † $P<0.05$ and ‡ $P<0.001$ as compared to non-transgenic mice by ANOVA using Student's t-test.

Function of locally generated angiotensin peptides

To test for function of locally-derived angiotensin peptides, hearts of single and double-transgenic mice were stained with the connective tissue-specific stain, Sirius Red. Results show a dramatic increase in perivascular and interstitial fibrosis in the hearts of double-transgenic mice (Figure 2.4), suggesting that locally-derived angiotensin peptides contribute to cardiac fibrosis.

Distribution of human renin captured by peripheral tissues

Immunohistochemistry was performed on tissues of transgenic animals expressing human renin in the liver (TTRhRen-A3) using an antibody with selectivity for human renin/prorenin (Figure 2.5). A dark, punctate staining pattern for human renin was seen in the hearts exclusively in transgenic animals which was restricted to cells in the periphery of small vessels (Figure 2.5A). This staining did not co-localize with vimentin (fibroblasts and some pericytes) and only partially with alpha smooth muscle actin (smooth muscle and some pericytes) and MAC-2 (macrophages) (refer to Appendices, Figure A.1.4 and A.1.5). Immunoelectron microscopy (Figure 2.6) revealed that the renin-containing cells in the heart were elongated perivascular cells in which dense cell bodies stained for human renin. Punctate, perivascular staining for human renin was also seen in the pituitary, testes, ovary, and lungs of human renin transgenic animals while human renin was not detected in any of these tissues in non-transgenic littermates (data not shown). Taken together with the finding that the heart does not express the human renin transgene (see above), these results suggest that renin captured from the circulation is stored in a discrete cell type of the heart and other vascular tissues.

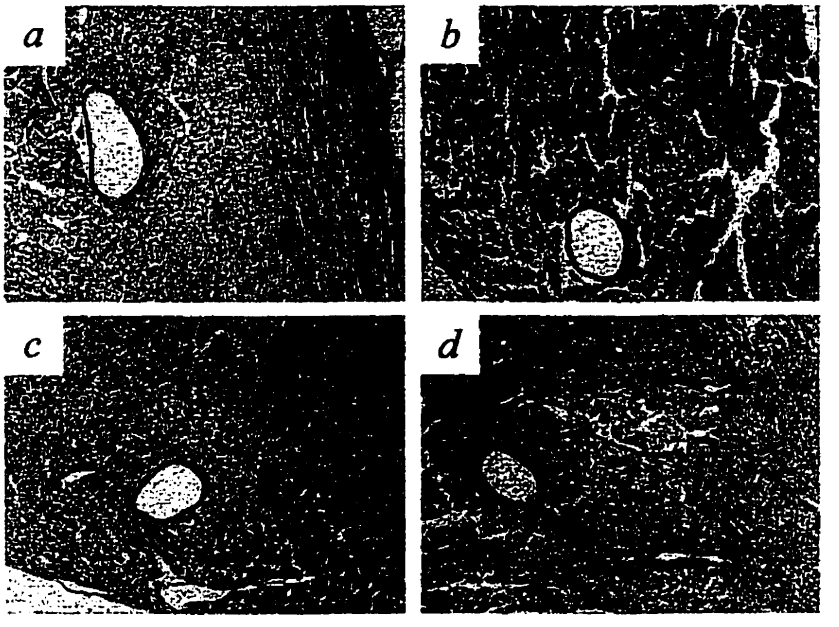

Figure 2.4: Sirius Red stain for connective tissue in the hearts of non-transgenic (A) mice, single-transgenic mice for liver-derived human renin (B), heart-derived human angiotensinogen (C) or double-transgenic mice (D). Note the dramatic increase of perivascular and interstitial fibrosis in double-transgenic mice. Original magnification at 40x.

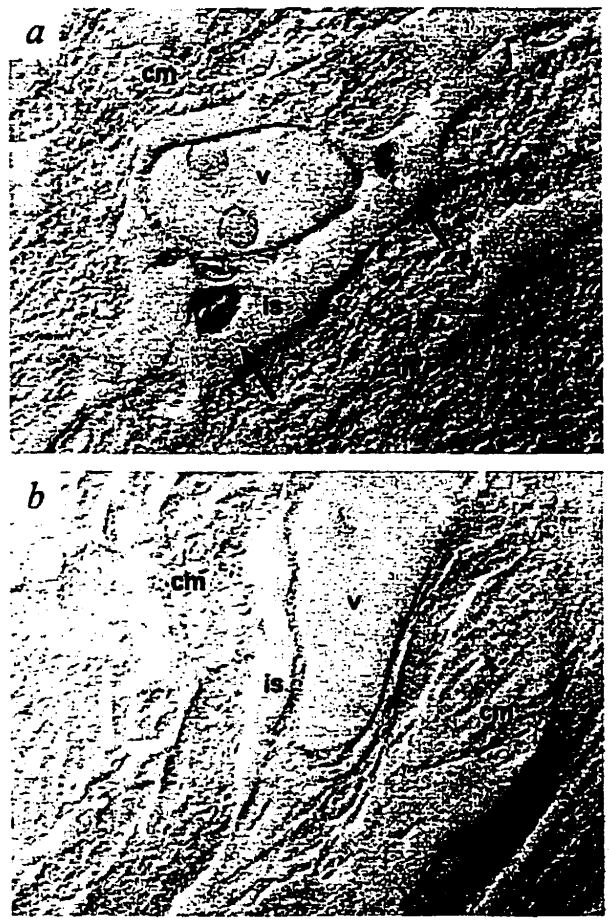

Figure 2.5 Immunohistochemical stain for human renin in the hearts of animals expressing human renin in the liver. Arrows show discrete cells staining for renin in transgenic animals (A), but not non-transgenic mice (B). v, vessel lumen. cm, cardiomyocytes. is, interstitial space. Original magnification at 160x.

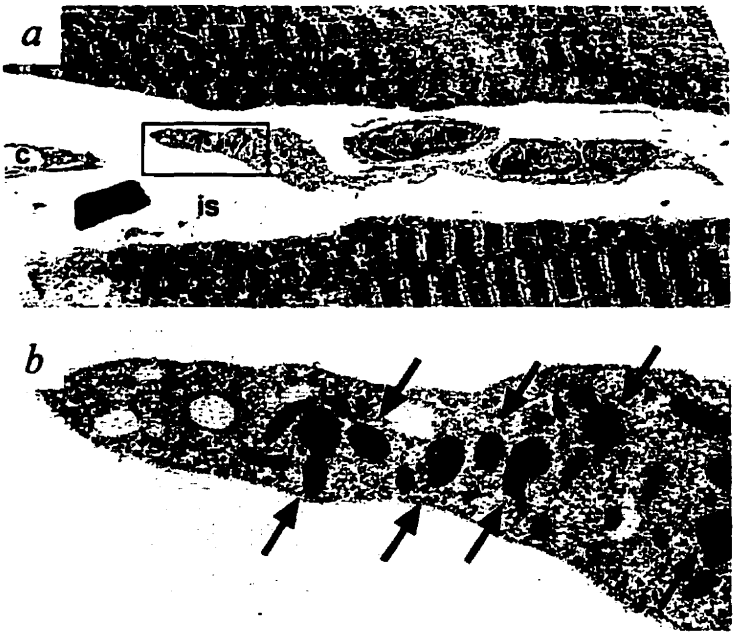

Figure 2.6 Immunoelectron microscopy of cells containing human renin within the hearts of animals expressing human renin in the liver. Renin staining is observed as discrete black dots (15 μm in diameter) corresponding to electron dense gold particles. Note the perivascular distribution of these cells (A; original magnification at 1500x), their localization in the interstitial space between muscle fibers and their content of renin-containing cytoplasmic inclusions (arrows in B; original magnification at 10,000x). c, capillary. cm, cardiomyocytes (muscle fiber). is, interstitial space. n, nuclei.

DISCUSSION

The current study provides the first *in vivo* demonstration that a chronic elevation in circulating renin leads to an increase in local synthesis of angiotensin peptides within a target tissue. Although human renin and angiotensinogen should have little biochemical interaction with their mouse homologues (25), the ensuing products of their reaction (Ang I, Ang II and metabolites) are identical in the two species. This should, in theory, allow the study of the human transgenes without interference from the mouse RAS and several studies have shown that over-expression of the human transgenes using their natural promoters does not lead to hypertension in transgenic mice (26-28). However, in our study, over-expression of human active renin in the liver led to hypertension which was clearly Ang II-mediated (responded to Ang II receptor antagonists, data not shown). The most likely explanation for this finding is the cleavage of the liver-derived mouse angiotensinogen by the human renin due to its high local concentration. This hypertension leads to some degree of cardiac hypertrophy which is not increased when these animals are mated to mice expressing human angiotensinogen in the heart. For this reason we were not able in the current study to test for the role of locally generated angiotensin peptides in the development of pressure-independent cardiac hypertrophy. However, the current study demonstrates that in animals with comparable levels of hypertension and cardiac hypertrophy (TTRhRen-A3 and TTRhRen-A3 X MHChAgt-2), high circulating renin leads to an increase in intracardiac angiotensin peptides and results in an increase in cardiac interstitial and perivascular fibrosis. Such a mechanism might explain in part why elevated circulating renin has been identified as a risk factor for myocardial infarction in hypertensive patients (9,10). Like these clinical studies, our results also suggest that the contribution of circulating renin to angiotensin peptide generation in the heart is independent of blood pressure.

Evidence of function of renin in the heart is accompanied by its detection in very discreet perivascular cells. In order to enter the heart, circulating renin would have to initially bind and traverse the endothelial cell layer in the lumen of blood vessels. Our current results suggest that this renin is subsequently transcytosed to the interstitium and stored in granular structures of perivascular cells. The identity of these cells is still

uncertain, although they partially co-localize with cells stained by an antibody to macrophages (MAC-2, refer to Appendices). It is uncertain whether the cells which concentrate renin in the heart are the actual site of renin catalytic activity: Attempts to stain the heart of double transgenic animals with antibody against Ang I have not been successful (data not shown) suggesting that the generated Ang I is either not stored or is generated in a diffuse compartment (perhaps the interstitial space). Indeed, de Lannoy *et al.* (29,30) have recently shown that angiotensin peptides generated in the isolated, perfused rat heart are derived primarily from the interstitial fluid. These investigators also noted little exchange of angiotensins between the interstitial and intravascular compartments, suggesting that the action of locally generated peptide was restricted to the interstitial space. Thus, the renin-staining cells seen in the hearts of our transgenic mice could either be storing renin for local release or be in the process of clearing the renin from the cardiac interstitium after its action on locally-derived angiotensinogen.

Two groups have characterized high affinity vascular renin-binding proteins in tissue membrane preparations from the rat (17,18). However, the properties of these proteins varied significantly between the two studies these studies raising the possibility that more than one type of renin-binding protein exists in the lumen of the vasculature. The identification of such binding proteins lends support to the existence of a tissue-restricted RAS that would control local levels of Ang II independently of the blood pressure modulating activity of the circulating RAS. Development of specific inhibitors to renin/prorenin binding proteins could, therefore, provide an effective way to block the local RAS and provide a new avenue for the treatment of various forms of cardiovascular disease.

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CHAPTER 3

Tissue activity of circulating prorenin

Gary Prescott, David W. Silversides and Timothy L. Reudelhuber

Submitted

In our previous study, we provided evidence for the uptake and local activity of circulating (pro)renin within tissues. However, we were not able to assess the presence and local activity of each form of renin. In this study, we specifically examined the uptake of circulating prorenin in tissues and assess its local activity within the heart. We focused this work on the possibility that local activation of prorenin may take place within tissue in an attempt to explain some of the pathologies associated with high circulating prorenin levels.

This is a multi-authored work in which Dr. David W. Silversides generated all transgenic mouse lines. Sui Mei Linda Chui and Daniel Methot provided the expression vectors used to generate the TTRhProren and MHChAgt mice lines. All the remaining of the work was carried out by myself under the supervision of Dr. Timothy L. Reudelhuber.

ABSTRACT

Both renin and its biosynthetic precursor, prorenin, are secreted into the circulation of mammals. Although the circulating levels of prorenin can exceed those of renin by as much as 100-fold in certain conditions, there is no evidence that prorenin contributes to the synthesis of circulating angiotensin peptide synthesis or increased blood pressure. In the current study, we have used a transgenic mouse model to demonstrate that circulating prorenin can contribute to synthesis of angiotensin peptides within tissues without increasing circulating angiotensin levels. This finding may explain some of the pathologies associated with high circulating prorenin levels.

INTRODUCTION

The rate-limiting reaction of the circulating renin-angiotensin system (RAS) is the cleavage of the decapeptide angiotensin I (Ang I) from circulating hepatic angiotensinogen by the kidney-derived aspartyl protease renin (Figure 3.1A). Ang I is subsequently processed by endothelial-derived angiotensin converting enzyme to the octapeptide Ang II which exerts its effects on vasoconstriction, aldosterone release and cell growth/apoptosis through its interaction with specific receptors (AT_1-R and AT_2-R). Although the RAS is an endocrine system, several tissues contain and/or synthesize components of the system (reviewed in (1-3)), raising the possibility that locally produced Ang II could act in an autocrine or paracrine fashion to regulate tissue function.

Renin is synthesized as a proenzyme precursor, prorenin. The active form of renin present in the circulation results from the proteolytic removal of the 43-amino acid N-terminal prosegment of prorenin. Although circulating active renin is derived exclusively from the kidneys, the kidneys and numerous other tissues also secrete prorenin into the circulation where it is normally present at 5-10 times the level of renin. Circulating prorenin has no detectable enzymatic activity as evidenced by a lack of increase in either circulating angiotensin II or blood pressure in animals injected with large quantities of prorenin (4,5). Nevertheless, several lines of circumstantial evidence link high circulating prorenin to end-organ damage: Dramatic elevations in circulating prorenin (to as much as 100 times the level of renin) are associated with renal damage in some diabetic patients (6-10). Transgenic rats with similar increases in circulating prorenin also exhibit renal damage and cardiac hypertrophy in the absence of an obvious increase in either circulating angiotensin peptides or hypertension raising the possibility that prorenin is contributing to these pathologies at a tissue level (11). Indeed, substantial evidence has accumulated that both prorenin and renin can bind cell surface "acceptors" present in the vasculature and in numerous tissues (12,13). In addition, there is evidence that prorenin can be activated in tissues or cultured cells by mechanisms that may not require the proteolytic removal of the prosegment (14-16). In the current study, we have created an *in vivo* model to directly test whether circulating prorenin can contribute to local generation of angiotensin peptides within tissues. In this model (Figure 3.1B), transgenic mice which release human prorenin

into the circulation exclusively from the liver were mated to mice expressing human angiotensinogen exclusively in the heart. The measurement of the product of the reaction, Ang I, in the hearts of double-transgenic mice serves as a direct measure of the ability of circulating prorenin to promote the activity of a local tissue RAS. Our results provide the first direct evidence that circulating prorenin contributes to the production of angiotensin peptides in the tissues of intact animals and supports a role for prorenin in some cardiovascular pathologies.

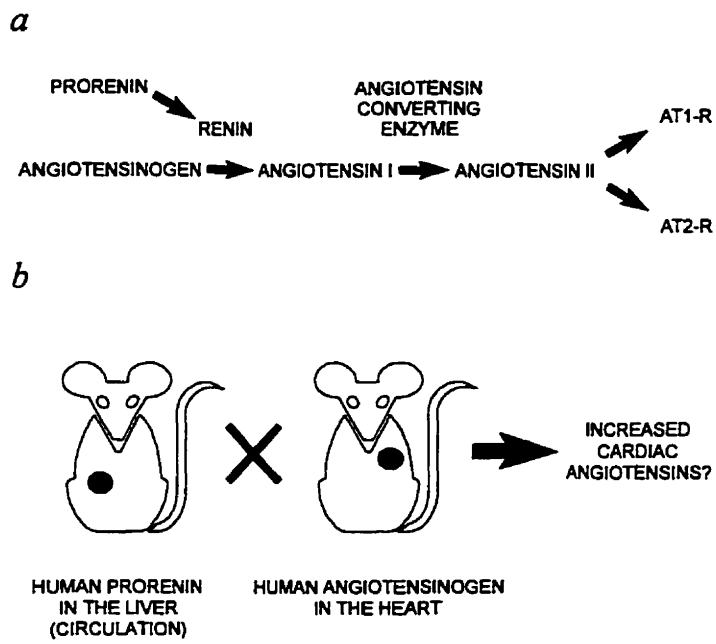

Figure 3.1 (A) Schematic diagram of the renin-angiotensin system. AT1-R and AT2-R; angiotensin II type 1 and type 2 receptors. (B) Strategy used in this study to test for the uptake of prorenin by the heart and its capacity to generate angiotensin peptide locally.

MATERIALS AND METHODS

Animals

All animal protocols were approved by the institutional Animal Protection Committee of the Clinical Research Institute of Montreal. Transgenic mice were generated in the FVB/N strain obtained from the Jackson Laboratory (Bar Harbor, Maine). Maintenance breeding was carried out in the same strain.

Transgene construction, generation of transgenic mice, and expression of the transgene.

To target the expression of human prorenin to the mouse liver, a 3 kb region of the transthyretin gene promoter was cloned upstream of the human prorenin cDNA. FVB/N mouse embryos were microinjected with the plasmid according to standard protocols (17) and all subsequent breeding was carried out in the FVB/N line. Genomic integration of the transgene was determined by PCR analysis of DNA obtained from tail biopsies and tissue-specific expression of the human transgene was verified by an RNAse protection assay from total tissue RNA as previously described (15). All animal tested were male at 10 weeks of age unless otherwise stated.

Physiological measurements

Plasma renin concentration (PRC) and total renin concentration (TRC) were measured as follows: Blood samples obtained by orbital puncture of mice lightly anesthetized with ether were collected into ice-cold microcentrifuge tubes containing EDTA and immediately centrifuged to isolate plasma. Plasma was stored at -20°C until assayed. Human PRC was determined by the rate of Ang I generation from an excess of human angiotensinogen. Under the assay conditions, mouse renin generated insignificant levels of Ang I from human angiotensinogen. Briefly, 0.25 µL (transgenic) or 5 µL (non transgenic) of plasma was incubated with 100 ng of purified human substrate (>95%; Sigma Chemical Co., St-Louis, Mo, USA) at 37°C for 0, 20, 40, and 60 minutes in a total volume of 150 µL of buffer, pH7.5. Reactions were stopped on ice and subsequent steps performed at 4°C. The Ang I generated was measured by radioimmunoassay (RIA). Total renin concentration

(TRC) was determined after incubation with trypsin (0.3 mg/mL; Boehringer Mannheim, Germany) at room temperature for 10 minutes in a total volume of 50 μL of buffer, pH8.0. Prorenin was calculated as the difference between total and active renin content.

Tail-cuff plethysmography (*BP-2000* system, Visitech Systems, Apex, NC, USA) was performed according to previously published procedures (18). Briefly, mice were trained to the apparatus for a total of 7 uninterrupted days and measurements were recorded and averaged for the following 3 days. The degree of cardiac hypertrophy was estimated by calculating the cardiac mass index (the ratio of cardiac ventricle wet weight to total body weight).

Ang I and II were measured by radioimmunoassay (RIA) of acid-soluble extracts of either plasma or heart tissue as previously described. Mice were anesthetized by intraperitoneal injection with 3 mg sodium pentobarbital (MTC Pharmaceuticals, Cambridge, Ontario) and 250 μL whole blood was collected by cardiac puncture in presence of inhibitor solution containing 1 μM remikiren (a specific human renin inhibitor received as a gift from F. Hoffmann-La Roche AG, Basel Switzerland), 1 μM captopril, and 10 mM EDTA (final concentration) and cleared immediately by centrifugation. Plasma samples (150 μL) were adjusted to 2 mL by addition of acid extraction buffer (80% ethanol, 0.1 M HCl) and again cleared by centrifugation at 13,000 X *g* for 30 minutes. Ethanol was evaporated and 2 mL of 1% ortho-phosphoric acid was added to each sample. Samples were again cleared by centrifugation, 2 mL of 1% ortho-phosphoric acid was again added. The samples were loaded onto Sep-Pak hydrophobic c18 cartridges (Waters Corp, Milford, MA, USA) which was subsequently washed with 2x 5 mL H_20. Angiotensin peptides were eluted with 3 ml of absolute methanol (Anachemia Canada Inc, Montreal, Quebec, Canada). Samples were then split in two equal aliquots for the separate measurement of Ang I and II. Lyophilized peptides were quantitated by RIA. The Ang I antibody used is specific for Ang I peptide with no detectable cross reactivity with Ang II or its metabolites whereas the Ang II antibody used (CD3) shows no cross-reactivity with Ang I (data not shown). For measurement of Ang I and II contained in heart tissue, animals were euthanized and excised hearts were pressed repeatedly onto blotting paper to remove excess blood before being flash frozen in liquid nitrogen. Frozen hearts were

pulverized with a mortar and pestle, and the powder was immediately homogenized in 2 ml of the acid extraction buffer. After clearing by centrifugation, the samples were treated as described above for the determination of Ang I and II content.

Organ histomorphology

For histology, the livers, kidneys and hearts of anesthetised transgenic and control animals were fixed *in situ* by organ perfusion using Bouin's fixative (0.9% picric acid, 10% formaldehyde, 5% glacial acetic acid). Organs were then quickly removed, post-fixed in Bouin's fixative for 5 hours, washed in 70% ethanol, and imbedded in paraffin.

