Renin-Synthesizing Cells in the Kidney and Beyond

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Renine-synthetiserende cellen in de nier en daarbuiten

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"Digo: o real não está na saída nem na chegada: ele se dispõe para a gente é no meio da travessia"

João Guimarães Rosa

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JUXTAGLOMERULAR CELL PHENOTYPIC PLASTICITY

AIMS OF THE THESIS

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ABSTRACT
Renin is the first and rate-limiting step of the renin-angiotensin system. The exclusive source of renin in the circulation are the juxtaglomerular cells of the kidney, which line the afferent arterioles at the entrance of the glomeruli. Normally, renin production by these cells suffices to maintain homeostasis. However, under chronic stimulation of renin release, for instance during a low-salt diet or antihypertensive therapy, cells that previously expressed renin during congenital life re-convert to a renin-producing cell phenotype, a phenomenon which is known as "recruitment". How exactly such differentiation occurs remains to be clarified. This review critically discusses the phenotypic plasticity of renin cells, connecting them not only to the classical concept of blood pressure regulation, but also to more complex contexts such as development and growth processes, cell repair mechanisms and tissue regeneration.

1- Introduction

The renin-angiotensin system (RAS) plays a central role in blood pressure regulation and fluid-electrolyte homeostasis. It involves an array of enzymes, peptides and receptors with endocrine, paracrine and autocrine functions, whose actions are able to correct small extracellular volume (ECV) variations by increasing or decreasing RAS activity. According to the classical, endocrine view of the RAS, also known as the systemic RAS, renin, synthesized in the kidneys, catalyzes cleavage of a 10-amino acid peptide, angiotensin I, from the N-terminus portion of the circulating angiotensinogen, a protein produced by the liver. This decapeptide is subsequently hydrolyzed by angiotensin I-converting enzyme (ACE), a dicarboxyl-peptidase ubiquitously present on the plasma membrane of endothelial cells, yielding the octapeptide angiotensin II, the active endproduct of the RAS cascade, which is responsible, among others, for sodium reabsorption in the proximal convolute tubule of the nephron, and for vessel constriction, especially arterioles within the renal and systemic circulation [1]. This traditional axis was completed later, with the discovery of aldosterone. Angiotensin II stimulates the zona glomerulosa in the adrenal cortex to release aldosterone, which promotes sodium reabsorption coupled with potassium exchange in the distal convolute tubule of the nephron. The whole axis, named renin-angiotensin-aldosterone system (RAAS), is now firmly linked to ECV adjustment and blood pressure regulation [2]. However, in the last three decades, the RAS has assumed an even bigger role, in that it also participates in the pathophysiology of renal and cardiovascular diseases, through its inflammatory and pro-fibrotic actions. As a consequence, RAS blockade now is a cornerstone in the treatment of renal and cardiovascular diseases [3, 4]. The RAS also contributes to normal and abnormal growth processes and, evolutionary, the growth-promoting actions of angiotensin precede its endocrine and paracrine effects, representing one of its most highly conserved functions [5].

2- Renin

In 1898, Tigerstedt and Bergman performed a set of experiments, where they observed a rise in blood pressure after injecting rabbit kidney extracts in the jugular vein of rabbits. They characterized the substance as derived from the renal cortex, soluble in water and alcohol, thermolabile, and named it "renin". This discovery remained dormant for more than 40 years, until a few decades ago research finally started to focus on renin [6]. Renin represents the first and the rate-limiting step of the RAS cascade. Overproduction and underproduction of renin result in severe disturbances of body fluid homeostasis. Precise regulation of renin release is therefore indispensable for proper RAS functioning [1, 7]. Renin is an aspartyl protease synthesized as an inactive zymogen, prorenin. The kidney secretes both renin and prorenin, but plasma levels of prorenin are, under normal conditions, about 10-fold higher than the plasma levels of renin [8]. Renin consists of 2 homologous lobes, with a cleft in between, which contains the active site. Prorenin has a 43-amino acid N-terminal propeptide, which covers the enzymatic cleft between the 2 lobes, thereby preventing the access of its specific substrate, angiotensinogen. Prorenin is a glycoprotein, which can be activated in 2 ways: proteolytically or non-proteolytically. The former is irreversible and involves removal of the propeptide by a processing enzyme, while the latter is reversible, inducible by certain conditions such as low temperature and pH, and results from a conformational alteration, i.e., unfolding of the propeptide from the enzymatic cleft [9].