Hematoxylin/eosin-stained tissue sections were examined for lesions and vascular hypertrophy by standard protocols. In addition, the hearts of non-, single- and double-transgenic mice were assessed for cardiomyocyte hypertrophy, interstitial and perivascular fibrosis by histological staining with Sirius Red.

For immunostaining of human prorenin sections (5 μm thick) of Bouin's fixed and paraffin-embedded tissues were mounted on 3-aminopropyltriethoxysilane (APTES)-coated slides (Sigma Chemical Co., St-Louis, Mo, USA), deparafinized, rehydrated and washed with H_2O. Non-specific antibody binding was blocked by incubation with 1% donkey serum in Tris-buffered saline (TBS; 50 mM Tris-HCl, 154 mM NaCl, pH7.4) for 1 hour at 25°C. Tissue sections were incubated with rabbit polyclonal antibody to human renin (BRI-6, 1:600; a generous gift from Daniel F. Catanzaro, Weill Medical College of Cornell University, NY) in TBS containing 5% Carnation milk powder. The sections were then incubated with a biotinylated donkey anti-rabbit IgG (1:200 dilution; Amersham, Oakville, Ontario), followed by streptavidin-horseradish peroxidase (HRP, 1:300 dilution; Amersham, Oakville, Ontario). Positive staining was detected using 0.025% diaminobenzidine (DAB) and 0.03% H_2O_2 for 8 minutes. The sections were dehydrated, mounted with Permount (Fisher Scientific Ltd, Nepean, Ontario), and photographed using Nomarski optics. No staining was detected when the first antibody was omitted (data not shown).

RESULTS

Expression of transgenes

Mice transgenic for the human prorenin cDNA under the control of the transthyretin promoter showed expression of the transgene in the liver as shown by RNAse protection assays (Figure 3.2). Although a small amount of expression was detectable in the stomach, expression was not detected in the other organs tested, including the heart and kidneys. Expression of the human angiotensinogen transgene was targeted to the heart using the alpha-myosin heavy chain promoter and the transgene expression pattern in these mice (MHChAgt-2) has been described previously (19).

Physiological and biochemical characterization of transgenic mouse lines

Expression of human prorenin in the liver of transgenic mice (TTRhProren-B7) leads to release and detection of human prorenin in the circulation of which >99% is the enzymatically inactive prorenin form (Table 3.1). Mice transgenic for human prorenin showed no elevation in blood pressure as compared to non-transgenic control littermates (Table 3.1). In fact, 10 week-old male mice showed a slight, but significant decrease in their blood pressure as compared to controls, however this difference was not apparent in female mice of the same age or in older (14 weeks) male mice (data not shown). The blood pressure seen in the double transgenic mice was identical to that seen in the mice expressing only the human prorenin in the liver (Table 3.1). The human angiotensinogen-expressing mouse line (MHChAgt-2) showed comparable blood pressure to non-transgenic animals. No significant differences were observed for the cardiac mass index between all of the mouse groups (Table 3.1).

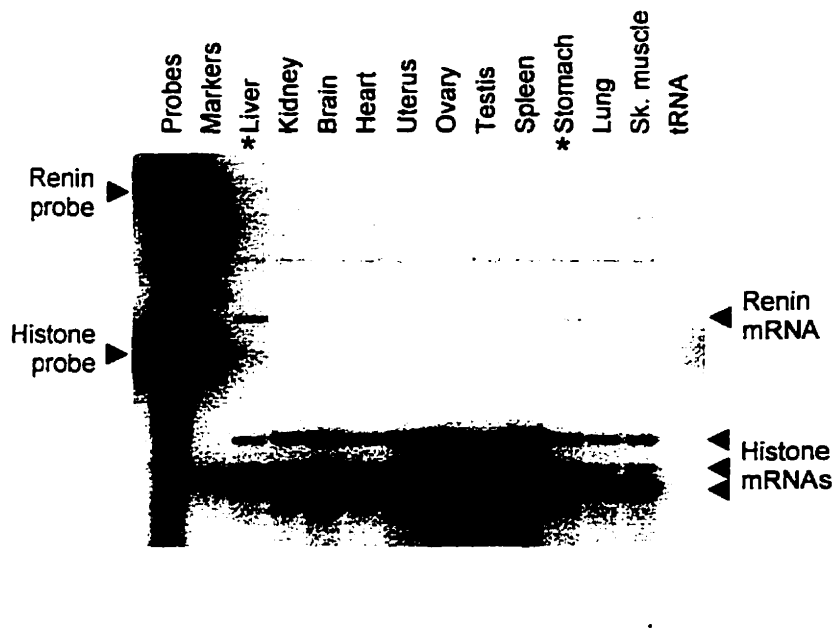

Figure 3.2 Tissue distribution of transgene expression as measured by RNase protection assay. Expression of human prorenin cDNA in various tissues of TTRhProren-B7 mice. Asterisks (*) denote sites of expression. Histone H4 mRNA is included as a normalization control.

Table 3.1 Physiological characterization of transgenic mice*

Mouse line	*Human protein expressed (tissue)*	*PRC (ng Ang I/ mL/h)*	*TRC (ng Ang I/ mL/h)*	*SBP (mm Hg)*	*CMI (1000x)*
non-transgenic	none	1.0±0.8	2.3±1.0	137±5	3.80±0.18
TTRhProren-B7	prorenin (liver)	1.5±0.7	50.8±5.6	124±9†	3.90±0.07
MHChAgt-2	angiotensinogen (heart)	nd	nd	135±6	4.03±0.23
TTRhProren-B7 X MHChAgt-2	prorenin (liver) + angiotensinogen (heart)	nd	nd	126±5†‡	3.97±0.20

*Values are mean ± SEM of single determinations on 5-12 individual animals. PRC = plasma renin concentration. TRC = total renin concentration. SBP = systolic blood pressure. CMI = cardiac mass index. nd = not determined. The level of prorenin can be roughly calculated by TRC-PRC. † $P<0.05$ as compared to non-transgenic mice by ANOVA using Student's t-test. ‡ difference not significant as compare to MHChAgt-2 mice.

Tissue uptake of human prorenin

Immunohistochemistry was performed on tissues of transgenic animals expressing human prorenin in the liver (TTRhProren-B7) using an antibody with selectivity for human (pro)renin. Punctate staining was observed in the heart of transgenic animals exclusively, in cells bordering the lumen of small blood vessels (Figure 3.3A). Specific staining was also observed in the pituitary and lungs (refer to Appendices, Figure A.1.2). However, no staining was detectable in sex organs (ovary, and testis).

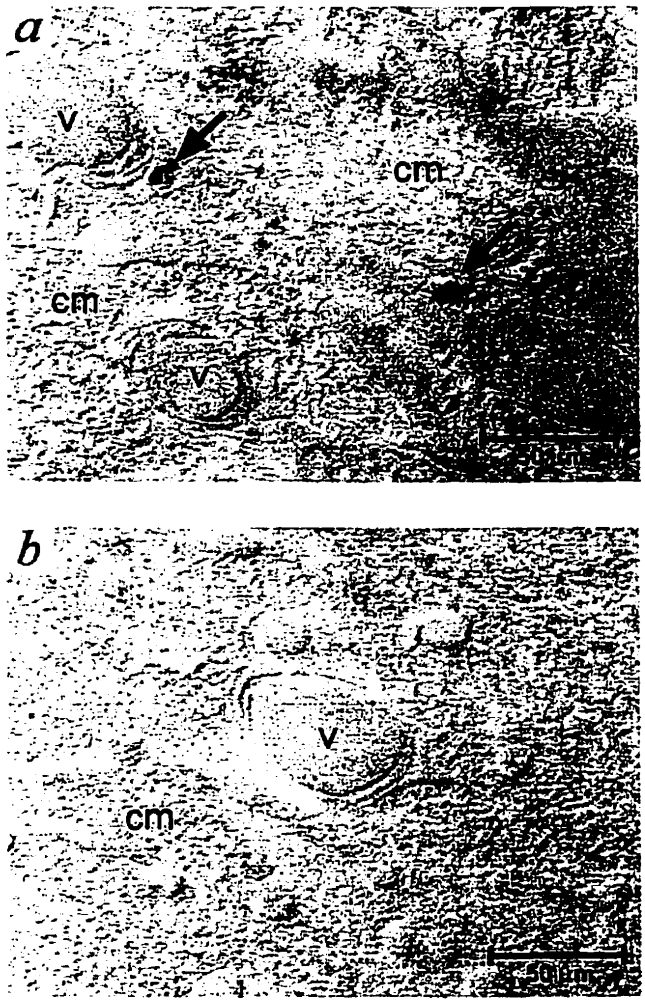

Figure 3.3 Distribution of circulating human prorenin taken up by the heart of transgenic animals. Arrows show discrete cells staining for prorenin in transgenic animals (A), but not non-transgenic mice (B). Immunohistochemistry was carried out using a human prorenin specific antibody (BRI-6, 1:600 dilution). v, vessel. cm, cardiomyocyte.

Enzymatic activity of prorenin taken up by tissues

To test whether prorenin taken up by tissues can contribute to tissue RAS activity, transgenic mice expressing human prorenin in the liver (TTRhProren-B7) were mated to mice expressing human angiotensinogen exclusively in the heart (MHChAgt-2). Double-transgenic mice were tested for circulating and cardiac content of Ang I (Figure 3.4). The results demonstrate that while the single-transgenic animals showed either low or undetectable levels of Ang I in the heart, double-transgenic mice exhibited a significant increase in cardiac Ang I content. Notably, the circulating levels of the Ang I did not increase in double-transgenic mice as compared to single- and non-transgenic controls, suggesting that enhanced production of angiotensin in the double-transgenic mice was restricted to the heart. Moreover, a significant decrease in plasma Ang I was observed for the prorenin-expressing mice. These results were reproduced in matings between additional founder lines of transgenic mice (data not shown) and are representative of a total of 3 independent experiments.

Histology of transgenic mouse tissues

Routine histological staining (hematoxylin/eosin) of hearts and kidneys of non-, single-, and double-transgenic animals revealed no apparent changes in heart physiology in double-transgenic mice compared with single- and non-transgenic mice (data not shown). Sirius red staining was used to assess any changes in interstitial and perivascular connective tissue in the heart. No differences were observed between groups of transgenic animal relative to the distribution and density of fibrosis (data not shown).

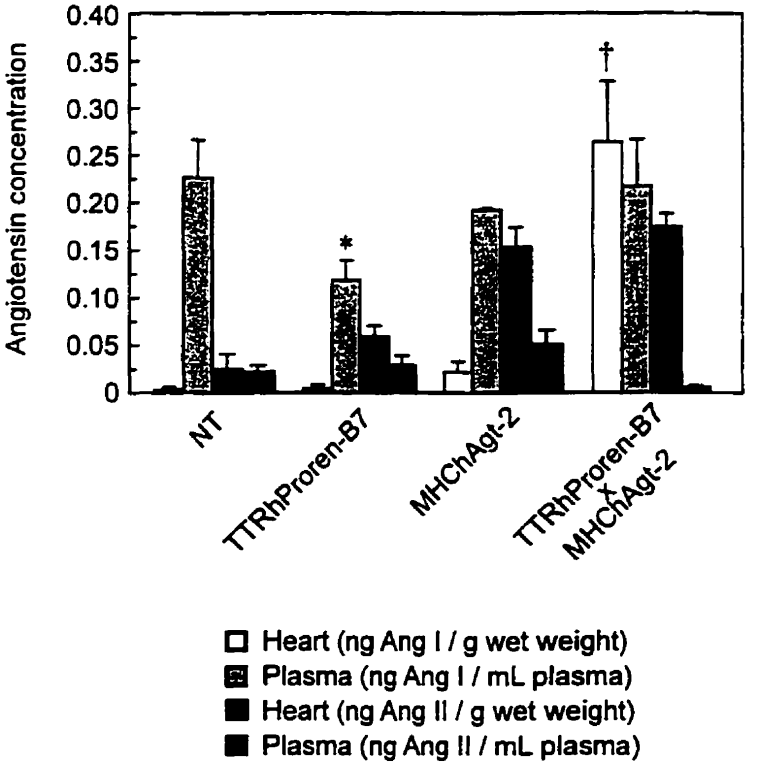

Figure 3.4 Angiotensin peptide measurement in plasma and hearts of non-, single- and double-transgenic animals. Three to five mice were used in each group. *P<0.05 as compared to non-transgenic mice by ANOVA using Student's t-test . †P<0.01 as compared to MHChAgt-2 mice by ANOVA using Student's t-test.

DISCUSSION

The present study provides the first *in vivo* demonstration that a chronic elevation in circulating prorenin leads to uptake of prorenin by tissues and an increase in local synthesis of angiotensin peptides within that tissue. Human renin and angiotensinogen have little biochemical interaction with their mouse homologues (20), although the ensuing products of their reaction (Ang I, Ang II and metabolites) are identical in the two species. This allows the study of the human renin and angiotensinogen transgenes without interference from the mouse RAS and makes it possible to target the effects to a given tissue (e.g. the heart). In our model, increased Ang I, the direct product of renin action on angiotensinogen, and was restricted to the heart of animals in which the substrate, angiotensinogen, was expressed. Since we observed no increase in plasma Ang I content in the double-transgenic animals as compared with single- and non-transgenic mice, we confirmed that the site of angiotensin peptide generation is within the heart, with no apparent leakage of those peptides in circulation. Notably, we failed to see an increase Ang II content in the heart of double-transgenic animals. While the explanation for this result is not known, it is possible that the levels of Ang I peptides generated do not permit significant conversion to and detection of Ang II. Furthermore, we did not observe any of the hallmarks of high cardiac Ang II including cardiac hypertrophy or increased fibrosis in the double transgenic mice demonstrating that a local increase in Ang I alone is not sufficient to induce cardiac pathologies. Thus, while we failed to demonstrate a physiological consequence of prorenin uptake and activity in the heart, it is possible that prorenin uptake plays a significant role in other tissues where it could encounter endogenous angiotensinogen. Such a possibility would need to be tested using native mouse prorenin as the human protein used in these studies does not have detectable activity on mouse angiotensinogen.

The nature of the activation step for circulating prorenin taken up by tissues is not known. Previous studies have demonstrated that human prorenin can be enzymatically active without the removal of it 43-amino acids prosegment within tissues (15). Cell culture studies (14,16) have shown that endothelial cells, cardiomyocytes and fibroblasts can bind and internalize not only renin, but also prorenin whereupon prorenin can be

activated to renin. It has been suggested that this finding explains why in the normal heart virtually no prorenin can be detected (21,22).

Several groups have reported on the existence of (pro)renin binding proteins and/or receptors (12,13,16,22-25). Nguyen *et al.* and Sealey *et al.*, with the use of radiolabelled (pro)renin, demonstrated high-affinity renin binding sites/receptors (K_d 1 nM) in human mesangial cells and in membranes prepared from multiple rat tissues including the heart and vasculature (13,23). As these sites bind prorenin and renin equally well, it is possible that they could account for prorenin uptake within tissues (13). The demonstration in this study that the prorenin being taken up can have enzymatic activity may serve to explain the cardiovascular pathologies observed in animals with high levels of circulating prorenin (11) and may point to a novel therapeutic target for their prevention in humans.

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CHAPTER 4

Blood pressure effect of circulating prorenin

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Submitted

In our previous study, we observed a hypotensive phenotype in some of the human prorenin expressing mice. This observation was similar with previous studies in which a significant decrease in blood pressure was observed upon injection or infusion of prorenin in experimental animals. However, it differs from study reported using a transgenic rat model over-expressing rat prorenin. In that model, rats are normotensive but develop some vascular pathophysiology. These observations led us to assess the implication of circulating prorenin in maintenance of blood pressure and on development of vascular pathologies. The present work describes results from transgenic mice harboring a chronic elevation of circulating prorenin. Our results fail to support a model in which circulating prorenin could act as a natural antagonist of renin binding in the vasculature and be responsible for the circulatory modifications and pathologies associated with its chronic elevations.

This is a multi-authored work in which Dr. David W. Silversides generated all transgenic mouse lines and Sandro Maschiotra, a laboratory technician, performed the RNase protection assays on TTRmProren and TTRmProren-mut mice tissues (Figure 4.3). Chantal Mercure, a laboratory technician, performed the blood pressure measurements of TTRmProren-mut mices. All the remaining of the work was carried out by myself under the supervision of Dr. Timothy L. Reudelhuber.

ABSTRACT

Although the physiological role of circulating prorenin (the inactive precursor of renin) is currently unclear, high prorenin levels are often associated with conditions of vasodilatation and/or vascular injury. It has been proposed that both renin and prorenin can be taken up by vascular cell types and in blood vessels where they might contribute to the activity of local renin-angiotensin systems. It has also been reported that both renin and prorenin bind to specific high-affinity acceptor sites in the vascular wall which might mediate their uptake into tissues. In the current study, we tested the hypothesis that chronically high circulating prorenin levels regulate blood pressure by acting as an endogenous competitor for renin binding and uptake in tissues. Transgenic mice overexpressing either native or active site mutated (enzymatically inactive) prorenin in the liver were generated. Levels of circulating prorenin were increased 40-100 times above normal in these transgenic mice whereas plasma renin concentration was either unchanged or decreased. Blood pressure measurements revealed either no difference or an increase (+26 mm Hg) in blood pressure for the native prorenin-expressing mice while the inactive prorenin mice showed no significant differences compared with control animals. No differences were detected in either heart rate or cardiac/renal histomorphology in transgenic animals. These results fail to support a role for prorenin acting as an endogenous competitor of renin binding and activity in the circulation.

INTRODUCTION

Circulating renin, a key enzyme in the renin-angiotensin system (RAS) plays an important role in the regulation of blood pressure and electrolyte homeostasis. Derived mainly from the kidney, it catalyzes the rate-limiting step of this enzyme cascade. Prorenin, the biosynthetic precursor of renin, is also present in the blood plasma and accounts for more than 90% of renin circulation in both man and rat. Prorenin is produced not only by the kidney but also by other tissues such as adrenal, testis, ovary, and placenta which contribute to its levels in the plasma (1-3).

Although the role of circulating renin in the regulation of blood pressure has been extensively documented, very few experiments have addressed the contribution of prorenin to cardiovascular regulation. Early experiments dealing with acute infusion of prorenin in animals showed no evidence of increased blood pressure, which was explained by the observed lack of prorenin activation in plasma (3-7). However, a transient reduction in blood pressure was reported in monkeys infused with recombinant human prorenin (6,8), and a prolonged reduction in blood pressure was seen in rats infused with recombinant rat prorenin (5). Supported by the existence of (pro)renin acceptor/receptor proteins in the heart and vasculature (9,10), the finding that prorenin as well as renin have a longer retention time in the circulation when infused in blood vessels (11), and that high prorenin has been associated with regional vasodilatation (12), some investigators (12) have proposed that renin activity in the circulation is enhanced by binding to the blood vessel wall. In this model, prorenin could act as a competitive ligand for renin binding in the vasculature where it might block renin activity in tissues of cardiovascular importance. To directly address this question, transgenic mice exhibiting chronic elevations of circulating prorenin were generated and tested for effects on blood pressure and tissue pathologies that might result from local hyperperfusion. Our results fail to support a role of chronically elevated prorenin as a regulator of cardiovascular function.

MATERIALS AND METHODS

Construction of the expression vectors

The expression vector for native mouse prorenin (TTRmProren) was constructed as follows. A cDNA for mouse Ren-1 was cloned downstream from a 3 Kb region of the transthyretin gene promoter (a gift from Dr. Robert Costa, The University of Illinois at Chicago) and upstream from the rabbit β-globin 3' non-translated region. Mouse prorenin incapable of exhibiting renin activity (TTRmProrenin-mut) was produced by converting an aspartic acid in the renin active site (position 32) to an asparagine (D/N +32). The substitution was carried out by site-directed mutagenesis using the overlap extension PCR technique described by Horton *et al.* (13). The following oligonucleotides were used: Mut-forward primer (5'-AAAGTCATCTTTAACACGGGTT-3'), Mut-reverse primer (5'-AACCCGTGTTAAAGATGACTTT-3'), Ext-forward primer (5'-CCCAAGCTTAGATGGACAGAAGGAGGATGCCTCTCTGGGCACTG-3'), and Ext-reverse primer (5'-ATGTCGGGGAGGGTGGGCACCTG-3'). All expression vectors were verified by sequencing of double-stranded DNA.

Cell culture and transient transfections

The human hepatocellular carcinoma HepG2 cells were grown at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum in a humidified incubator at 5% CO_2. Cells were plated at a density of 1 X 10^6 cells in 35-mm wells and transfected 6 h later with 20 μg of the appropriate expression vector using the Lipofectamine® Reagent method (Life Technologies, Inc).

Biosynthetic labeling of proteins

Transiently transfected HepG2 cell were replated in 25-mm wells 48 h after transfection. The next day, the cells were depleted of methionine for 1 h in methionine-free Dulbecco's modified Eagle's medium containing 10% dialyzed fetal calf serum, labeled with 300 μCi of [^{35}S]methionine per well, and incubated in complete medium for 18 h. At the end of this period, culture supernatants were then immunoprecipitated with a rabbit anti-human

prorenin (BRI-6) and protein A-Sepharose. Immunoprecipitated proteins were fractionated by SDS-PAGE, and gels were subjected to fluorography.

Generation and maintenance of transgenic mouse lines

FVB/N mouse embryos were microinjected with appropriate expression vectors according to standard protocols (14). Breeding of all mice was carried out in the FVB/N line keeping all transgenic animals heterozygous for the transgene. Presence of transgene was determined by PCR analysis of DNA obtained by tail biopsies. All animals studied were at 8-12 weeks of age. All control animals used were non-transgenic littermates. All animal protocols were approved by the institutional Animal Protection Committee of the Clinical Research Institute of Montreal.

Determination of transgene expression levels

Transgene expression was quantitated by an RNAse protection assay from total tissue RNA of liver samples as previously described (15). The probe used spans the rabbit ß-globin 3' non-translated region. Intensity of the bands was estimated by scanning densitometry of autoradiograms using an Alpha Imager 2000 Digital Imaging System (Alpha Innotech Corp.). Transgene expression levels were compared by normalizing the intensity of the prorenin band to the corresponding histone H4 band intensity. Relative levels (fold increase of expression level) were based on the ratio of individual expression level to the lowest level measured (TTRmProren-3 mice).

Determination of plasma renin and prorenin concentration

Plasma samples were obtained by orbital puncture as described previously (16). Plasma renin concentration (PRC) and total renin concentration (TRC) were determined by renin enzymatic assays followed by radioimmunoassay (RIA) of generated Ang I. Both assays were performed as described previously (16) with the following modification: 0.1 µL plasma samples (diluted in water, 50 µL final volume) and 50 µL semi-purified sheep angiotensinogen (equivalent to 8000 pg Ang I) were used for the renin assays.

Blood pressure measurements

Blood pressure measurement was performed by tail-cuff plethysmography (*BP-2000* system, Visitech Systems, Apex, NC, USA) as described previously (16). Briefly, mice were trained to the apparatus for a total of 7 uninterrupted days and measurements were recorded for the following 3 days.

Angiotensin peptide measurements

Ang II was measured by RIA of acid-soluble extracts of either plasma or kidney according to previously described methods (16). Briefly, 0.2 ml plasma extracted from blood drawn by cardiac puncture in presence of peptidase inhibitors (1 μM remikiren, 1 μM captopril, and 10 mM EDTA final concentration) were used for Ang II measurement in plasma. Both kidneys were collected, washed in phosphate buffered saline (pH7.4) and tested separately for angiotensin peptide content.

Organ histomorphology

For the histological examinations, the livers, kidneys, lungs, testis and hearts of anesthetized transgenic and control animals were fixed *in situ* by organ perfusion using Bouin's fixative, collected and then imbedded in paraffin. Tissue sections (6-7 μm) were produced. One set of tissue sections was stained with hematoxylin/eosin according to standard protocols and examined for apparent physiological abnormalities (i.e. vascular lesions and hypertrophy). A second set of tissue sections was stained with Sirius Red as described previously (16) to assess differences in interstitial and perivascular fibrosis.

RESULTS

Transgene construction and characterization

The transgenes we have constructed place the mouse Ren-1 cDNA under the control of the transthyretin promoter. In mice, this promoter drives the transgene expression in the liver as previously demonstrated (17). In order to test for the contribution of enzymatic activity in any eventual physiological role of circulating prorenin, we made identical constructions in which one of the aspartic acids in the active site of renin (aspartic acid 32) was converted to asparagine by site-directed mutagenesis.

To verify the synthesis and release of the recombinant prorenins encoded by these transgenes, cultured hepatic cells transfected with the appropriate vectors was metabolically labeled with [^{35}S]methionine and culture supernatant were immunoprecipitated with an antibody specific for prorenin and renin. As shown in Figure 4.1, bands corresponding to mouse prorenin were observed in the supernatant of cells transfected with the native and mutated mouse prorenin expression vectors. This result indicates that the expression vectors direct the proper synthesis and release of prorenin from transfected cells. The effectiveness of the D/N +32 mutation in eliminating renin enzymatic activity was confirmed by performing renin assays of culture supernatant collected from transfected cells (data not shown).

Generation of transgenic mice

Out of seven founder lines generated for the native mouse prorenin, only two (e.g. TTRmProren-3 and TTRmProren-7) were found to release significant amounts of prorenin into the plasma (as indicated by TRC values) with no detectable increase in plasma renin concentration (PRC) (see below). Of the three founder lines generated for mutated mouse prorenin, only two (TTRmProren-2mut and TTRmProren-3mut) were shown to express the transgene in liver with levels comparable or higher than TTRmProren mouse lines (see below). The relative level of transgene mRNA expressed in liver of transgenic mice varied over a range of approximately 4-fold (Figure 4.2).

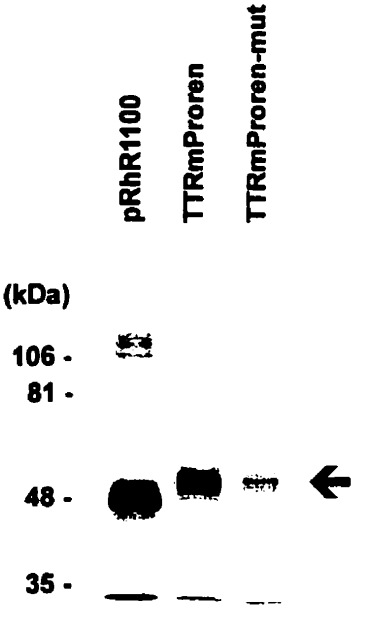

Figure 4.1 Detection of labeled mouse prorenin in culture supernatant from cells transfected with the native (TTRmProren) or active site-mutated mouse prorenin (TTRmProren-mut) vectors by immunoprecipitation. Arrow indicates the expected migration of mouse prorenin. A human renin expressing vector (pRhR1100) was used as a positive control.

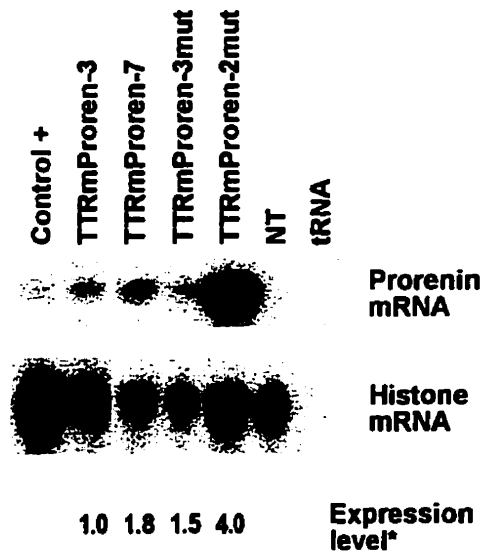

Figure 4.2 Transgene expression in liver of founder lines expressing native (TTRmProren-3 and –7) or mutated mouse prorenins (TTRmProren-2mut and –3mut) as measured by RNase protection assay. Histone H4 mRNA is included as a control for RNA loading. Total RNA from transfected cells (Control +) and non transgenic animals (NT) were used as controls. Expression levels (bottom) correspond to relative fold increase of individual expression levels compared with lowest level measured (TTRmProren-3).

Expression of native mouse prorenin (Ren-1) in the liver of transgenic mice leads to a 10-fold increase in total renin concentration (active renin and prorenin) in both transgenic mouse lines studied (Table 4.1) which corresponds to an increase in prorenin in the order of 40-fold in the TTRmProren-3 mouse line and 50-fold in the TTRmProren-7 mice. Plasma active renin concentration measurements (PRC) revealed a significant decrease in circulating active renin in transgenic TTRmProren-7 mice compared with non-transgenic littermates whereas no significant differences were observed for the TTRmProren-3 mice (Table 4.1). This latter observation suggests a downregulation of the endogenous renin in this transgenic mouse line.

Table 4.1 Physiological characterization of transgenic mice*

Mouse line	*Geno-type*	*PRC (ng Ang I/mL/h)*	*TRC (ng Ang I/mL/h)*	*SBP (mm Hg)*	*Pulse (beats/min)*	*n*
TTRmProren-3	NT	1779±899	2309±888	132±2	646±12	8
	TG	1314±567	22561±4085†	124±3	645±14	8
TTRmProren-7	NT	3158±757	4045±1198	130±3	649±17	10
	TG	1617±125†	46771±5097†	156±3†	656±17	10
TTRmProren-2mut	NT	nd	(~3177)‡	136±3	711±15	7
	TG	nd	(~34000)‡	138±4	696±8	5
TTRmProren-3mut	NT	nd	(~3177)‡	136±3	711±15	7
	TG	nd	(~90000)‡	143±5	715±13	5

* Results represent the mean (± SEM) values of single determinations. PRC, plasma renin concentration (active renin). TRC, total renin concentration (prorenin + active renin). SBP, systolic blood pressure. NT, non transgenic littermate mice. TG, transgenic mice. †$P<0.001$, compared to control animals (NT) determined by ANOVA using Student's t-test. ‡Expected value based on relative expression level of prorenin mRNA determined by RNase protection.

Since the mutant mouse prorenin (TTRmProren-mut) lacks enzymatic activity, determination of PRC and TRC by renin assays is not possible. However, an estimation of the plasma prorenin concentration in those animals can be made based on determination of the relative mRNA expression level of the individual mouse lines. Assuming that plasma level of circulating prorenin is proportional to it mRNA expression level in liver (as suggested by the correlation seen in the animals expressing native prorenin) TTRmProren-mut2 and –mut3 mice are expected to have plasma prorenin levels in the order of 125x and 46x, respectively, as compared to control animals (Table 4.1).

Biochemical and physiological characterization of transgenic animals

Blood pressure measurements on all transgenic mouse lines showed either no difference (TTRmProren-3, TTRmProren-2mut, and TTRmProren-3mut) or a moderate increase (TTRmProren-7) in their mean systolic blood pressure as compared with control non-transgenic littermates. We failed to observe any significant decrease in blood pressure in any of the transgenic mouse lines. Heart rates of the transgenic mice were also unaffected as compared to control animals (Table 4.1).

To assess the contribution of prorerin to the activity of the circulating RAS in the moderately hypertensive TTRmProren-7 mouse line, we measured Ang II peptide concentration in both plasma and kidney in these mice and in control non-transgenic littermates. As shown in Figure 4.3, both plasma and kidney Ang II concentrations were significantly lower in the TTRmProren-7 transgenic mice as compared to control animals. This result suggests that chronically increased levels of circulating prorenin do not contribute to increased circulating RAS activity. The hypertension observed in these animals also does not appear to be a consequence of an increase in intrarenal Ang II content.

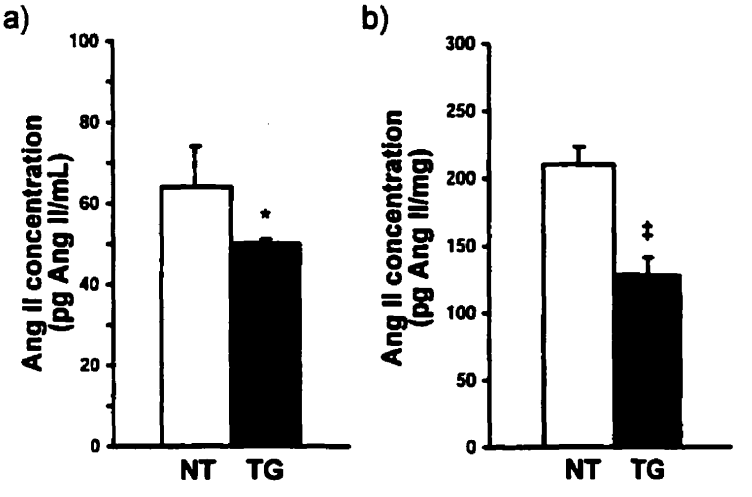

Figure 4.3 Ang II concentration in plasma (a) and whole kidney (b) in TTRmProren-7 (TG) and control animals (NT). Values are mean (± SEM) of single determinations on 5-6 individual animals. *P<0.01, †P<0.001 compared to control animals determined by ANOVA using Student's t-test.

Routine histological staining (hematoxylin/eosin) performed on major organs of native mouse prorenin transgenic animals and control revealed no apparent changes in tissue morphology in transgenic mice compared with control animals (data not shown). No vascular lesions were observed. Sirius staining was used to assess any changes in interstitial and perivascular fibrosis in tissue and failed to show any differences in transgenic mice compared with control animals. These results reveal no pathophysiological consequences of chronically increased plasma prorenin in our transgenic mice.

DISCUSSION

In this study, we report the creation of an *in vivo* model to assess the role of prorenin in the regulation of blood pressure. Based on the hypothesis that prorenin in circulation may be acting as an endogenous competitor for renin binding to the vascular wall, we predicted that sustained increase in plasma prorenin levels should lead to an hypotensive phenotype in our model. Since we failed to observe a significant decrease in mean systolic blood pressure in all transgenic mouse lines studied, we provide evidence refuting the latter hypothesis. However, our *in vivo* model has demonstrated that, in intact animals, 1) prorenin is not converted to active renin in the circulation as noted by the lack of observed increase plasma renin concentration in transgenic animals, and that 2) prorenin has no significant intrinsic enzymatic activity in the circulation as suggested by a lack of an increase in plasma Ang II concentrations. These data confirm previous reports (7) indicating that circulating prorenin does not participate, either directly or indirectly, to circulating RAS activity.

Somewhat surprisingly, one of our lines of transgenic mice with high levels of circulating native prorenin (line TTRmProren-7) was hypertensive in spite of the fact that it exhibited no increase in circulating active renin or Ang II. Although we do not know that exact explanation for this increase in blood pressure, our results strongly suggest that the hypertension is a result of RAS action in one or more tissues and not in the circulation. There is evidence for the existence of tissue RAS in many organs and tissues (18), some of which participate directly in the regulation of blood pressure. Local activation of the RAS in brain, kidney or adrenal can lead to hypertension independent of circulating RAS activity (19-21). Prorenin taken up by tissues would presumably have to be activated locally to contribute to angiotensin formation and hypertension. Notably, our results do not show an increase in renal Ang II content in the hypertensive line TTRmProren-7 line in spite of the fact that the intrarenal RAS has been implicated in blood pressure regulation (20,22). Nevertheless, several studies have shown that prorenin taken up by either cultured cells or by tissues in whole animals can be converted to active renin by either proteolytic or non-proteolytic mechanisms (7,15,23,24). The current study suggests that this prorenin might contribute to an increase in blood pressure.

These results also raise the possibility that a blood pressure-lowering effect of circulating prorenin (by renin uptake competition) could be masked by a concurrent blood pressure increasing action of circulating prorenin (by contribution to a tissue RAS). To rule out this possibility, we generated transgenic mice with high circulating levels of active site-mutated prorenin which, although still capable of binding to a renin acceptor, would not be able to contribute to the activity of a tissue RAS. Our results show no effect of chronically high levels of mutated prorenin, suggesting that competition for a renin uptake protein is not a major contributor to blood pressure modulation.

Previous reports have proposed that circulating prorenin could also have a role in vascular pathologies independent of blood pressure. Veniant *et al.* (25) reported that transgenic rats overexpressing prorenin exhibit renal vascular lesions and cardiac hypertrophy without significant hypertension. In our transgenic mice expressing native prorenin, we observed no apparent pathophysiology associated with chronically elevated circulating prorenin. The difference in these results may be related to the difference in plasma prorenin levels between the previously reported rat model (up to 400-fold increase) and our present model (a maximum of a 100-fold increase). Alternatively, it may simply be that prorenin doesn't play the same role in mice compared to rat relative to it effects on cardiovascular system.

In conclusion, the present study fails to support a model in which circulating prorenin could act as a natural antagonist of renin binding in the vasculature leading to a decrease in blood pressure. Our failure to show any consequences of increased circulating prorenin in our mice raises the possibility that chronic elevations in circulating prorenin are not responsible for the circulatory modifications and pathologies with which they are commonly associated.

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CHAPTER 5

General discussion

The experimental work presented in this thesis has furthered our understanding of the role of circulating RAS components relative to its participation in circulating and tissue RAS activity, in several respects: 1) our results studying the uptake of circulating (pro)renin in tissue has confirmed the presence *in vivo* of blood-borne (pro)renin in several tissues, 2) results from *in vivo* renin enzymatic assay studies revealed tissue activity of circulating prorenin which strongly suggested a role for circulating prorenin in local RAS activity, 3) the potential physiological implication of such local activity has been highlighted by subsequent physiological studies providing evidence for distinct role of tissue RAS such as promoting cardiac fibrosis, and 4) results from studies assessing a role for prorenin in circulation tend to refute the hypotheses that circulating prorenin contributes to the systemic control of blood pressure and to the pathophysiology of the vasculature. However, our results raise the possibility that local activity of prorenin might affect the long-term maintenance of blood pressure.

1 Our experimental models

Before discussing the results gathered from the current studies, it is essential to understand the premise behind the design of our experimental models.

1.1 Study of uptake and activity of circulating (pro)renin in tissues

Using the gene-targeting approach, transgenic mice harboring the human (pro)renin gene in the liver and the human angiotensinogen in the heart were produced (Figure 2.1, panel B and Figure 3.1, panel B). On the premise that human (pro)renin is constitutively released into the circulation, is accessible to all tissues, and will share enough similarity to the mouse (pro)renin to behave like the latter, uptake of (pro)renin can be determined by precise immunological localization of human (pro)renin within tissue. Upon confirmation of it localization within tissue, local renin activity can be determined *in vivo* by providing its substrate to the tissue (i.e. the heart) (Figure 5.1). Measurement of tissue angiotensin concentration should demonstrate if (pro)renin is active within tissue.

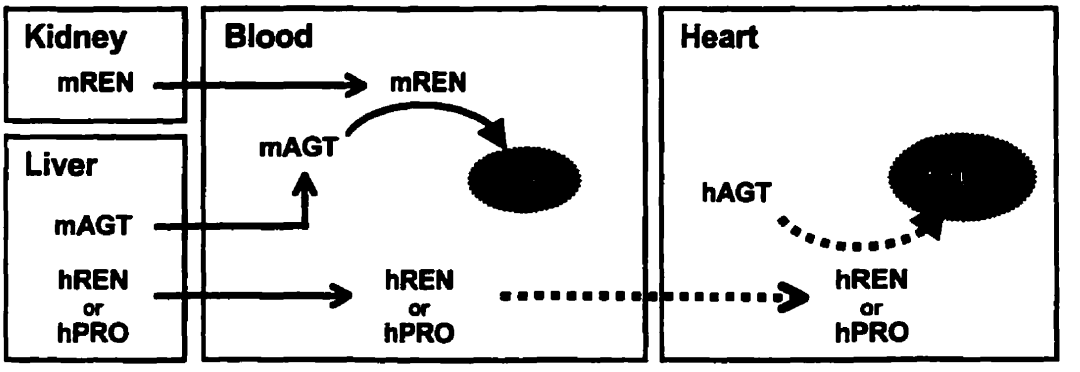

Figure 5.1 Diagramatic representation of the experimental approach used to assess (pro)renin uptake and activity in tissue. Full arrows (⟶) represent actual processes. Interrupted arrows (⋯▸) represent processes under investigation. RAS components: mouse renin (mREN), human renin (hREN), human prorenin (hPRO), mouse angiotensinogen (mAGT), human angiotensinogen (hAGT), and angiotensin I (Ang I).

1.2 Study of the function of prorenin in circulation

To study the function of prorenin in circulation, we used the same gene-targeting approach as for the previous model to produce transgenic mice harboring the native mouse prorenin gene (Ren-1) or an inactive mouse prorenin (Ren-1 with D/N +32 substitution) in the liver. On the premise that the native and inactive mouse prorenin are constitutively released into the circulation and its plasma levels in excess compared to the endogenous renin, the role of circulating prorenin relative to it implication in control of blood pressure can be assessed by precise measurement of the blood pressure of transgenic mice. Moreover, histologic examination of major tissues of transgenic mice should allow the determination of the possible role of prorenin in the pathophysiology of the vasculature.

2 Uptake of circulating (pro)renin by tissue *in vivo*

The origin of renin in cardiovascular tissues is a controversial issue. A growing body of evidence favors the view that renin uptake is the major mechanisms used to provide renin to these tissues. However, direct evidence for this process is still lacking. Described in

Chapter 2 and 3, studies assessing the presence of circulating (pro)renin in tissue by immunohistochemistry provided direct evidence for the uptake of circulating (pro)renin by tissue such as the heart. Moreover, detection of human renin enzymatic activity *in vivo* (indicated by angiotensin peptides concentration in tissue expressing the natural substrate of human renin) provided further evidence for its presence in the heart (refer to Section 3).

2.1 Nature of (pro)renin uptake by tissue

Uptake mechanisms

The simple fact of observing the presence of human (pro)renin in discreet perivascular cells within tissues indicates that circulating (pro)renin has the ability to enter a tissue by crossing the endothelial cell layer forming the blood vessel wall. The mechanism of (pro)renin uptake by tissue *in vivo* is still not known. However, based on our observations, this mechanism may require two consecutive steps: passage of (pro)renin from the intravascular compartment to the tissue interstitial compartment, and then binding and/or internalization of (pro)renin in perivascular cells. The first step, which represents the crossing of the blood vessel wall (i.e. endothelial cells layer) could be a diffusion of (pro)renin through fenestrations, fused vacuoles, cell junctions, or by vesicle transcytosis. All those processes have been observed to take place in capillaries and larger blood vessel (241-243). Alternatively, crossing of the blood vessel wall can be achieved by a receptor-mediated process. Binding to acceptor/receptor proteins, (pro)renin could be brought within a given tissue by transcytosis. Present in the interstitial compartment, (pro)renin might enter cells via a similar process (i.e. receptor-mediated endocytosis). In support of an active process of (pro)renin uptake in tissue (and cells), cell membrane proteins specifically binding (pro)renin have been identified and shown to be present on endothelial and cardiac cells (202-206). In addition, recent evidence from *in vitro* cell culture has shown that human prorenin and renin can be internalized in cardiomyocytes, fibroblasts or endothelial cells through a mannose-6 phosphate receptor mechanisms (202,203). Moreover, these studies have demonstrated that upon internalization, prorenin was proteolytically activated.