3 – Juxtaglomerular Cells (JGC)

The JGC of the kidney are the only source of renin and the main source of prorenin in the circulation [1, 10]. Several extrarenal tissues, such as the adrenal gland, ovary, testis, placenta and retina, additionally produce and secrete prorenin [8]. Indeed, after a bilateral nephrectomy, prorenin, but not renin, can still be detected at low levels in blood [11]. Following its synthesis, prorenin enters the Golgi apparatus to be glycosylated, and then proceeds to one of two different pathways. The first one involves clear vesicles containing prorenin which are secreted constitutively, while the second pathway involves prorenin tagging for regulated secretion, which is accompanied by proteolytic prorenin-renin conversion, and renin storage in dense cytoplasmic granules [10], awaiting release in response to various stimuli, such as a sudden fall in blood pressure, a low salt diet, or β-adrenergic stimulation [12].

JGC are part of the juxtaglomerular apparatus, a tight structure situated on the kidney glomerular hilum, composed mainly of specialized epithelial cells (macula densa cells of the ascending limb of the nephron loop), extraglomerular mesangial cells, the terminal portion of the afferent arterioles and the proximal portion of the efferent arterioles. JGC are within these vessels and exhibit an epithelioid appearance, with a prominent endoplasmic reticulum, Golgi apparatus, and renin granules, but also containing myofibrils and adhesion plaques, typical of smooth muscle cells [13].

Efforts to characterize JGC have historically been hampered by several factors: JGC lose their secretory granules and, concomitantly, their ability to proteolytically activate prorenin when placed in culture [14]; JGC make up approximately 1/1000 of the total cell mass of the kidney, making it difficult to isolate sufficient quantities of pure cells for further characterization [15].

4 – JGC Progenitors and their Embryology

JGC represent one type of the so-called repertoire of renin-producing cells (RPC), a group of cells that at some moment in their lifespan produce renin. During kidney ontogeny in mammals, renin expression changes according to a pattern (Figure 1). It first appears in the undifferentiated metanephric blastema before vessel formation has begun. Once the vascularization begins, around the 14th embryonic day in mice and rats, RPC are distributed along the walls of the arteriolar vessels. As the process evolves, renin starts to be expressed in other cells along the distal portions of the newly formed vessels. Gradually, RPC disappear from the larger vessels, shifting toward the afferent arterioles, and, finally, assuming their position on the juxtaglomerular apparatus [12]. Gomez et al. showed that the ontogeny of renin expression by RPC depicts its own phylogenetic evolution pattern [16].

The expression of the renin gene is tissue-specific and developmentally regulated [17]. Renal renin concentrations are high in early life, decreasing progressively as kidney maturation evolves [18]. During this process, RPC are associated with assembling and branching of the developing kidney vasculature [19], and the ablation of these cells in mice during development results in a distinct kidney phenotype with peculiar vascular abnormalities [20]. *In vivo*, vascularization of the kidney is synchronized with epithelial nephrogenesis [21]. Epithelial branching morphogenesis is critical for the formation of various organs, including the vasculature and kidneys [22]. The definitive kidney in mammals originates from a complex interaction between the ureteric bud and the metanephric mesenchyme, both derived from the intermediate mesoderm. These reciprocal actions lead the ureteric bud to elongate and bifurcate toward the metanephric mesenchyme, forming on its tip, an aggregate of mesenchyme cells, the cap mesenchyme. This condensate of cells generates a vesicle, which continues to form a comma-shaped body and later, a S-shaped body that gives rise to most of glomerulus and tubular epithelia. As nephrogenesis progresses, the newly formed S-shaped body is fused to the ureteric bud, from which the collecting ducts and ureter originate [23, 24].

Cap mesenchyme expresses the transcriptional factors Six2 and Cited1 and gives rise to Bowman's capsule, podocytes, the proximal and distal convolute tubules and the loop of Henle. There is an outer layer of loose mesenchyme cells, adjacent to the cap mesenchyme, which expresses different transcriptional factors: cKit (endothelial precursors) or forkhead box D1 (FoxD1, stromal cells). The latter factor is responsible for the generation of RPC, mesangial cells and all mural cells, including vascular smooth muscle cells (VSMC), perivascular fibroblasts and pericytes [25-27].