In chapter 2, we provided evidence that (pro)renin uptake is independent of blood pressure based on the results:

1) Anti-hypertensive treatment did not lead to decreased renin activity in heart of double transgenic animals compared with vehicle treated animals (Table 2.2),

2) Anti-hypertensive treatment did not lead to a significant decrease in renin immunoreactivity in the heart of transgenic animals compared with vehicle treated animals (data not shown).

These results suggest that (pro)renin uptake is not mediated by simple diffusion of plasma constituents to the interstitial space, and bulk (non-specific) endocytosis into perivascular cells since variations in blood pressure have been demonstrated to affect the movement of blood-borne substances across the vessel wall (244). However, if circulating (pro)renin entry in the interstitial space is mediated mainly by diffusion, binding and/or uptake of interstitial (pro)renin in perivascular cells as well as it local enzymatic activity have to be specific and limited processes.

Uptake selectivity

Based on the presence of an IR-human (pro)renin signal in TTRhProren-B7 mice, we can state that prorenin, the precursor form of renin can enter tissue to be detected locally. The precise nature of the renin form detected within tissue is still to be determined since it is conceivable that prorenin is proteolytically processed and/or associated with proteins within the tissue. Since both form of human renin are present in plasma of human active renin-expressing mice (refer to Section 6.2) and since we were unable to distinguish each renin form by immunohistochemistry (refer to Section 2.1), the entry of human renin in tissues could not be confirmed in the TTRhRen-A3 mice. This result raises the possibility that the observed IR-human (pro)renin in tissue of the latter mouse line may derive strictly from circulating prorenin, not renin. This hypothesis has been previously presented by Sealey and Rubattu (216) in an attempt to define a role for prorenin in circulation and tissues. In support of this hypothesis, selective uptake (or extraction) of circulating prorenin has been reported for the heart (192,193) and kidney (194). However, several lines of evidence argue against this hypothesis: renin binding proteins identified to date have been

demonstrated to have comparable affinity for both renin forms (204), and renin uptake has been demonstrated in tissue including the heart upon infusion or injection of pure active renin (185,186,197-199).

Taking into account the relative plasma level of human active renin and prorenin in TTRhRen-A3 and TTRhProren-B7 mice (refer to Section 6.3), comparison of tissue distribution and staining intensity of IR-human (pro)renin between those mouse lines raises the possibility that *in vivo* the uptake is selective for prorenin in heart, liver and pituitary, while being selective for active renin in gonads (i.e. ovary and testis) (refer to Section 1 of Appendices). Selectivity in (pro)renin uptake could be mediated by differences in affinity of (pro)renin binding proteins. Alternatively, if the (pro)renin uptake by tissue is not selective for one particular renin form, the difference in tissue distribution and staining intensity could be explained by differences in density of (pro)renin binding proteins in various tissues.

2.2 Cell-type(s) responsible for the uptake of human (pro)renin

As mentioned in chapter 2 and 3, and presented in Appendices (Section 1), the cell-type responsible for the capture of (pro)renin in the heart and other tissue is still unknown. However, (pro)renin uptake has been observed in Kupffer cells (liver) and proximal tubule cells (kidney). These observations are in accordance with previous reports demonstrating that corresponding tissues are the main site of circulating (pro)renin accumulation in the body (113,245-253). Those cells have been demonstrated to be responsible mainly for the clearance of circulating (pro)renin (246,250,251). However, one study reports conversion of circulating prorenin to renin by those tissues (248) raising the possibility that circulating (pro)renin may participate in a local RAS in those tissue.

In the heart and testis of our transgenic animals, IR-human (pro)renin was observed in cells adjacent to capillaries or small arterioles (refer to Appendices). In these particular tissues, the morphological study performed by electron microscopy suggests that one cell-type is responsible for the uptake of circulating (pro)renin in those tissues and that it may be the same cell for both tissues. However, comparison of the staining intensity between human active renin-expressing mice (TTRhRen-A3) and human prorenin-expressing mice

(TTRhProren-B7) may suggest a difference in the cell affinity for each renin form in these tissues (refer to Appendices).

Although we were unable to identify the cell-type staining for the human (pro)renin in the heart, our studies permitted us to eliminate the cardiomyocytes, endothelial cells, and vascular smooth muscle cells as potential candidates. Based on the morphological characteristics of cells staining for human (pro)renin (described in Appendices, Section 1), the following candidate cells can be considered:

1) Macrophage/monocyte cells:

Macrophage/monocyte cells are responsible for the clearance of plasma and interstitial proteins. Those cells have been shown to be able to infiltrate tissue to become resident. Macrophage/monocyte cells are important in inflammation processes in cardiovascular tissue (254,255). Since we demonstrated that a small portion of the human (pro)renin stained cells are resident macrophages (partial co-localization of IR-human (pro)renin with MAC-2), we may consider the possibility that non-resident macrophages or monocytes are responsible for most of the (pro)renin uptake in the heart and other tissue such as the testis. In support of a role of macrophage/monocyte cells in (pro)renin uptake and local activity, human monocytes have been shown to contain a substantial amount of Ang I and Ang II (256), and renin activity and immunoreactivity have been detected in resident alveolar macrophages/monocytes (257). In an attempt to localize these cells in the heart, antibodies for MAC-1 (CD11b) and F4/80 were used in immunohistochemistry on cardiac tissue sections. Using different tissue fixatives and immunohistochemical procedures, we were not able to obtain a clear immunoreactive signal (data not shown). More cell markers specific for macrophages and monocytes are now available (i.e. BM8, MP23, MOMA1, MOMA2, M5/114, BMDM1 and NLDC145 (258)) and could be used to test this possibility.

2) Pericytes:

Pericytes are cells define mainly by their strict localization to capillaries and post-capillary venules. Pericytes are morphologically, biochemically and physiologically heterogeneous (231). However, pericytes possess certain common characteristics: i) pericytes regulate endothelial proliferation and differentiation; ii) pericytes are contractile and the contraction may either exacerbate or stem endothelial junctional inflammatory leakage; iii) pericytes function as a progenitor cell; iv) pericytes synthesize and secrete a wide variety of vasoactive autoregulating agonists; v) pericytes synthesize and secrete structural constituents of the basement membrane and extracellular matrix; vi) pericytes are involved in specific microvascular diseases. Their tissue localization and morphological characteristics make pericytes good candidates for the cell-type responsible for (pro)renin uptake in the heart. However, the lack of specific cell markers for pericytes make their identification quite difficult. Still, one histological stain specific for pericytes has been developed by Zimmermann in 1923, and uses a silver impregnation stain for light microscopy (259). Unfortunately, this staining is not compatible with the (pro)renin staining and co-localization techniques used in our studies making the co-localization impossible.

3 Activity of circulating prorenin within tissue

Our experimental model has been designed to assess renin activity *in vivo*. By providing its natural substrate to target tissue, presence of renin enzymatic activity can be determined by measuring the concentration of angiotensin peptides in that particular tissue. As demonstrated in chapter 2 and 3, both human active renin (TTRhRen-A3) and human prorenin (TTRhProren-B7) mice have been shown to possess some human renin activity within their heart which depends on the presence of human angiotensinogen locally. From our studies, we are the first to provide direct evidence for the local activity of circulating prorenin within tissues (refer to Chapter 3). Previous studies where isolated or recombinant prorenin was infused or perfused in whole body or in isolated tissue preparation failed to observe activation of externally provided prorenin by tissue as indicated the lack of observed alteration of blood pressure, increase in plasma renin concentration or activity, or increase in tissue angiotensin peptides concentration (220,260). However, one study reports

a possible physiological consequence of increased circulating prorenin in rat that might suggest the possible local activity of circulating prorenin (222).

3.1 Local activity of circulating (pro)renin is independent of blood pressure

In chapter 2, we demonstrated that local activity of circulating (pro)renin in the heart is independent of blood pressure. This is based on results obtained from animal treated with losartan, an AT_1 receptor antagonist used to normalize blood pressure. We have to be careful in interpreting results from studies performing drug treatment especially when the drug used interacts directly with components of the endocrine system studied. A previous study has reported the increase of both Ang I and Ang II (7- and 2-fold, respectively) in heart of rats treated with losartan when compared with vehicle treated hypertensive animals (139). In our mouse model, treatment with losartan led to a significant increase in plasma Ang I, but not cardiac Ang I in non transgenic mice (Figure A.2). This can be explained by inhibition of negative feedback mediated by the AT_1 receptor on JG cells, resulting in stimulation of renin synthesis and secretion from kidney. For the other transgenic mouse lines (human renin, human angiotensinogen and double transgenic mice), no increase in plasma Ang I is observed, suggesting that treatment with losartan in those mice seems not to completely release the inhibition of renin synthesis and release from kidney. These results suggests that in those latter mouse lines, control of renin release is not limited by circulating Ang II or blood pressure effects. We may consider treating the animals with non-RAS anti-hypertensive drug such as diuretic, natriuretic or beta-adrenergic receptor blockers. However, the choice of the drug used as an anti-hypertensive is still difficult since studies looking at effects of those drugs on circulating and tissue RAS activity have demonstrated that all of them have profound effects on RAS activity, directly or indirectly. However, since we demonstrated that uptake and local activity of circulating prorenin do occur in the absence of increased blood pressure in TTRhProren-B7 mice (refer to chapter 2), we provide direct evidence that (pro)renin uptake by tissues is independent of blood pressure.

3.2 Nature of prorenin activation in tissue

The nature of the activation *in vivo* of prorenin taken up by tissues is still not known since we were not able to determine the renin forms present in the heart and therefore, to provide evidence for the nature of the activation step for circulating prorenin taken up by tissues (refer to Section 6.1). However, activation of prorenin could be mediated by proteolytic or non proteolytic processes:

Non proteolytic activation

The prosegment of prorenin is responsible for the inhibition of renin. Alterations of prorenin structure which result in displacement of the prosegment can lead to it reversible activation. Plasma prorenin has less than 10% of the full activity of renin, but its activity *in vitro* is increased by lowering the pH or temperature, or lipids (96-98,100,261). Since no significant variations in temperature can occur *in vivo*, the cryoactivation of prorenin is not likely to account *in vivo*. However, acidification has been suggested for the activation of prorenin in interstitial space or in cells (upon internalization, in endosomes) (262). Evidence for non proteolytic activation of prorenin has been provided recently by *in vivo* renin enzymatic assays in mouse expressing both prorenin and angiotensinogen in pituitary (262). Alternatively, it has been proposed that prorenin activity in tissue could be mediated by it close association with binding proteins (216). However, no direct evidence has been provided for this latter hypothesis.

Proteolytic activation

The proteolytic process, characterized by the irreversible activation of prorenin, requires the presence of convertases (processing enzymes) at tissue site. At present, the kidney is the only organ that is known to convert prorenin to renin in human and mammals. Nevertheless, some conversion of prorenin has been 0reported to take place in discrete extrarenal tissues in rodents, such as the adrenal and submandibular gland (263,264). Proteolytic activation of prorenin by trypsin, plasmin, kallikrein, cathepsin B, and other proteinases has been demonstrated *in vitro* (95,235,265-267). However, none of these enzymes have been demonstrated to convert prorenin *in vivo*. Previous reports suggest that, in primates, the circulating prorenin taken up by the liver and kidney is converted to renin

without secreting renin back into the circulating (248). In addition, recent studies assessing the uptake of renin and prorenin by cardiac cells *in vitro* have demonstrated that prorenin was proteolytically activated upon internalization (202,203). We need to be careful in interpreting results from those studies since they are based on tissue extracts. Prorenin is sensitive to lysosomal proteases released during the extraction and proteolytic conversion to active renin is likely to occur.

In conclusion, the nature of the activation of prorenin taken up by tissue is still to be determined *in vivo*. To test the possibility that proteolytic removal of the prosegment of prorenin is require for the activation of prorenin is tissue, transgenic mice harboring the human prorenin gene with a non cleavable prosegment as used by Methot *et al.* (262) could be generated and assessed for it local activity in the heart as performed in the present studies.

3.3 Is tissue activity of circulating (pro)renin limited to the heart?

Our *in vivo* renin enzymatic assays were designed to assess the local activity of circulating (pro)renin in the heart. However, taking advantage of the ectopic expression of the human angiotensinogen transgene in the lung and kidney, we measured the concentration of angiotensin peptides in different tissue of mice expressing both human active renin and angiotensinogen (TTRhRen-A3 x MHChAgt-2). Preliminary data are presented in Figure A.3. While plasma and kidney Ang I concentrations in double transgenic animals are similar or decreased compared with control mice, Ang I concentration in the lung is increased in the mice harboring both transgenes. Although the difference in Ang I concentration are not quite significant due probably to the small sample size, these results raise the possibility that circulating (pro)renin is also active within the lung. However, no increased Ang II concentration is observed in that tissue suggesting that if increased local generation of Ang I does occur, the local concentration of Ang I is either too low to contribute to local Ang II concentration or that the site of Ang I generation is not in close proximity to tissue ACE. Interestingly, no increase in Ang I or Ang II concentration is observed for the kidney. Since the substrate for human renin is present locally, this may indicate that human (pro)renin is not present locally within the kidney. Alternatively, the human (pro)renin may be present within the kidney but not in close proximity of the human

angiotensinogen site of expression. The latter possibility is worth consideration since the cell-type responsible for the human angiotensinogen expression in the kidney of the MHChAgt-2 mouse line is not known. Considering the facts that IR-human (pro)renin is readily detectable in the heart and lung, and present at much lower concentrations in the kidney, we found a direct correlation between its presence determined by immunohistochemistry and its presence denoted by *in vivo* enzymatic assays. This suggest that its presence in tissue is directly correlated with its local activity.

3.4 Site of local (pro)renin activity (i.e. Ang I generation)

Tissue angiotensin generation may occur in interstitial fluid, on the cell membrane, or within cells (Figure 5.2).

Interstitial fluid

According to studies performed in the isolated rat heart, circulating renin and angiotensinogen are able to reach the interstitial space (186). This raises the possibility of angiotensin generation within this fluid. Indeed, during combined renin/angiotensinogen perfusion of the Langendorff heart, the levels of Ang I and Ang II in interstitial fluid were found to be two to three times higher than the levels measured simultaneously in the intravascular compartment (185,186). Data from *in vivo* studies in the dog also demonstrated that the cardiac interstitial angiotensin levels are higher than plasma levels of these peptides (268). Since diffusion of intact Ang I and Ang II from intravascular compartment to the interstitial compartment is marginal (most likely because of rapid metabolism of angiotensins in the vascular wall), the high interstitial levels can be taken as evidence for interstitial angiotensin generation (185,186).

Cell membrane

In support of a role for membrane-bound renin in local generation, it has been observed that that both *in vivo* and *in vitro* the amount of Ang I released by the heart via coronary effluent was too high to be explained by the renin-angiotensinogen reaction occurring in intravascular fluid during coronary passage (186,269,270). Moreover, in the isolated Langendorff heart preparation perfused with renin and angiotensinogen, Ang I release via

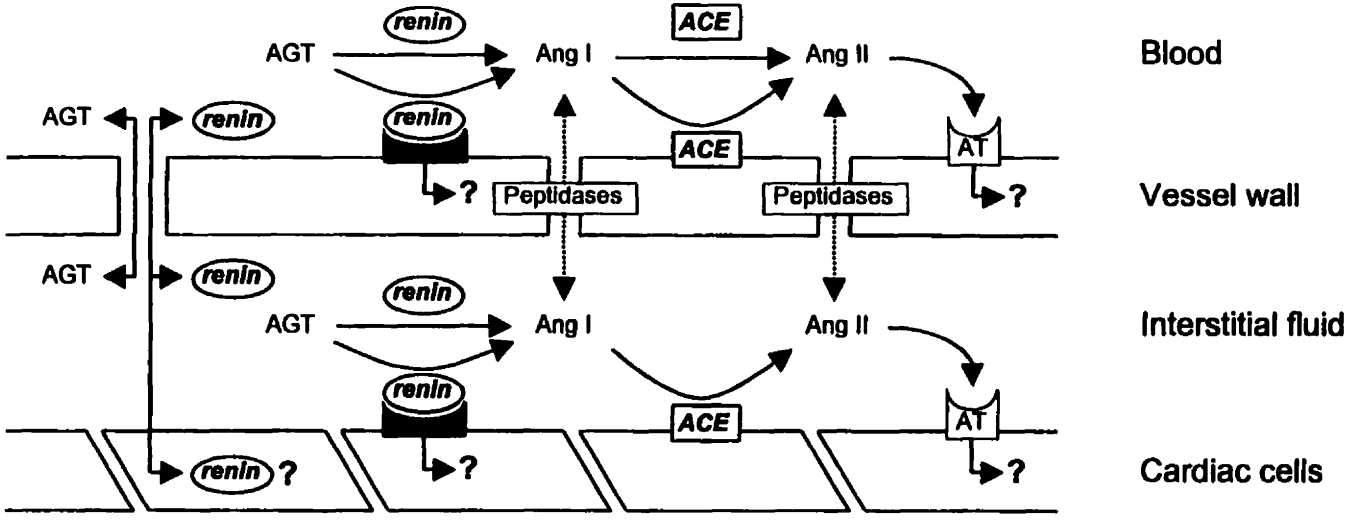

Figure 5.2 Proposed scheme of angiotensin (Ang) I and II production in the heart (based on Danser *et al.* (271)). Intravascular and interstitial compartments as well as blood vessel wall and cardiac cells (endothelial cells, myocytes, fibroblasts, macrophages) are depicted. Circulating renin and angiotensinogen (AGT) both enter the interstitial fluid compartment. Renin may also bind to the vascular wall and cardiac cells. ACE is present on endothelial cells and cardiac cells. Ang I and II are metabolized by peptidases while passing through the vascular wall. Ang I and II in the interstitial fluid are mainly generated outside the vascular fluid compartment. Tissue Ang I and II generation may occur not only in the interstitial fluid or on the cell surface but also within cells, for instance after renin uptake by the cells. Binding of Ang I to the AT_1 receptor is followed by intracellular signaling.

coronary effluent reached a steady-state level long after renin and angiotensinogen had reached a steady state in this fluid (186), and angiotensin release continued after discontinuation of the renin perfusion (198). These data suggest that tissue-bound renin rather than extracellular fluid renin is responsible for the high Ang I level in coronary effluent. Endothelial cells, vascular smooth muscle cells, cardiomyocytes and cardiac fibroblasts may all be involved in the binding process since acceptor/receptor for renin has been demonstrated to be present on those latter cells (202-206).

Intracellular compartment

Direct evidence for intracellular angiotensin generation is not available. Renin dialysis into cultured cardiomyocytes leads to a decrease in the conductance of the adjacent myocytes (54). The reduction of conductance was amplified when renin was infused together with angiotensinogen and attenuated when renin inhibitor was co-administered, suggesting that these effects are mediated by renin-dependant Ang II formation within the cell. In support of an intracellular angiotensin generation, renin and prorenin have been demonstrated to be internalized in endothelial and cardiac cells (202,203). These studies report that prorenin becomes activated upon internalization. From these findings, Danser *et al.* (271) proposed that, concurrently with (pro)renin, angiotensinogen could be taken up from the interstitial fluid via bulk fluid endocytosis to generate angiotensin intracellularly. In addition, evidence for intracellular generation of Ang II in the rat juxtaglomerular cells has been provided (272). In this case, the mechanisms proposed involved the action of endogenous renin on internalized, exogenous angiotensinogen.

In our study, additional experiments have been performed in an attempt to localize the site of renin activity within the heart. Heart sections from double transgenic mice for human active renin and angiotensinogen (TTRhRen-A3 x MHChAgt-2) were stained with an antibody recognizing Ang I and staining were compared with control mice. We failed to detect any significant Ang I immunological signal in those mice possibly due to technical difficulty of properly fixing angiotensin peptides in tissue. The size and amino acid composition of angiotensin peptides (refer to Table 1.1), and their tissue location are some reasons that might explain the lack of observed Ang I staining in our study. It is possible that if (pro)renin activity occurs in the interstitial fluid, Ang I would not accumulate at the

site of synthesis but would rather diffuse throughout the heart. Fixative treatment would wash the angiotensin away or alternatively, the immunological signal would be too diffuse and faint to be distinguishable from background signal. We have to note that angiotensinogen-expressing mice (MHChAgt-2) harbor an increase in cardiac Ang I and Ang II content as compared with non- or single-transgenic for human active renin (refer to Figure 2.3 and 3.4). In those mice, heart sections stained for Ang I give diffuse staining of cardiomyocytes. This staining was considered as background staining for the double transgenic mice since it was intended to localize the site of Ang I generated from human (pro)renin, not from endogenous mouse peptidases.

However, based on the localization of circulating (pro)renin determined by electron microscopy, we speculate that renin and prorenin enzymatic activity may take place intracellularly and/or in the interstitial fluid. It is unlikely that (pro)renin generates Ang I bound to cell membrane since we failed to observed staining of (pro)renin on any cell surface of cardiac and testicular tissues (refer to Appendices). In addition, unlike the model proposed by Danser *et al.* (271) for the intracellular generation of angiotensin peptides in endothelial cells and cardiomyocytes, we failed to observe the intracellular localization of (pro)renin in those particular cells suggesting that *in vivo*, circulating (pro)renin present in interstitial fluid is not taken up by those cells to generate angiotensins intracellularly.

3.5 Cardiac fibrosis is enhanced by local (pro)renin activity

Cardiac myocytes are surrounded by a fine network of collagen fibers that are generated primarily by cardiac fibroblasts (273). In pathologic situations involving hypertrophic reaction of cardiomyocytes, such as pressure overload or postmyocardial infarction, cardiac fibroblasts proliferate and extracellular matrix proteins accumulate disproportionately and excessively (274). This process, called reactive fibrosis, leads to increased ventricular stiffness, and hence diastolic, and then systolic, dysfunction of the heart (275). In addition, fibrosis may disrupt the cardiac conducting system (276,277). Thus, cardiac fibrosis is recognized as a key process that links pathologic cardiac hypertrophy to heart failure.

Research increasingly shows that Ang II is involved in cardiac fibrosis. Thus, treatment with ACE inhibitors or AT_1 receptor antagonists attenuates the cardiac fibrosis that occurs in experimental myocardial infarction (164,278,279), renovascular hypertension (280), and genetic hypertension (281). More directly, chronic infusion of Ang II in rats was shown to induce cardiac fibrosis through the AT_1 receptor (274).

Many clinical and experimental studies show that ACE inhibitors or AT_1 antagonists have more beneficial effects on the heart than expected, resulting solely from their blood pressure-lowering effect (279,282,283). Studies also point to additional mechanisms, other than increasing cardiac afterload, for Ang II-dependent cardiac fibrosis (274,284,285). Therefore, it is believed that Ang II has a direct local effect on cardiac tissue, independent of its systemic hemodynamic effect. Other evidence supporting this notion comes from *in vitro* studies showing that Ang II directly promotes DNA and protein synthesis in cardiac fibroblasts (286-291).

In chapter 2, we demonstrated that local increase in angiotensin peptides (Ang I and Ang II) in the heart resulting from local human renin-angiotensinogen reaction, promote an increase in interstitial and perivascular fibrosis in mice of comparable blood pressure. Moreover, in chapter 3, we demonstrate that local increase in Ang I alone (in the absence of cardiac hypertrophy and increase in cardiac Ang II) does not lead to enhance cardiac fibrosis. These findings represent the first direct evidence for a role of locally generated Ang II in the heart.

Is the observed cardiac fibrosis independent of the presence of cardiac hypertrophy? Our studies did not permit us to answer this question since the founder line used (TTRhRen-A3) harbors a hypertensive and hypertrophic phenotype in the absence of increased cardiac Ang II concentration (refer to Table 2.1, and Figure 2.1). However, results from a study assessing the direct effect of local Ang II in the heart strongly suggest that Ang II alone can induce directly cardiac fibrosis but not hypertrophy (Sjors van Kats, personal communication). Moreover, in some experimental models, a synergy between cardiac hypertrophy and the effect of Ang II on cardiac fibrosis have been observed while in others, cardiac fibrosis seems to be independent of cardiac hypertrophy (292). These

results tend to indicate that cardiac fibrosis can be promoted by the effect of local Ang II alone, but can be enhanced by the presence of physiological conditions such as hypertension and/or cardiac hypertrophy. Differences observed between experimental models can be due to differences in mechanisms of action of Ang II, the type of cardiac fibrosis and the nature of the hypertrophic phenotype.

Type of cardiac fibrosis observed in our model

It is common to distinguish between two different types of fibrosis, namely, reparative and reactive fibrosis. Reparative fibrosis occurs as a reaction to a loss of myocardial material (due to necrosis to apoptosis, after myocardial ischemia or senescence), and it is mainly interstitial. In contrast, reactive fibrosis is observed in the absence of cell loss as a reaction to inflammation and is primarily perivascular. Reactive fibrosis extends further into the neighboring interstitial space (293). During cardiac remodeling, reactive and reparative fibrosis usually coexist (294).

Our model develops cardiac hypertrophy in presence of hypertension but in absence of an apparent increase in plasma and cardiac Ang II as indicated by TTRhRen-A3 mice (refer to Table 2.1, and Figure 2.1). This hypertrophy is probably the consequence of increased work load. In our model, the cardiac hypertrophy is associated with the observation of scars in the myocardium of both TTRhRen-A3 and TTRhRen-A3 x MHChAgt-2 mice (Figure 2.4, panel B and D compared with panel A and C). When Ang II is produced locally as in TTRhRen-A3 x MHChAgt-2 mice (and comparable to the myocardial infarction), enhance perivascular and interstitial fibrosis is observed. While the increased work load leads to hypertrophy with a modest increase in perivascular fibrosis (i.e. reactive fibrosis) as observed in TTRhRen-A3 (Figure 2.4, panel B), local Ang II generation in presence of the hypertrophic phenotype leads to observation of an increase in reactive fibrosis and presence of reparative fibrosis as compared to TTRhRen-A3 mice. These observations provide direct evidence of a role of local Ang II generation in the induction of both reactive and reparative fibrosis in the heart. Our observations are in agreement with previously described experimental model in which reparative fibrosis has been observed with endogenous activation of RAS, created by unilateral renal artery ischemia (295).

Possible mechanisms of induction of cardiac fibrosis by Ang II

Several mechanisms are possible for the induction of cardiac fibrosis by Ang II. Ang II can directly act on fibroblasts via AT_1 receptor to promote collagen deposition (169) or indirectly, by inducing the release of ET-1, TGF-β, PDGF, bFGF or IGF-1 by cardiac cells, which in turn can induce procollagen synthesis and/or prevent collagen degradation by fibroblasts (296-299). Alternatively, since Ang II has been demonstrated to be involved in inflammatory processes by recruiting macrophages from circulation to tissue sites, local increase in Ang II can increase the number of macrophages present at tissue sites while inducing release of TGF-β from those cells (300). This possibility is less probable since we failed to observe a significant increase in MAC-2 positive cells (resident macrophages) in heart of TTRhRen-A3 x MHChAgt-2 as compared with single- and non-transgenic animals (data not shown). However, more cell markers specific for macrophages should be used to fully assess this possibility.

Alternatively, it has been demonstrated that increase plasma levels of aldosterone can induce cardiac fibrosis (301). Since we did not investigate the expression of human renin and angiotensinogen in the adrenal in our transgenic mouse model, it is still possible that local RAS activity in this particular tissue would lead to increased synthesis and release of aldosterone in plasma of double transgenic mice and induce fibrosis in the heart (endocrine effect of increase plasma aldosterone). Moreover, since there is evidence now that aldosterone can also be synthesized and regulated in the myocardium (293), local Ang II can indirectly promote fibrosis by inducing synthesis and release of cardiac aldosterone.

In conclusion, local Ang II generation can promote perivascular and interstitial deposition of collagen characteristic of reactive and reparative fibrosis. The mechanisms of induction of fibrosis by Ang II and the dependence of this process on hypertrophy are still to be determined. Nevertheless, our studies present direct evidence for a role of circulating (pro)renin in the enhancement of cardiac fibrosis that may contribute to the pathogenesis of congestive heart failure and tissue remodeling. In support of a physiological role of circulating renin in promotion of cardiovascular diseases, both prospective and retrospective clinical studies of hypertensive patients have shown a strong correlation

between elevated circulating renin levels in hypertensive patients and the risk of myocardial infarction independent of blood pressure (213,214,302).

4 Function of circulating prorenin

Several hypotheses have been formulated to explain the presence of prorenin in circulation and in several tissues. Circumstantial evidence has pointed out the possibility that prorenin may participate directly in the regulation of blood pressure by acting as an endogenous competitor to renin binding or may be associated with the development of some vascular pathophysiology such as vascular lesions.

4.1 Role on the maintenance of blood pressure

As described in chapter 4, the study assessing the role of circulating prorenin relative to it involvement in maintenance of blood pressure tends to refute the hypothesis that prorenin play a role as an endogenous competitor of renin binding to blood vessel wall. This is based on our failure to observe a decrease in blood pressure in transgenic mice compared with non-transgenic animals (refer to table 4.1). However, since the implication of tissue binding relative to the systemic and local renin activity is not known in mice and given that we did not provide direct evidence for the presence of inactive prorenin in plasma in our inactive mouse prorenin mouse lines (TTRmProren-mut; refer to Section 6.6), further experiments are needed to confirm our findings.

4.2 Role on the vascular pathophysiology

Elevated plasma prorenin has been previously associated with the presence of vascular pathophysiology observed in some type of diabetes (303-307) or experimental model (222). However, it is still not known if prorenin is directly implicated in this pathophysiology. As mentioned in chapter 4, we assessed the pathophysiological effects of chronic elevation of plasma prorenin based on histology studies looking at possible vascular lesions and cardiac fibrosis. In all transgenic mouse lines studied, we failed to observe apparent renal vascular lesions, cardiac fibrosis, or other vascular pathophysiology indicative of a possible pathophysiological effect of prorenin on the vasculature of the transgenic mice. Our findings are in disagreement with the previously described model of Veniant *et al.* (222). In

their model, transgenic rats overexpressing rat prorenin in the liver, leading to a 400-fold increase in plasma prorenin, renal vascular lesions and cardiac hypertrophy was observed in the absence of increased blood pressure. From these observations, they suggested that long-term exposure to elevated plasma prorenin is vasculotoxic. However, since the blood pressure measurements were performed under anesthesia, it is still possible that their model was hypertensive and that the observed cardiac hypertrophy and severe renal lesions are the results of this hypertensive phenotype (refer to comments on condition and method of blood pressure measurement in Section 7). We may add that one possible explanation is that during the inflammatory process, neutrophils produce abundant enzymes that possibly activate prorenin and thereby enhance local Ang II levels (308). The species differences and plasma level of prorenin (40 to 50-folds in our model compared with 400-fold in the rat) may also account for the differences in the phenotypes observed.

In conclusion, we provide evidence that circulating prorenin does not play a role on the systemic control of blood pressure and on development of vascular pathophysiology. However, considering our previous findings, one function of circulating prorenin may be to provide potential renin activity to various target organs as proposed by Osmond *et al.* (215).

5 Comparison with previous models

5.1 Study of uptake and activity of circulating (pro)renin in tissues

Prior to our studies, the uptake and local activity of circulating (pro)renin have been directly assessed *in vivo* by one group of investigators directed by Dr Friedrich C. Luft (Germany) using the following models:

1) In isolated perfused hearts from rats overexpressing the human angiotensinogen (198), human renin uptake and local activity have been studied. This study shows that the human renin can be taken up from circulation by the heart and remains active much longer than its presence in circulation. The prolonged release of Ang II led to long-lasting coronary vasoconstriction.

2) In isolated perfused hindlimbs from rats overexpressing human angiotensinogen (199,220), human renin and prorenin uptake and local activity in vascular wall have been studied. This study showed that human renin derived from the circulation binds to the vascular wall of himdlimbs and remains active much longer than its presence in circulation. However, this study failed to demonstrate prorenin uptake and local activity. By co-infusion of renin with an excess prorenin, these investigators demonstrate that prorenin is not an endogenous antagonist for the long-lasting effects of renin in the vascular wall. Moreover, prorenin does not affect acute renin-related effects on blood pressure.

Our findings are in agreement with these models relative to uptake and local activity of renin, and on the role of circulating prorenin relative to control of blood pressure. However, our findings differ relative to the uptake and local activity of prorenin since they failed to demonstrate prorenin uptake and local activity in the hindlimbs (220). Given that they did not test for human prorenin uptake in the perfused heart model (198), it may be possible that prorenin uptake and local activity occur selectively in cardiac tissues, but not in skeletal muscles. This could be attributed to differences in tissue affinity and/or activity of circulating prorenin. However, several differences between their models and ours may account for this divergence (refer to Section 5.3).

Other similar models have been used to demonstrate renin uptake and local activity *in vivo* using an isolated perfused tissue preparation (185,186,197,309) or by injecting/infusing renin in living animals (190,191). Although all those models provided some evidence for renin uptake and activity in tissues, no assessment for the uptake and local activity of prorenin has been reported. Thus, our model using the human prorenin-expressing mice represent the first model developed to directly assess the uptake and local activity of circulating prorenin in tissues *in vivo*.

5.2 Study of the function of prorenin in circulation

Very few studies have been reported to assess the function of circulating prorenin *in vivo*. With the exception of the transgenic rat model overexpressing rat prorenin in the liver (discussed in Section 4.1), all studies have been performed by injecting or infusing prorenin

in intact animals (260,310-312). Lenz *et al.* (311,312) reported normal cardiac and renal functions upon bolus infusion of recombinant human prorenin into anesthetized cynomolgus monkey. However, these investigators observed a transient fall in blood pressure following prorenin infusion. Similarly, Hu *et al.* (310) presented evidences for an anti-hypertensive effects of circulating prorenin. In their model, rats were implanted with chronic arterial and venous catheters and infused with recombinant prorenin and/or a hypertensive dose of Ang II for 3 to 7 days. Blood pressure was monitored continuously. The prorenin infusion had no effect on cardiac or renal functions, however, significant falls in blood pressure were observed after two hours infusion lasting up to three days. Surprisingly, the prorenin infusion markedly reduced the blood pressure of Ang II-dependent hypertensive rats. Thus, they concluded that prorenin is an active vasodilatator component of RAS.

Our findings differ from those studies since we failed to observe an hypotensive phenotype in our model (refer to Chapter 4 and Section 4.1). Many differences between their models and our may account for the difference in observed phenotype. Most of these differences are discussed below (refer to Section 5.3). Alternatively, these differences may come from the fact that we used mouse as a model since all studies were performed using either monkey or rat models. Measuring blood pressure at one particular time (9-11 weeks) in our study, the animals had time to physiologically adapt to altered physiological conditions (refer to comments on compensation mechanisms in Section 6.4) whereas in all studies reported, recording of blood pressure was performed during and after infusion/injection of prorenin, limiting such adaptation.

5.3 Limitations of previous reported models:

Non physiological conditions of animals studied

Most if not all studies are based on measurements and/or instrumentations of animals models under anesthesia. Observation of variation in blood pressure may depend on the presence and type of anesthetic used, and on the method of recording variation in blood pressure (see comment in Section 3.1). In all our models, measurements of blood pressure

were performed on conscious but restrained animals excluding possible variation inherent to the use of anesthetics.

Invasive procedures (catheterization/canulation) and non-physiological conditions (buffered perfused animals) used to deliver (pro)renin to circulation

Surgical intervention can induce factors present locally or systemically that can influence biological and physiological processes. Moreover, since the mechanism of uptake of circulating (pro)renin in tissue is still not known, presence of blood constituents may be required *in vivo* for observing the uptake and/or activation of circulating prorenin. In our models, renin and prorenin are produced and delivered endogenously in circulation. This process does not require any external interventions. Thus, in that regard, the present studies did take place in better physiological conditions than any previous studies.

Source of (pro)renin used

Several previous studies used exogenous renin and prorenin of different sources. Some studies used recombinant (pro)renin (260,310-312) while other used (pro)renin extracted from tissues (185,186,190,191). Using exogenous sources of (pro)renin raise the possibility of altering the tertiary structure of (pro)renin (folding and/or glycosylation state) due to manipulations and storage conditions of isolated (pro)renin. These alterations may account for the difference in uptake and local activity of (pro)renin observed.

Recent report of uptake of renin and prorenin in cardiac cells demonstrated that the internalization of (pro)renin is mediated by mannose-6 phosphate receptors (202). This finding suggested that presence of sugar composition may be require for the uptake of (pro)renin *in vivo*. Moreover, the glycosylation may be implicated in modulation of (pro)renin activity (i.e. affinity/specificity to angiotensinogen) (see comments in Section 2.1 of Chapter 1). In our models, human renin and prorenin are expected to be glycosylated and to harbor a sugar composition comparable to the endogenous mouse (pro)renin although it is of a different origin.

Acute versus chronic effects of (pro)renin.

In all studies in which (pro)renin was delivered by infusion/perfusion, results were limited to the acute effects of increase plasma (pro)renin. This approach allow to observe transient effects of (pro)renin as exemplified by Lenz *et al.* (311,312) studies (refer to Section 5.2) but limit the studies to short term effects. In our model, (pro)renin is constitutively released in circulation and plasma levels are chronically elevated. This feature is inherent to the use of the TTR promoter. Our approach permitted us to assess the long-term effects of circulating (pro)renin, allowing slow biological processes to occur and some physiological adaptation to develop upon sustained stimulation. This allowed us to assess the uptake and local activity of circulating (pro)renin in the heart at steady-state, and the implication of increased cardiac Ang II on cardiac physiology. Thus, this feature may account for some of the differences observed for the uptake and local activity of prorenin. If the uptake and/or local activation of prorenin are slow or limited processes *in vivo*, studies based on acute elevation of plasma prorenin may not provide evidence for such phenomenon.

In conclusion, we provided the first true *in vivo* model for study of the uptake and local activity of circulating (pro)renin. Our experimental models offer many great advantages compared with previous reported model: our studies were conducted on intact conscious animals, our studies were performed in better physiological conditions, the biological processes (i.e. uptake and activity) could be monitored *in vivo* at steady state, and long-term as well as local and systemic effects of circulating (pro)renin could be measured.

6 Limitations of our experimental models

Although our experimental models harbor some great advantage, they are still subject to some limitations. This section discuss the major limitations encounter during our studies.

6.1 Detection of human (pro)renin by immunohistochemistry

Inability of using the HA tag in vivo

The expression vectors for human (pro)renin transgene were designed with a protein tag (HA) fused to the C-terminus of human (pro)renin (Figure 5.3). This tag, corresponding to a highly immunogenic epitope derived from human influenza virus hemagglutinin (313),

would allow the specific detection of the transgenic fusion protein, avoiding the possible cross-reaction with endogenous (pro)renin or related proteins. Although preliminary studies performed *in vitro* showed expression and detection of our fusion proteins in transfected tissue culture cells using an antibody specific for the HA-tag, we failed to detect our transgene product *in vivo* (i.e. immunohistochemistry on transgenic tissue sections) using the same antibody (data not shown). In addition, in an attempt to determine the relative abundance of each renin form present in cardiac tissue of TTRhProren-B7 and TTRhRen-A3 mice and thus, assess the nature of the activation of circulating prorenin in tissue, we failed to immunoprecipitate and detect the human (pro)renin by immunohistochemistry from plasma and tissue sample of TTRhRen-A3 mice (data not shown). It is thus possible that *in vivo*, the HA-tag portion of the human (pro)renin fusion protein is proteolytically absent or simply not accessible for detection. The former possibility is more likely to occurred since we failed, in denaturing conditions to immunoprecipitate the fusion protein using an anti-HA tag. In these conditions all epitopes (including the HA tag) should be accessible to the antibody.

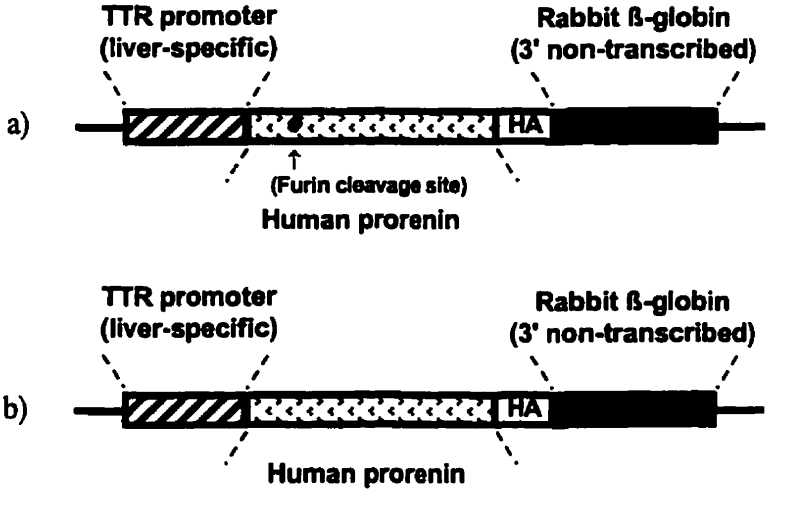

Figure 5.3 Schematic diagram of the expression vectors used to direct the liver-specific expression of human active renin (a) and human prorenin (b) in the mouse.

The inability to use the HA tag limited our studies by several ways: For the detection of human (pro)renin in transgenic tissue, we had to rely on a less specific antibody (see below) making the interpretation of the observed immunological signal more difficult. Moreover, we were unable to determine the presence and the relative abundance of each renin form in tissues of our transgenic models, therefore to provide evidence for the nature of the activation of circulating prorenin within tissues (refer to Section 3.2).

Discrimination between human and endogenous mouse (pro)renin

We have demonstrated the presence of human (pro)renin in heart and other tissues by immunohistochemistry (Chapter 2 and 3, and Appendices) using an antibody recognizing the human (pro)renin. The antibody used (BRI-6, from Dr Catanzaro) was specific for the human (pro)renin, demonstrating minimal cross reactivity with mouse renal (pro)renin (Figure A.1.3). Staining for mouse (pro)renin was restricted the JG cells in kidney of control mouse (Figure A.1.3, panel B) since no immunoreactive (IR-) (pro)renin signal was observed in other tissue tested and other kidney cell-types (data not shown). This finding was expected since mouse and human renin should harbor very similar tertiary structure as predicted by it respective amino acid sequence, and that JG cells of kidneys are one of the most abundant sources of (pro)renin in mice (87,175). Thus, there is still the possibility that some of the immunohistochemical signals detected in tissue sections of transgenic mice using this antibody correspond to mouse (pro)renin and/or structurally-related proteins such as cathepsin. However, results from immunohistochemical studies comparing different antibodies specific for human (pro)renin tend to confirm that the IR-human (pro)renin observed is the human (pro)renin (data not shown).

Discrimination between human renin and prorenin

Since the antibody used to detect human (pro)renin (BRI-6) has been reported to recognize common epitopes to both renin and prorenin forms, we could not assess the relative abundance of each renin form in tissues of transgenic animals. Thus we cannot provide direct evidence of presence of one particular form of renin in tissues. However, we can assume that both forms of renin are present in tissue, particularly in the heart since evidence of both diffusion and uptake of both forms has been provided (review by Danser

et al. (271)). Usage of selective antibody against the prosegment of prorenin should permit the discrimination of each renin form and assessment of the relative abundance of each form within tissue.

6.2 Presence of human prorenin in human active renin-expressing mice model

The expression vector for human active renin transgene was designed from the human prorenin transgene vector by adding a furin site at the juncture of the prosegment and the active renin molecule (Figure 5.3, panel A). Furin being an ubiquitous protease, removal of the prosegment of prorenin should occur in the secretory pathway of expressing cells (314). In our models, while the transgenic mouse line expressing the human prorenin (TTRhProren-B7) harbor only prorenin (99%) in circulation (Table 3.1), the human active renin-expressing mouse line (TTRhRen-A3) possess significant amounts of circulating human prorenin (14% of total human renin concentration) in addition to the active human renin (Table 2.1). One possible explanation for this observation is the incomplete conversion of prorenin by furin in the liver. Alternatively, non processed prorenin may be release from ectopic expression sites, contributing to the presence of human prorenin in plasma of transgenic animals. Regardless of the possible causes contributing to the presence of a significant amount of human prorenin in this particular mouse model, subsequent biochemical and physiological observations made from these mice cannot be attributed solely to the presence of the human active renin alone. Taking into account that we were not able to discriminate between each renin form due to the antibody used and that we were unable to use the HA tag *in vivo* (refer to Section 6.1), we could not provide direct evidence for the uptake and local activity of human active renin in our present models. However, we provided evidence for the uptake of circulating prorenin by tissues and its local activity (refer to Chapter 3).

6.3 Method of generation of transgenic mouse lines

Inherent to the procedure used to generate our transgenic founder lines (i.e. random integration of the expression vector in the mouse genome), the site of transgene integration as well as the copy number of integrated transgene varies between each individual founder line generated. Since the locus in which the transgene is integrated potentially contain

regulatory elements that might affect the transcriptional regulation of the inserted transgene (i.e. repression or activation), it is common using this method to observe ectopic expression of transgenes and variations in its expression levels between founder mouse lines. These variation between lines that is dependent on chromosomal integration position is referred to as "position effect variegation" (315).

Ectopic expression of the integrated transgenes

Ectopic expression of our transgenes has been observed in all mouse line tested. As demonstrated by the tissue distribution of the transgenes expression, ectopic expression has been detected in brain of human active renin mice (TTRhRen-A3; Figure 2.2, panel A), in stomach of human prorenin mice (TTRhProren-B7; Figure 3.2), and in kidneys and lungs of human angiotensinogen mice (MHChAgt-2; Figure 2.2, panel B). In theory, these sites should not provide significant transgene product expression leading to its release in circulation and/or interference with our studies. However, this provides additional variations that can account for the observed altered mouse phenotype (refer to Section 7). Moreover, these ectopic expression sites limited our studies on the uptake of (pro)renin since we had to exclude those tissues (ectopic expression sites) from our studies.

Variation in transgene expression levels

Variations in the transgene expression levels have been observed between the different transgenic mouse lines. For the mouse lines used in our studies (TTRhRen-A3 and TTRhProren-B7), comparison of relative concentration of renin and prorenin in plasma of both transgenic mouse lines indicate that TTRhRen-A3 carry ~65 times more human active renin and ~9 times more human prorenin in the circulation as compared to the plasma concentration of human prorenin in TTRhProren-B7. Thus, the interpretation of observed differences between those two mouse lines was rendered more difficult. For example, to evaluate the uptake selectivity for each renin form (refer to Section 2.2), we had to rely on the intensity of the immunohistochemical signal which is semi-quantitative and take into account the relatively large difference in renin and prorenin plasma concentration. If we were to compare mice harboring similar renin and/or prorenin plasma levels, our interpretation of observed difference would have been more reliable and accurate.

To overcome the lack of specificity in transgene integration, a homologous recombination-based gene targeting procedure has been developed (316,317). By targeting the integration of transgenes into a specific locus, variation in the transgene copy number and the position effects are precisely controlled. However, since the occurrence of the homologous recombination event is quite rare, this approach is more time and labor extensive.

6.4 Heterologous promoter used

Tissue-specific gene targeting

Potentially the most powerful as well as the most limiting aspect of generating transgenic animals is the promoter sequences used to regulate expression of the transgene. Production of transgene product in the preferred spatial pattern, at an appropriate time, and at effective concentrations is characteristic of ideal regulatory sequences. Such ideal regulatory sequences do not exist. As previous reports demonstrated, the transthyretin (TTR) promoter used in our study allowed specific expression of its associated transgene to the liver (318). However, some low expression of the transgene should also occur in the brain and kidney. These secondary sites of transgene expression have the potential to interfere in our studies as discussed previously (refer to Section 6.3).

Constitutive expression

Constitutive expression of a transgene, especially if it deleteriously affects the tissues to which it is targeted, often results in prenatal or postnatal death or causes a variety of compensatory changes (319) in the overall gene expression pattern of the tissue. These changes could result in unexpected phenotypes that may not reflect the true biological functions of the transgene (320-323). Furthermore, the transgene behaves as a self-antigen, inducing negative selection of reactive T cells in the thymus and causing the animals to become immunologically tolerant to the transgene. Therefore, constitutive expression of a transgene can impair it functional or physiological role *in vivo*.

To circumvent these problems, several system have been developed by which the expression of a transgene can be induced at desired time points and otherwise be kept

completely silent for an extended period of time. The use of inducible promoters, such as heat shock, metallothionein, and murine mammary tumor virus promoters, that can be regulated by temperature, zinc, or dexamethasone have been used (321,322,324). However, these system are frequently associated with a high basal level of expression, a less than impressive induction of the transgene, a relative lack of specificity, and a possible toxicity of the induction method. Conditional gene expression *in vivo* has been achieved using a variety of model systems. One of them takes advantage of the cre-lox recombination system by which a transgene can be activated and an endogenous gene deleted in a tissue-specific and time-dependent manner (325). However, this system requires the exogenous delivery of the cre gene (usually by an adeno- or retrovirus), and the induction is irreversible. Recently several drug- or ligand-inducible systems have been developed *in vitro* and, to some extent, *in vivo* (326,327). These systems involve the use of a chimeric transcriptional activator that reversibly activates a target gene in response to the administration of the inducing agent (i.e. ecdysone, tetracycline). One of the systems that uses the intrinsic properties of the Escherichia coli tetracycline resistance operon has been applied widely to the generation of cell lines with tightly regulated gene expression in response to tetracycline (322). Thus, we might consider using such inducible promoter to study the short-term effects of circulating (pro)renin and therefore, minimize the possible compensatory changes that might occur in the present model.

6.5 Confirmation of presence of inactive mouse prorenin in plasma of transgenic animals

In the TTRmProren-mut mouse lines, we could only predict the presence and amount of inactive mouse prorenin in circulation. Thus, there is still the possibility that mutated mouse prorenin does not reach the circulation. It is possible that the mutation in the inactive mouse prorenin causes an alteration in the folding of prorenin, resulting in degradation of transgenic products *in vivo* before release from hepatocytes. However, since we observed the presence of the transgenic product in the medium of cells transfected with the expression vector used to produced the transgenic mouse lines (Figure 4.2), we expect the inactive mouse prorenin to be released from hepatocytes and be present in circulation. However, another possibility might still be that inactive prorenin present in circulation is

cleared more rapidly from the circulation than native mouse prorenin or that altered tertiary structure prevents inactive mouse prorenin to bind (pro)renin binding prorenin.

To determine the presence and quantity of inactive prorenin in plasma of transgenic animals, we could take advantage of the c-Myc tag present at the C-terminus or prorenin (Figure 5.4). We would need to develop specific RIA, ELISA or quantitative Western blot for our transgene products using the c-Myc tag. Alternatively, use of direct RIA for mouse prorenin with TRC/PRC measurement may permit prediction of plasma levels of inactive prorenin in those mice. Such a direct RIA for mouse prorenin has been described previously (328).

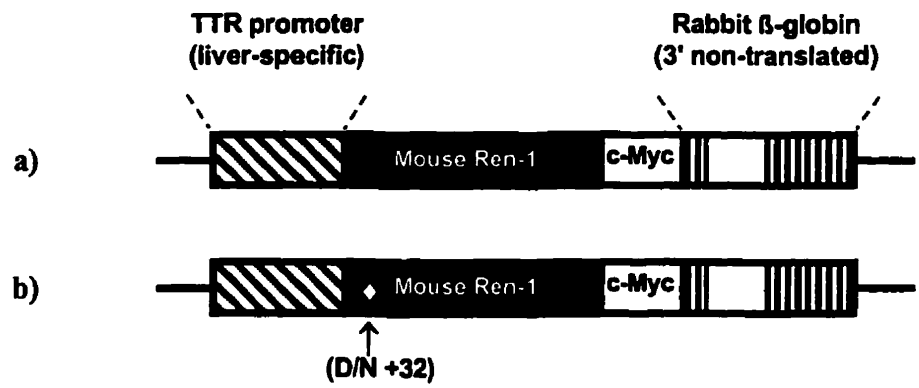

Figure 5.4 Schematic diagram of the expression vectors used to direct the liver-specific expression of native mouse prorenin (TTRmProren; A) and active site mutant of mouse prorenin (TTRmProren-mut; B) to the mouse.

7 Finding the causes of hypertensive phenotype observed in human active renin-expressing mice.

Human active renin-expressing mice (TTRhRen-A3) harbor a hypertensive phenotype clearly mediated by Ang II since treatment with an AT_1-receptor antagonist results in normalization of blood pressure in those mice (Table 2.2). This observation suggests that

the human (pro)renin is cross reacting directly with the mouse angiotensinogen. Although the species specificity of human renin is well known *in vitro* (Table 5.1), the precise kinetic properties of renin *in vivo* are actually not known. Thus, human renin may exhibit differences in species specificity *in vivo* compared with *in vitro*. Another possibility could be the induction of the endogenous RAS components by the human (pro)renin resulting in increase endogenous RAS activity. The latter possibility is less likely to happen since it is well established that renin is downregulated by an increase in plasma renin level.

Table 5.1 Values of kinetic constants of recombinant human renin (rhRN) and mouse submandibular gland renin (mSMGRN) acting on mouse angiotensinogen *in vitro**.

	K_m (nM)	K_{cat} (h^{-1})	K_{cat}/K_m (nmol h^{-1})
rhRN	1.04	3.10×10^{-1}	3.13×10^{-1}
mSMGRN	2.47	7.14	2.89

* Reproduced from Fukamizu *et al.* (225).

Genetic variations?

Physiological characterization of an independent transgenic mouse line expressing human active renin (TTRhRen-A1) has demonstrated comparable increase in blood pressure as for TTRhRen-A3 mice (data not shown). Since each independent founder mouse line is the result of random genome integration of the transgene, the site of integration of the transgene in the mouse genome can not be a cause. Identical disruption or activation of endogenous genes is unlikely to occur in two independent founder lines. The site of genome integration of transgene is therefore not a possible cause for the observed phenotypes.

The genetic background is also to be considered. Phenotypic variation resulting from allelic segregation in an outbred or random bred population can detract from a given transgenic model. It is clear that while blood pressure values are consistent with a given inbred line of mice, significant variation is observed between different lines. But, since all

our transgenic mice are inbred mice genetically identical to control mice (with the exception of the presence of transgene), we can exclude the possibility of variation of the genetic background as a cause.

Increased plasma RAS activity?

Cross reactivity of human renin with mouse angiotensinogen in plasma of transgenic animal has been proposed as cause of the observed hypertension *in vivo*. Measurement of plasma Ang I and II concentration in TTRhRen-A3 mice has demonstrated that plasma Ang I and II level were decreased compared with control which tend to exclude this possibility (Figure 2.3 and A.4). Moreover, these results tend to demonstrate a decrease in circulating RAS activity. Although we failed to clearly determine the PRC of mouse renin in transgenic animal (data not shown), immunohistochemical staining of renin in kidney of both control and transgenic mice demonstrate a significant decrease in mouse renin expression in our hypertensive mouse (Figure A.1.3). This indicates that the decrease in circulating RAS activity in TTRhRen-A3 mice is more likely due to downregulation of mouse renin expression. Thus, we provide evidence that circulating RAS activity is not the cause of the hypertensive phenotype in TTRhRen-A3 mice.

Increased tissue RAS activity?

Evidence for the implication of local RAS, independent of circulating RAS, in control of blood pressure has been previously reported. Action of Ang II in brain, adrenal, adipose tissues and the kidney has been shown to lead to increase blood pressure (329-331). In an attempt to localize the potential implication of a local RAS in the control blood pressure in our hypertensive mice, we extracted and measured angiotensin peptides from different tissues of TTRhRen-A3 mice and compared levels with control normotensive mice as previously described in Chapter 2. As pointed out in Figure A.4, the heart, liver and kidney of TTRhRen-A3 harbor a decrease in Ang I and II concentration compared to control which suggest that no significant activation of a local RAS take place in those tissues. However, a slight increase in Ang I and II concentration in testis of transgenic animals raise the possibility that this particular tissue may be implication of in the control of blood pressure. In support for this possibility, we provided evidence for significant uptake of

human (pro)renin in this tissue (refer to Section 2 and Appendices). Although, to our knowledge, no one has reported evidence for the direct implication of this organ in control of blood pressure, increased plasma testosterone has been shown to cause a hypertensive phenotype in rats (332). This raises the possibility that a sustained local effect of circulating human (pro)renin in TTRhRen-A3 testis induces synthesis and release of androgen (testosterone) in circulation which in high concentration can affect renal function leading to increase blood pressure. To test this possibility, blood pressure could be measurement before and after castration in transgenic and control mice. If a local activation of tissue RAS in the testis is responsible for the observed hypertensive phenotype, removal of this particular tissue in transgenic animals should harbor blood pressure comparable to controls. Alternatively, treatment of transgenic and control animals with flutamide, an androgen-receptor antagonist might be considered.

Another tissue susceptible to be implicated is the brain. Several lines of evidence have been provided for the implication of the central nervous system in the regulation of blood pressure. In our model, inherent to the use of TTR promoter, ectopic expression of transgene has been observed in that particular tissue (Figure 2.2, panel A). This makes the brain the prime tissue candidate for an activated local RAS. As observed in Figure A.4, concentration of Ang I and II in the brain of both transgenic and control mice were at the lower limit of detection. So we are unable to rule out this possibility. Further experiments would be needed to assess this possibility such as measuring the plasma and urine catecolamine levels which is recognized as a good marker for the activation of the brain RAS.

Alternatively, the adrenal gland has been demonstrated to influence blood pressure through release of aldosterone. Since we did not investigate this tissue relative to the transgene expression and angiotensin level, this tissue could also be implicated. Measurement of plasma aldosterone and tissue Ang II levels in TTRhRen-A3 and comparison with levels found in non-transgenic should rule out this possibility. In support of a role of the adrenal in control of blood pressure, transgenic rats harboring the mouse Ren 2 gene develop fulminant hypertension associated with high enhanced adrenal renin activity and plasma aldosterone levels whereas plasma and kidney renin levels are low, and

renal renin gene expression is essentially undetectable (221). However, lack of observed significant increase in cardiac fibrosis in TTRhRen-A3 mice as compared with non transgenic mice tend to suggest that increase plasma level of aldosterone is less likely to be the cause (refer to Section 3.5). To test this possibility, adrenectomy or treatment with spironolactone, an aldosterone receptor antagonist might be considered.

From our results it is still not possible to determine the cause of the hypertensive phenotype observed in our human active renin mice. However, we provide clear evidence that the observed rise in blood pressure is not caused by increased circulating RAS activity or tissue RAS present in the heart, liver or kidney. Further experiments are needed to assess the possible implication of testis, brain and adrenal in the regulation of blood pressure in our model.

Differences with previous transgenic models

Differences in blood pressure phenotype have been observed between previously reported transgenic mouse models: transgenic mice harboring the human renin gene with part of it natural promoter (333-336) and transgenic mice carrying the rat renin under control of mouse metallothionein I promoter (expression mainly in the liver) (337) are normotensive while transgenic rats carrying the mouse renin (Ren-2) develop fulminant hypertension (338). It is important to note that most, if not all, transgenic mice harboring human RAS component express the latter at low levels due principally to the fact that its expression is under regulation of its natural promoter (downregulated as for the endogenous renin). However, in the Ren-2 transgenic rats model and our human active renin model, plasma levels of active renin are chronically elevated due to constitutive unregulated expression of transgene. This lack of feedback regulation of circulating renin is probably an important factor in the observation of a hypertensive phenotype in our mice.

As pointed out by some studies, the observation of a hypertensive phenotype in mice is dependent directly on methodology used to measure blood pressure in those animal. Sinn *et al.* (336) observed no difference in systolic pressure in many transgenic mouse groups using tail cuff measurement or an indwelling carotid catheter under ketamine anesthesia, but when the same mice (used for the tail cuff measurement) were measured using an

indwelling carotid catheter under conscious, unrestrained conditions, they reproducibly observed a significant increase in blood pressure. Similarly, in an attempt to perform single blood pressure measurements using the tail cuff method, we observed loss of the hypertensive phenotype in our mice when measured under ketamine anesthesia (data not shown). Moreover, it has been observed that blood pressure in some hypertensive mouse models can be accurately measured via tail cuff, while others cannot (339), suggesting that results from blood pressure measurement can differ depending on the hypertensive mouse models studies, the physiological condition of the mice during blood pressure recording and the recording method used. It may be difficult to directly compare results from different laboratories unless the methods for recording blood pressure are identical and the nature of the blood pressure phenotype comparable.

8 Summary and conclusion

In summary, we have developed two transgenic mouse models which permit us to assess the uptake and local activity of circulating (pro)renin and the role of circulating prorenin in the control of blood pressure and development of pathophysiology of the vasculature. With the first model, we provided the first *in vivo* demonstration that a chronic elevation in circulating renin and/or prorenin can leads to an increase in local synthesis of angiotensin peptides with a target tissue. Moreover, we provided the first *in vivo* demonstration of circulating prorenin uptake by tissue leading to it local activation *in situ*. Although the uptake mechanisms and nature of prorenin activation are still not known, we provide evidence for uptake of circulating prorenin by perivascular cells both in the heart and testis. We have demonstrated that (pro)renin uptake and local activity in tissue are independent of blood pressure. Morphological studies suggest that prorenin uptake may take place in macrophages and possibly pericytes. We demonstrated that in animals with comparable levels of hypertension and cardiac hypertrophy, high circulating (pro)renin can lead to an increase in cardiac interstitial and perivascular fibrosis characteristic of reactive and reparative fibrosis. Finally, with our second model, we provide evidence that circulating prorenin does not play a role on the systemic control of blood pressure or in development of vascular pathophysiology.

Thus, the present studies have furthered our understanding of the role of circulating renin and prorenin relative to its participation in circulating and tissue RAS activity. In circulation, renin participates in the systemic RAS activity. By catalyzing the first step leading to the generation of Ang II, the main RAS effector peptides, renin is a key regulator of the short-term maintenance of blood pressure. Renin in circulation is also taken up by tissue such as the heart to potentially participate in local RAS activity. Although its importance is questioned, local activity of renin may contribute to the physiological adaptation of cardiovascular tissues providing long-term proper maintenance of blood pressure. Prorenin, also present in circulation harbor no apparent role in circulation. Being taken up by tissues, circulating prorenin may provide local renin activity within target tissues. It high and stable concentration in circulation may provide basal tissue RAS activity.

9 Future prospects

Future experiments can be performed in the context of the present studies:

1. To confirm the uptake of circulating (pro)renin *in vivo* and prove that human (pro)renin behave as the endogenous (pro)renin in the mouse, a study could be performed, as presented in chapter 3 using the mouse prorenin-expressing mouse lines (Chapter 4).

The benefits of such studies are: i) would permit the study on endogenous prorenin instead of heterologous prorenin (i.e. tissue uptake, local activity, physiological effects), ii) would permit to assess the implication of the active site in the uptake process (i.e. use of the inactive mouse prorenin-expressing mouse lines), iii) would permit to assess directly the physiological implication of an elevated circulating prorenin on tissue RAS activity without the need to supply the substrate to target tissue (i.e. physiological conditions similar to diabetes and others diseases).

However, such a study would have some limitations: i) the transgene product would be hard to discriminate from the endogenous prorenin. We would need to rely entirely on the presence and accessibility of the c-Myc tag for immunohistochemical localization and for quantification of transgenic product in plasma and tissues (refer to comments on the HA-

tag detection in Section 6.1); ii) increased chance of reactivity between the transgene product and endogenous RAS as compared to the present study.

2. Based on previous reports demonstrating the uptake of prorenin by mannose-6 phosphate in endothelial and cardiac cells *in vitro* (202,203), we could determine if uptake of (pro)renin required the presence of sugar (glycosylation). By producing mice expressing the human prorenin with mutation in the two putative glycosylation site, uptake and local activity could be assess as presented in chapter2.

3. To determine the cell-type responsible for taken up (pro)renin in tissue, more cell marker should be tested on tissue section. Alternatively, since staining is intracellular, cell type could be determined by FACS analysis of isolated cardiac or testis cells of transgenic animals. Alternatively, by identifying the organelles in which renin is present should give clues about it function and the cell type possessing this type of organelle.

4. To define the nature of prorenin activation in tissue, a mutant form of prorenin in which its prosegment is non cleavable (as described by Methot *et* al. (262)) could be use to test if the local tissue activity *of* prorenin *in vivo* dependent of the proteolytic removal of its prosegment.

5. To determine the precise cause of the hypertensive phenotype in TTRhRen-A3, complete physiological characterization of transgenic mice could be performed: Measurement of plasma and urine electrolytes/metabolites to evaluate renal and cerebral function; measurement of catecolamines, aldosterone, testosterone, NO, endothelin, ANF, and other effector molecule known to be implicated in hypertension. Also, we might consider using new technology available for the measurement of blood pressure such as telemetry (24 hours recording), catheterization (local blood pressure), sonography/echography (vascular/cardiac physiology).

Claims to original research

We provided the first *in vivo* model to assess the uptake and local activity of circulating (pro)renin by tissues.

We are the first to provide direct evidence for i) uptake of circulating renin and prorenin by tissues *in vivo,* ii) local activity of circulating prorenin within tissues, iii) local Ang II generation can lead to increased fibrosis (reactive and reparative) in the heart. Moreover, the role of prorenin in the circulation is limited to providing renin activity in tissues.

APPENDICES

1 Localization of circulating (pro)renin by immunohistochemistry

In chapter 2 and 3, we reported the localization of circulating human (pro)renin in several tissues of TTRhRen-A3 and TTRhProren-B7 mice lines and briefly discuss the localization of the immunoreactive (IR-) human (pro)renin in the heart of transgenic animals (refer to Figure 2.5, 2.6, and 3.3). However, since most results were not shown and thoroughly discussed, the following sections present further analyses of the localization of circulating human (pro)renin relative to it tissue distribution, co-localization with specific cell markers, and subcellular localization by electron microscopy. Results from these analyses provide clues about the precise nature of the observed (pro)renin uptake and site of enzymatic activity. This may also define the possible relation between its localization and local activity.

Tissue distribution and cellular localization of circulating human (pro)renin

The tissue distribution the IR-human (pro)renin is presented in Figure A.1.1 to A.1.3, and is summarized in Table A.1.1. Cellular localization of the staining in tissue is described in Table A.1.2.

Comparison of the tissue distribution of the IR-human (pro)renin between human active renin expressing mice (TTRhRen-A3) and human prorenin expressing mice (TTRhProren-B7) revealed similarities and differences. While distribution of IR-human (pro)renin was similar in the heart, liver and pituitary, being associated with some capillaries or small arterioles (heart and pituitary), or hepatocytes and Kupffer cells (liver), it differed in the lung, ovary and kidney. In TTRhRen-A3 mice, IR-human (pro)renin is localized in cells in close proximity of the smooth muscle cell layer boarding the bronchioles of the lung (Figure A.1.1, panel C) while its is located in cells present in alveolus in TTRhProren-B7 mice (Figure A.1.2, panel C). Unlike the former, the latter cell distribution is comparable to distribution of resident macrophages (MAC-2 staining) in control mice (data not shown). As for the ovary, cell distribution was restricted to cells outside the follicles in TTRhRen-A3 mice (Figure A.1.1, panel E) while some staining could be observed in cells within follicles in TTRhProren mice (Figure A.1.2, panel E, dark arrow). For the kidney, the IR-human (pro)renin signals were present in proximal tubule of

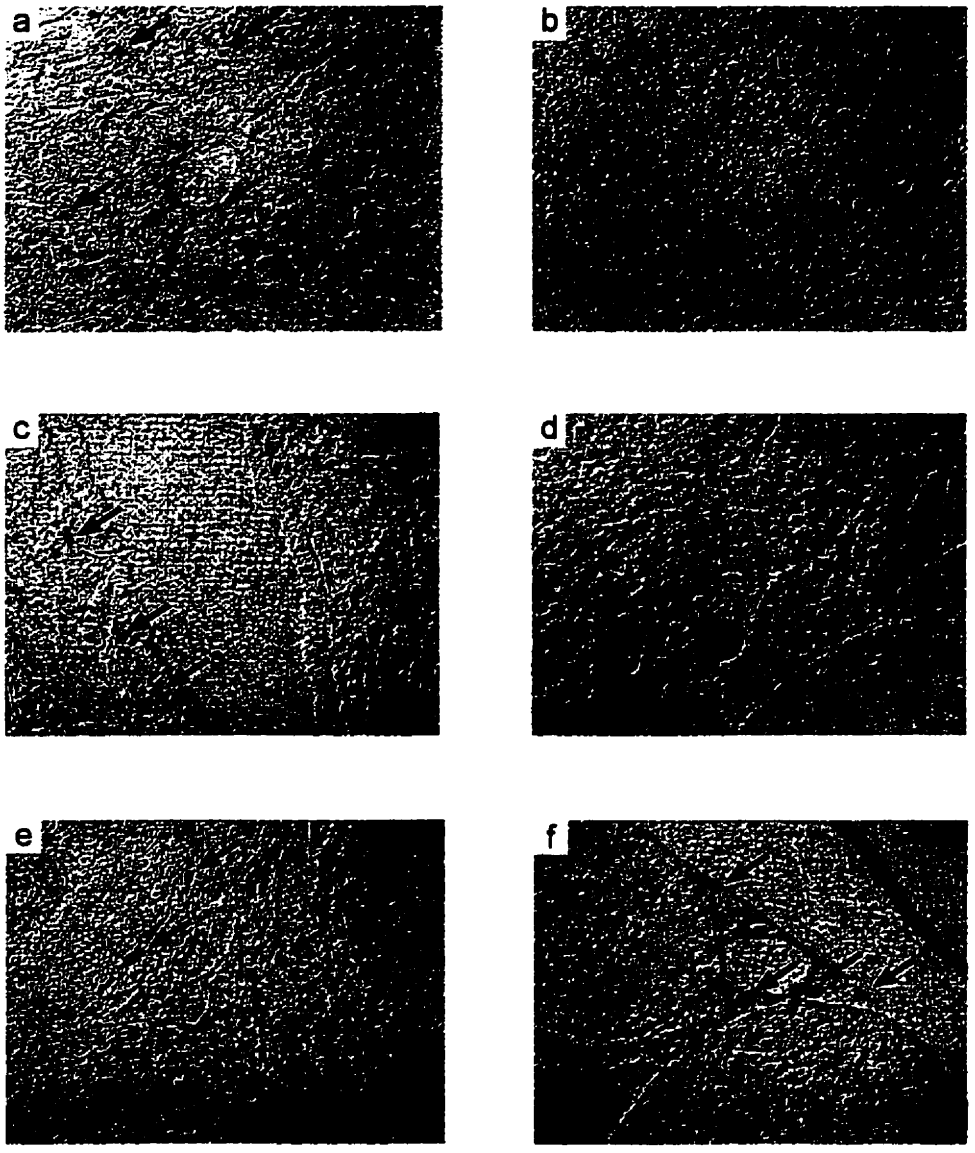

Figure A.1.1 Tissue distribution of immunohistochemical staining of human (pro)renin in heart (a), liver (b), lung (c), pituitary (d), ovary (e), and testes (f) of TTRhRen-A3 transgenic animals. No staining observed in non-transgenic animals. Arrows show discrete cells staining for (pro)renin. Original magnification at 40x.

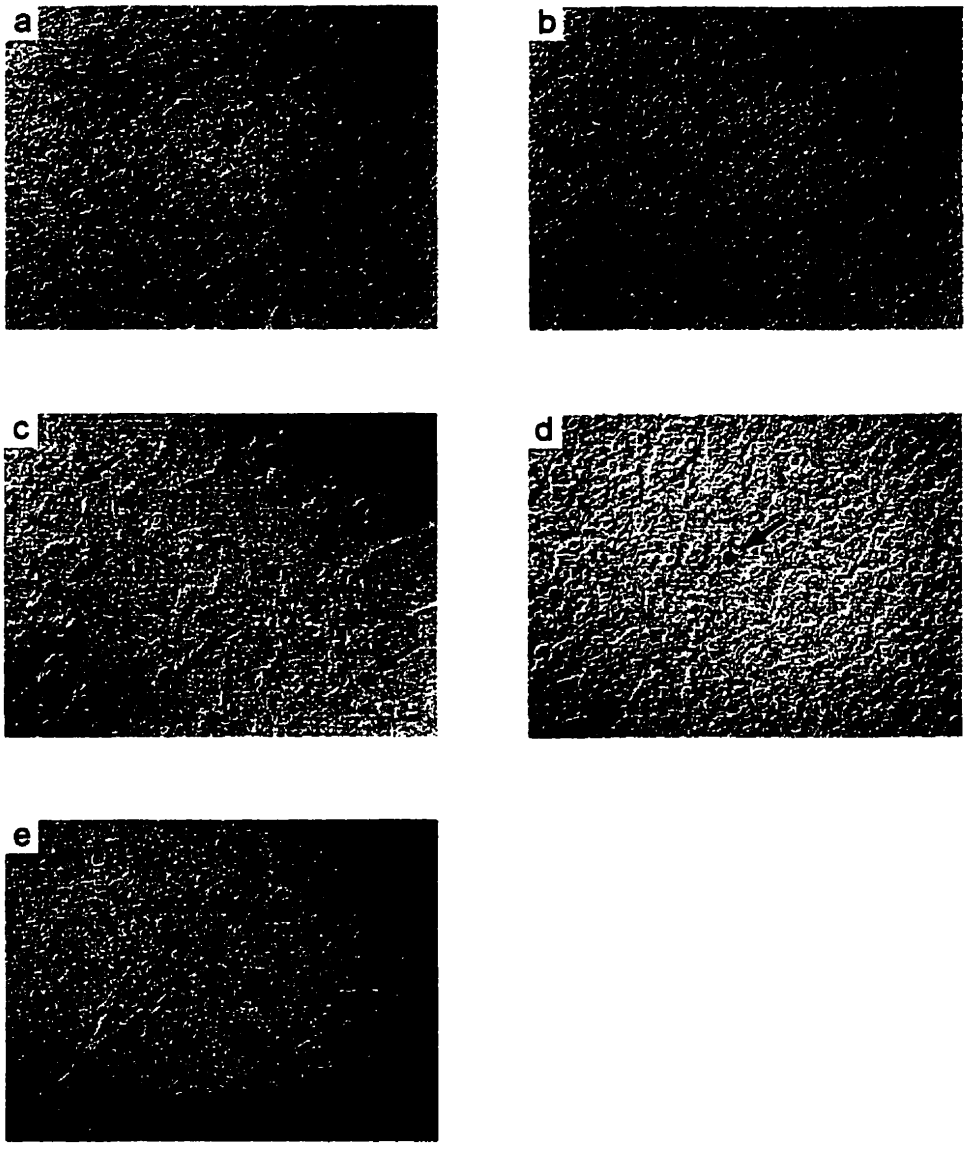

Figure A.1.2 Tissue distribution of immunohistochemical staining of human (pro)renin in heart (a), liver (b), lung (c), pituitary (d), and ovary (e) of TTRhProren-B7 transgenic animals. No staining observed in non-transgenic animals and in testes of transgenic animals. Arrows show discrete cells staining for (pro)renin. Original magnification at 40x.

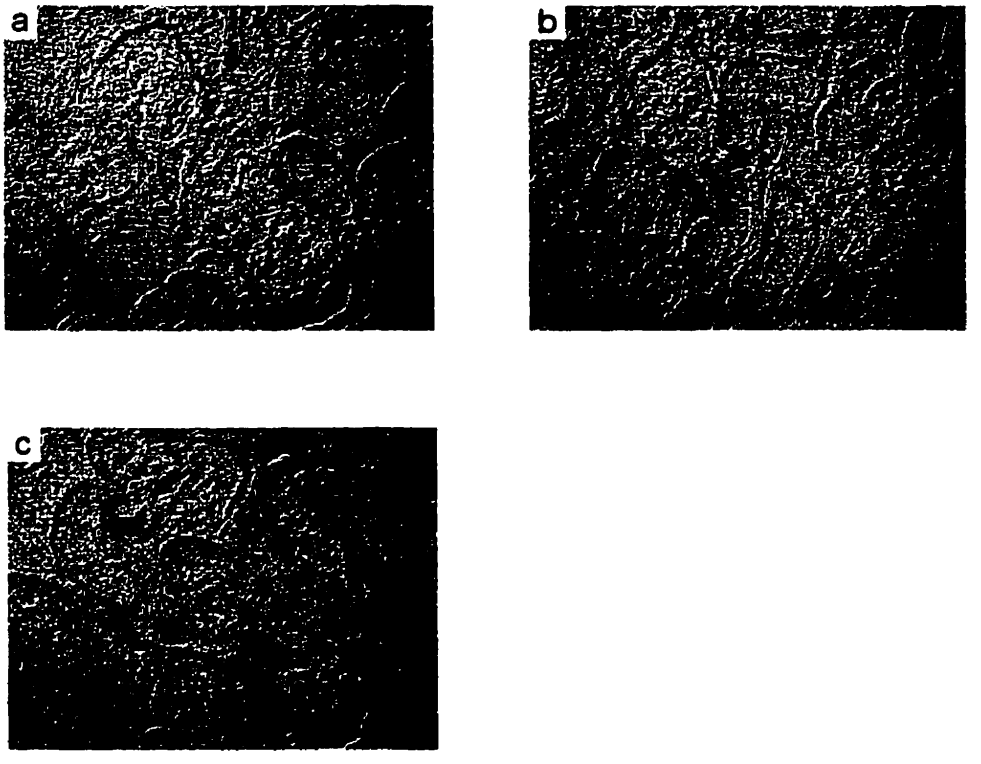

Figure A.1.3 Immunohistochemical staining of (pro)renin in kidney of TTRhRen-A3 (a), non-transgenic (b), and TTRhProren-B7 (c) animals. Arrows show staining of the juxtaglomerular cells. Original magnification at 160x.

Table A.1.1 Distribution and relative intensity of immunoreactive human (pro)renin in tissue of TTRhRen-A3 and TTRhProren-B7 transgenic mice*

Tissue	*TTRhRen-A3*	*TTRhProren-B7*	*Compared distribution*†
Heart	++++	++	Same
Liver	+++	++	Same
Lung	+++	+/-	Different
Pituitary	++	+	Same
Ovary	+++++	+/-	Different
Testis	+++++	-	NA
Kidney	+/-	-	Different

*Based on localization of human (pro)renin by immunohistochemistry using the rabbit anti-human (pro)renin BRI-6 (refer to Chapter 2 and Figure A.1.1 to A.1.3). Relative intensity of IR-human (pro)renin signal display by "+" and "–" signs. NA, not applicable. †Refer to Table A.1.2.

Table A.1.2 Description of the localization of IR-human (pro)renin staining in human active renin-expressing mice (TTRhRen-A3) and human prorenin-expressing mice (TTRhProren-B7).

	TTRhRen-A3	*TTRhProren-B7*
Heart	Dark staining of perivascular cell associated with some capillaries and small arterioles. No staining in aorta.	Idem
Liver	Diffuse staining corresponding mainly of hepatocytes. Dark staining of Kupffer cells.	Idem
Lung	Dark staining of cells associated with connective tissue adjacent to the smooth muscle cells layer surrounding the epithelium of bronchioles.	Faint staining of cells localized in alveolus. Staining harbor similar distribution of resident macrophage (MAC-2).
Pituitary	Few but dark staining of cells associated with blood vessels of the anterior lobe.	Idem
Ovary	Dark staining of cells associated with theca cells layer and connective tissue. Not staining in follicles (primordial or atretic)	Few but dark staining of cells both in follicles (atretics) and associated with connective tissue.
Testes	Dark staining in cells associated with blood vessels and adjacent to Leydig cells.	No staining observed.
Kidney	Faint staining of the proximal tubule cells (not shown).	No staining other than the JG cells (mouse renin staining).

TTRhRen-A3 transgenic animals (data not shown) while, in TTRhProren-B7 mice, no staining other then the JG cells (cross-reactivity with mouse (pro)renin) is (Figure A.1.3, panel B and C). These observations indicate differences in the cell-type responsible for the uptake of human (pro)renin and possibly in the affinity of the cell to each form of renin.

Comparison of the relative intensity of IR-human (pro)renin signal indicated that gonads (ovary and testis) are the tissues harboring the most IR-human (pro)renin in all tissue tested of TTRhRen-A3 (Figure A.1.1). However, we observe the opposite in TTRhProren-B7 mice (Figure A.1.2). Considering the relative difference in plasma concentration of both renin form between our transgenic mouse lines (refer to Chapter 5, Section 1), these observations may indicate selective uptake of active form of renin by the gonads as opposed to tissues such as heart, liver and pituitary which may uptake selectively prorenin. Alternatively, considering the differences in blood pressure phenotype between those two transgenic mouse lines, this difference in IR-human (pro)renin signal intensity may represent a consequence of an increase in interstitial space (pro)renin concentration due to an increase vascular permeability of those particular organs in hypertensive animals. It has been demonstrated that an increase in blood pressure lead to increase in capillary permeability to plasma constituents (i.e. proteins and metabolites) (244). The latter possibility is less probable since normalization of blood pressure in the TTRhRen-A3 mice does not lead to significant decrease intensity of IR-human (pro)renin in those tissue (data not shown). Similarly, if the uptake mechanism is mediated by (pro)renin binding proteins, differences in density of latter proteins between the two transgenic mice lines can account for the difference in immunological staining intensity. In support of this possibility, (pro)renin binding proteins have been characterized previously.

Co-localization of IR-human (pro)renin with specific cell markers

Co-localization of the IR-human (pro)renin with markers for smooth-muscle cells (α-smooth muscle actin), for fibroblasts (vimentin) and macrophage (MAC-2) has been performed (refer to Chapter 2). Few IR-human (pro)renin signals seems to co-localized with markers for smooth muscle cells and macrophages (Figure A.1.4 and A.1.5, respectively), suggesting that the majority if not all of (pro)renin containing cells are not

smooth muscle cells or resident macrophage in the heart. However, the respective localization of the latter cells and the human (pro)renin-containing cells indicate a close proximity between them. As for cardiac fibroblasts, the vimentin staining, which is characterized by a diffuse extracellular distribution around fibroblasts, was not informative due to it faint immunoreactivity (data not shown). However, we have noted that distribution of IR-human (pro)renin in heart of mice carrying the human active renin and human angiotensinogen (TTRhRen-A3 x MHChAgt-2) is closely associated with regions of collagen accumulation (data not shown) suggesting that the (pro)renin-containing cells may still represent fibroblasts or it precursors (myofibroblasts or possibly pericytes).

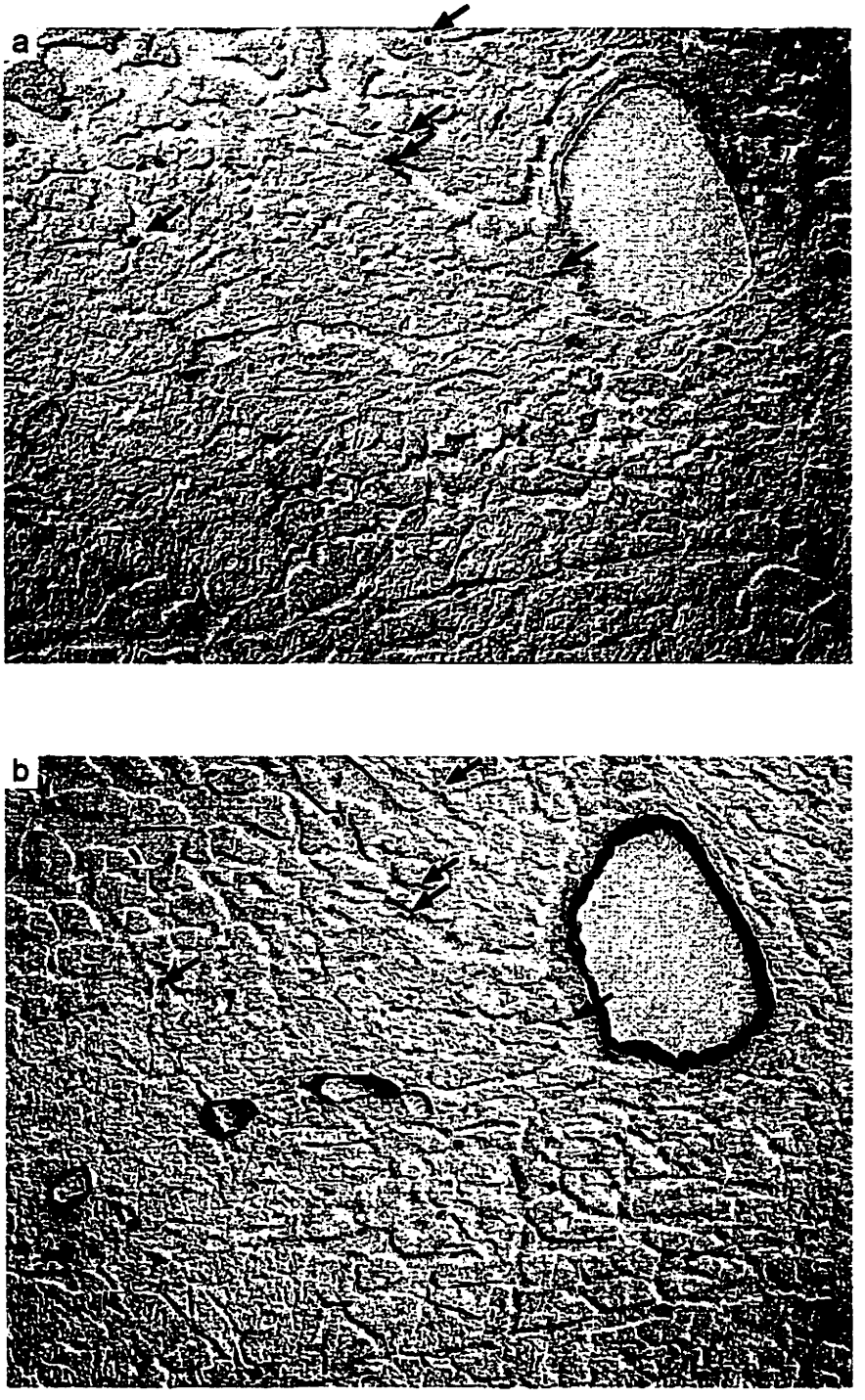

Figure A.1.4 Co-localization of IR-human (pro)renin (a) with α-smooth muscle actin (b) in the heart of TTRhRen-A3 mice. Arrows show possible co-localization of human (pro)renin with the cell marker. Original magnification at 40x.

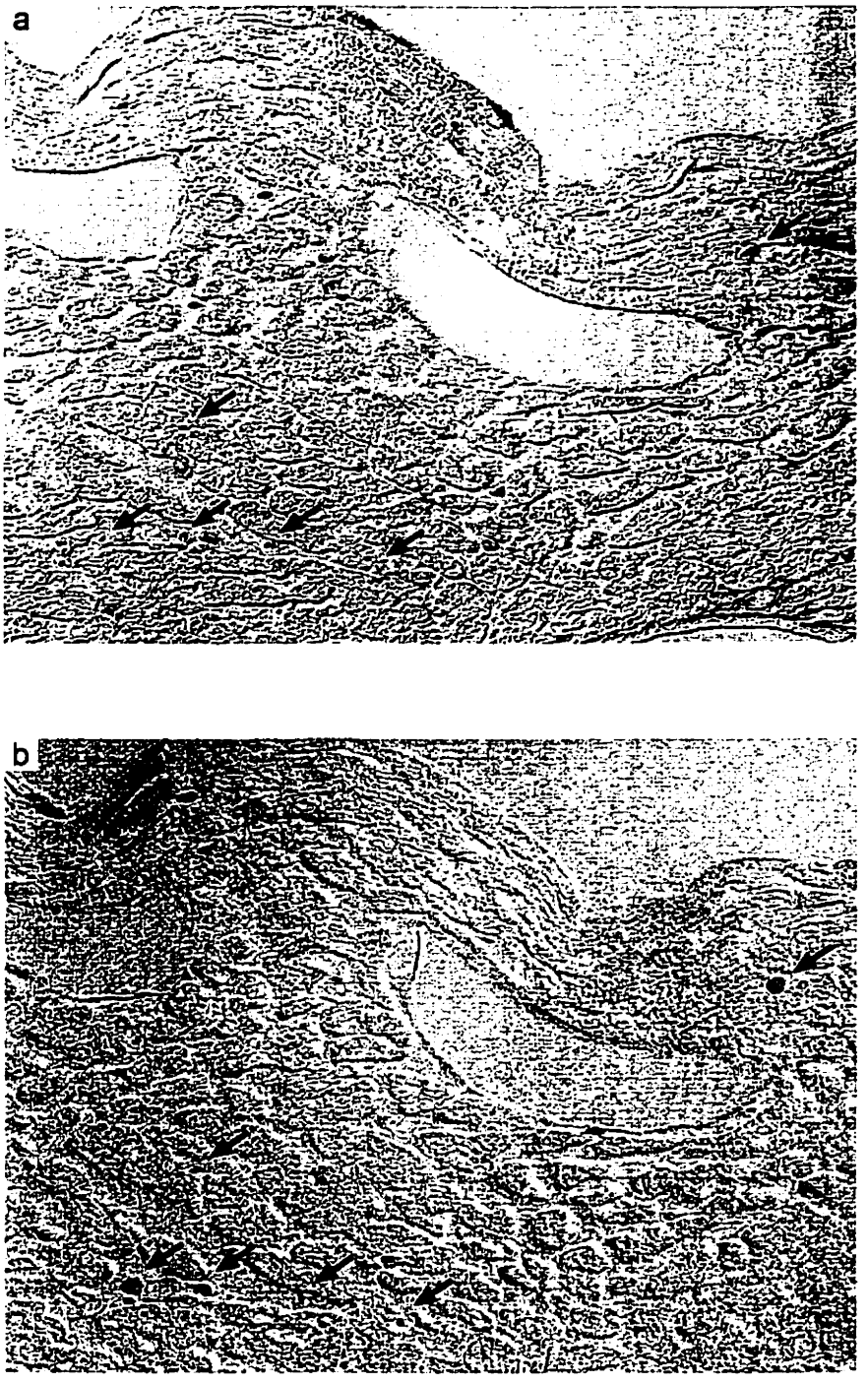

Figure A.1.5 Co-localization of IR-human (pro)renin (a) with MAC-2 (b) in the heart of TTRhRen-A3 mice. Arrows show possible co-localization of human (pro)renin with the cell marker. Original magnification at 40x.

Subcellular localization of IR-human (pro)renin

As presented in Figure 2.6, IR-human (pro)renin has been localized in vesicle-like organelles of cells in close proximity of capillaries in the heart. To compare the cell-type responsible for the uptake of (pro)renin between tissue, additional studies have been performed on testis of same animal (TTRhRen-A3 mice) using the electron microscopy. Although the testis harbors tissue organization quite different then the heart, localization of the IR-human (pro)renin in testis is identical to the heart, being limited to vesicle-like organelles of cells in close proximity of capillaries. However, in testis, the human (pro)renin-containing cells are in proximity of steroids granules-containing cells characteristic of Leydig cell. These observation suggest that mainly one cell type is responsible for uptake of (pro)renin both in heart and testis. All IR-human (pro)renin stained cells observed in both tissue harbor these morphological characteristics:

- Localized in close proximity to small capillaries (heart) or slightly bigger vessel (testis),
- Cell not in direct contact with the endothelial cell of blood vessel, a thin layer of connective fiber (basal lamina) separating them,
- Cell contains a lot of dense vesicle-like organelle, but no apparent contractile fibers (myofibril) present in cytoplasm.

Moreover, we failed to observed staining associated with cell membrane in the electron microscopy. This indicates the possibility that (pro)renin does not bind renin-acceptor/receptor proteins previously identified by Campbell and Valentijn (205), Nguyen *et al,* (206), and Sealey *et al.* (204). We are careful in interpreting these observations since we don't have direct evidence that human (pro)renin behave like the mouse (pro)renin in vivo. Moreover, there is also the possibility that potential membrane bound (pro)renin is washout from the interstitial space during the whole body perfusion performed. However, this latter possibility cannot explain the absence of intracellular IR-human (pro)renin signals observed in vascular endothelial cells or cardiomyocytes. Thus, these results indicate that uptake of (pro)renin don't take place in those latter cell type *in vivo*.

2 Effect of losartan treatment (8 d) on plasma and cardiac Ang I concentration in TTRhRen-A3 x MHChAgt-2 mice.

The mice expressing active human renin in the liver (TTRhRen-A3) are hypertensive as compared to their non-transgenic littermates. To test whether renin was taken up from the circulation by a pressure-dependent mechanism, double-transgenic animals were treated with an anti-hypertensive agent (losartan; 30 mg/kg/day in drinking water) for a period of 1 week to normalize their blood pressure and their cardiac and circulating levels of Ang I were compared to that of vehicle treated littermates. Results (Table 2.2, Fig.) demonstrate that even though anti-hypertensive treatment led to a significant reduction in the blood pressure of the double-transgenic animals, there was no decrease in the ratio of cardiac to circulating Ang I in the treated animals as compared to untreated littermates. These data suggest that the contribution of circulating renin to the cardiac RAS is not mediated by a pressure-dependent mechanism.

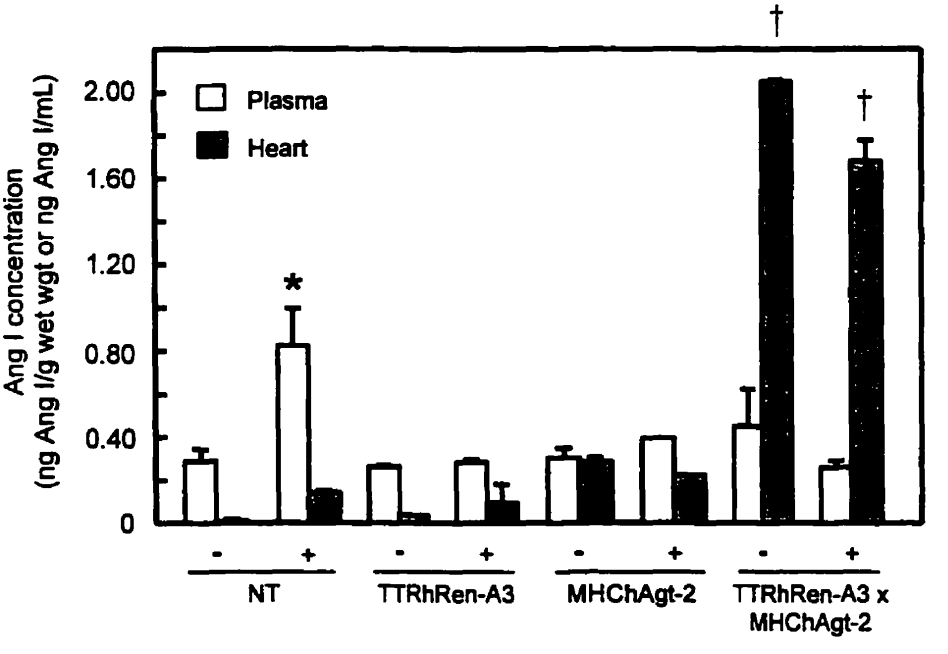

Figure A.2 Concentration of Ang I in plasma and heart of non-, single- and double-transgenic animals (TTRhRen-A3 x MHChAgt-2) after 8 days of treatment with losartan (+) or vehicle (-) (refer to Materiel and Methods of Chapter 2). *P<0.05, †P<0.001 as compared to non-transgenic mice by ANOVA using Student's t-test.

3 Are there other sites of angiotensin synthesis from presence of circulating human (pro)renin and local human angiotensinogen?

To investigate the possibility that the observed in vivo enzymatic activity of circulating human (pro)renin is limited to the heart, we collected kidneys and lungs tissue in addition to the heart and plasma of human active renin-expressing mice (TTRhRen-A3) crossed with human angiotensinogen-expressing mice (MHChAgt-2). Procedure was identical to method reported in chapter 2 with the exception that ketamine/acpromazine anesthetic cocktail was used instead of pentobarbital. Compared with pentobarbital anesthetic, use of this cocktail tends to increase plasma Ang I (by a factor of 2) and slightly lower cardiac Ang I concentration in all animals (data not shown).

Preliminary data are presented in Figure A.3. While plasma and kidney Ang I concentrations in double transgenic animals are similar or decreased compared with control mice, Ang I concentration in the lung is increased in the mice harboring both transgenes. Although the difference in Ang I concentration are not quite significant due probably to the small sample size, these results raise the possibility that circulating (pro)renin is also active within the lung. However, no increased Ang II concentration is observed in that tissue suggesting that if increased local generation of Ang I does occur, the local concentration of Ang I is either too low to contribute to local Ang II concentration or that the site of Ang I generation is not in close proximity to tissue ACE. Interestingly, no increase in Ang I or Ang II concentration is observed for the kidney. Since the substrate for human renin is present locally, this may indicate that human (pro)renin is not present locally within the kidney. Alternatively, the human (pro)renin may be present within the kidney but not in close proximity of the human angiotensinogen site of expression. The latter possibility is worth consideration since the cell-type responsible for the human angiotensinogen expression in the kidney of the MHChAgt-2 mouse line is not known. Considering the facts that IR-human (pro)renin is readily detectable in the heart and lung, and present at much lower concentrations in the kidney, we found a direct correlation between its presence determined by immunohistochemistry and its presence denoted by *in vivo* enzymatic assays. This suggest that its presence in tissue is directly correlated with its local activity.

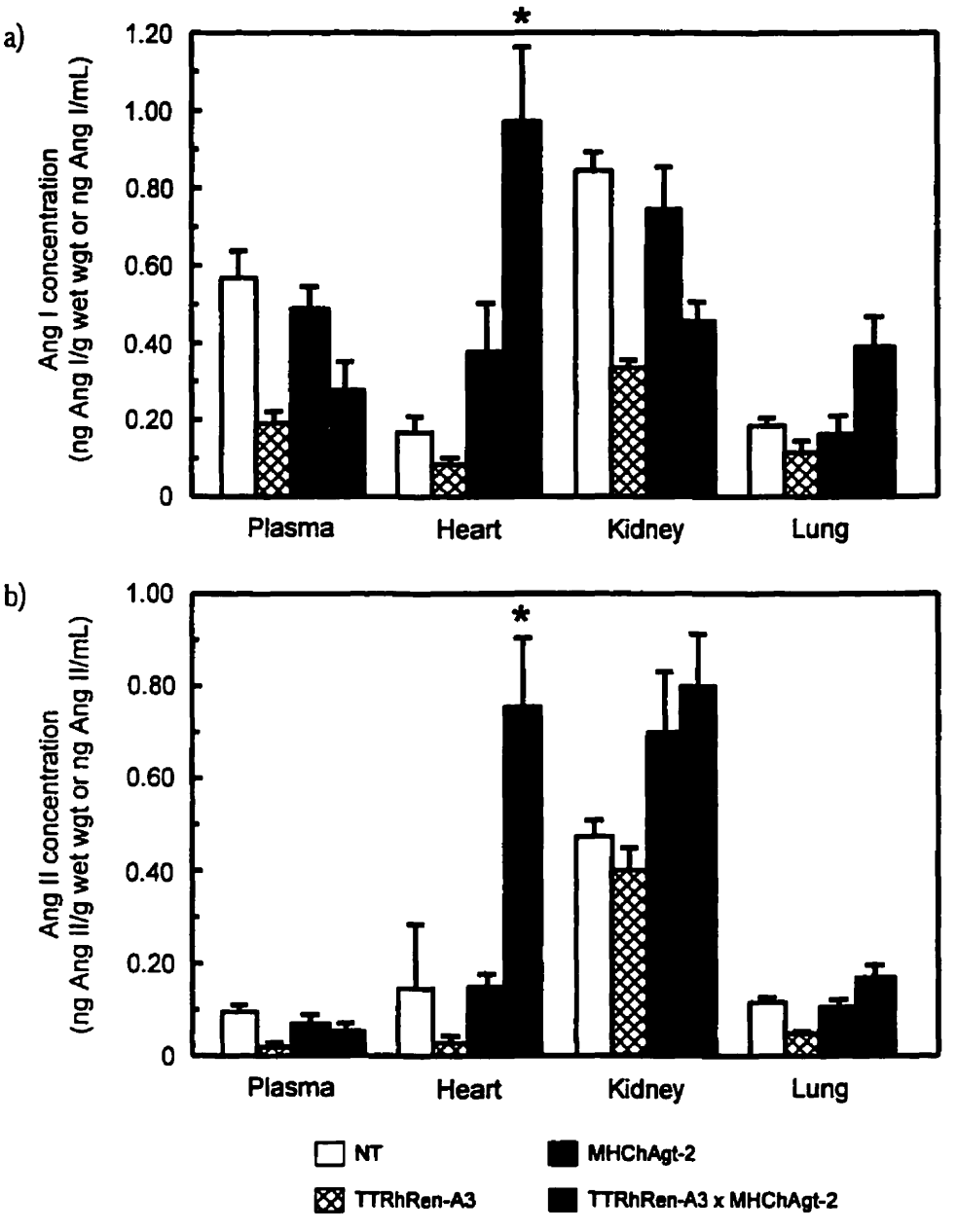

Figure A.3 Concentration of Ang I (a) and Ang II (b) in plasma, heart, kidney and lung of non-, single- and double- transgenic animals (TTRhRen-A3 x MHChAgt-2). *P<0.001 as compared to non-transgenic mice by ANOVA using Student's t-test.

4 Is tissue RAS of human (pro)renin responsible the observed hypertensive phenotype in TTRhRen-A3 mice?

To investigate the possibility that the hypertensive phenotype observed in TTRhRen-A3 mice may be the results of an increase tissue RAS, we collected plasma and tissue (heart, brain, liver, kidney and testis) from TTRhRen-A3 and non-transgenic mice. Angiotensin peptides were extracted and measured as describe in Material and Method of Chapter 2, using ketamine/acpromazine anesthetic cocktail instead of pentobarbital (refer to Appendices, Section 3).

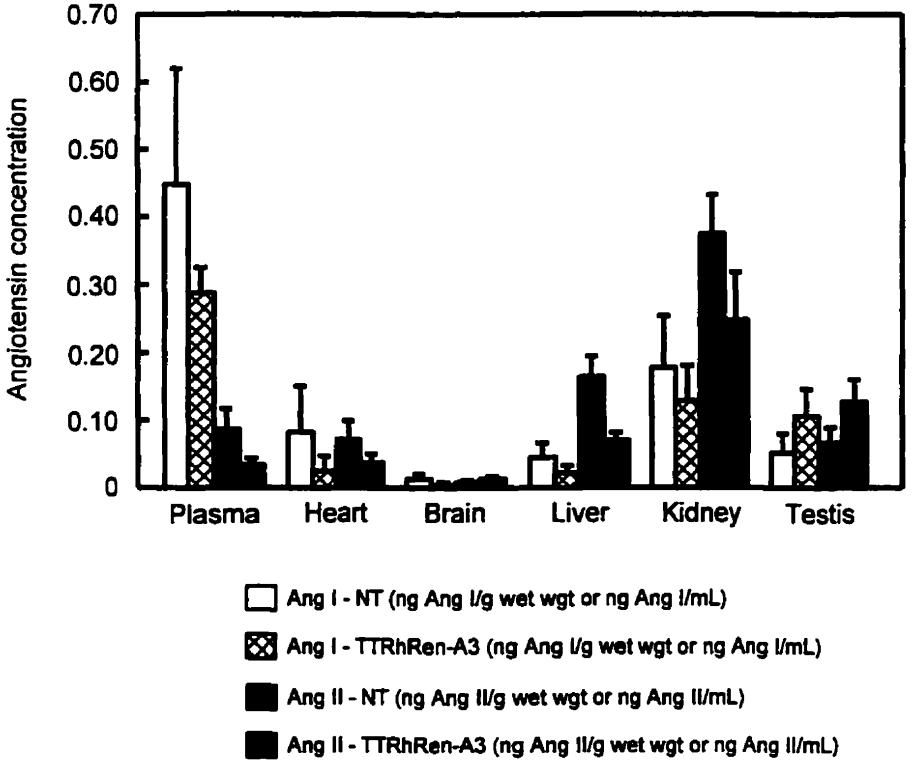

Figure A.4 Concentration of Ang I and Ang II in plasma and tissues of non-transgenic (NT) and TTRhRen-A3 mice.

As pointed out in Figure A.4, the heart, liver and kidney of TTRhRen-A3 harbor a decrease in Ang I and II concentration compared to control which suggest that no significant activation of a local RAS take place in those tissues. However, a slight increase in Ang I and II concentration in testis of transgenic animals raise the possibility that this particular tissue may be implication of in the control of blood pressure. In support for this possibility, we provided evidence for significant uptake of human (pro)renin in this tissue (refer to General dicussion, Section 2 and Appendices, Section 1). Although, to our knowledge, no one has reported evidence for the direct implication of this organ in control of blood pressure, increased plasma testosterone has been shown to cause a hypertensive phenotype in rats (332). This raises the possibility that a sustained local effect of circulating human (pro)renin in TTRhRen-A3 testis induces synthesis and release of androgen (testosterone) in circulation which in high concentration can affect renal function leading to increase blood pressure.

From our results it is still not possible to determine the cause of the hypertensive phenotype observed in our human active renin mice. However, we provide clear evidence that the observed rise in blood pressure is not caused by increased circulating RAS activity or tissue RAS present in the heart, liver or kidney. Further experiments are needed to assess the possible implication of testis, brain and adrenal in the regulation of blood pressure in our model.

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