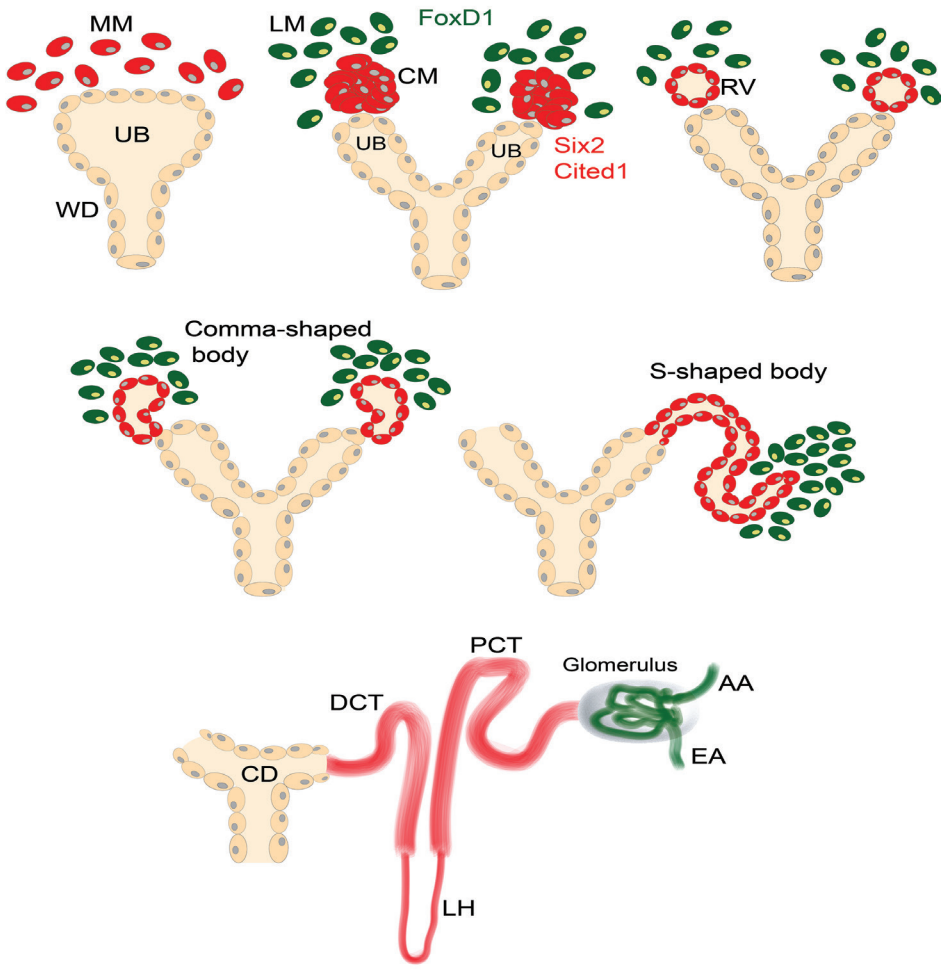

Figure 1. Schematic illustration of nephrogenesis. The ureteric bud (UB) interacts with the methanephric mesenchyme (MM), resulting in the subsequent formation of the cap mesenchyme (CM) and loose mesenchyme (LM). The CM harbors the transcription factors Six2 and Cited1 (in orange), and will give rise to the tubular system (proximal convolute tubule (PCT), loop of Henle (LH) and distal convolute tubule (DCT); the LM harbors the transcriptions factor FoxD1 (depicted in green), and will give rise to most of the glomeruli and vascular cells from the afferent arteriole (AA) and efferent arteriole (EA). The UB will evolve to from the collecting duct (CD) and ureter. Modified from [21, 24, 33].

5- The Recruitment Phenomenon

JGC lineage involves differentiation of the above metanephric mesenchymal cells. This complex process generates hemangioblasts, which will evolve to endothelial cells, and RPC precursors, the latter harboring the transcriptional factor FoxD1. During kidney ontogeny, these precursors will give rise to JGC and a subset of VSMC [25, 27]. In the adult, stress events that threaten body homeostasis, such as hypotension or extracellular fluid depletion, are normally corrected through renin release by JGC. Nevertheless, if this response does not suffice, or if renin expression is chronically stimulated, VSMC along the preglomerular arterioles undergo metaplasia to a renin cell phenotype in order to also synthesize renin, a phenomenon known as "recruitment" [28] (Figure 2). Importantly, the upregulation of renin synthesis and release following a homeostasis threat is due to a rise in the number of RPC (hyperplasia) rather than an elevation on the renin production per cell (hypertrophy) [29, 30].

The recruitment phenomenon is an indispensible mechanism to maintain homeostasis. In the 1970s, Cantin et al. observed metaplasia of VSMC into JGC in the arteries and arterioles of ischemic kidneys [31]. Nowadays, it is known that this transformation involves differentiation of a non-renin-expressing cell into a phenotype that is able to synthesize renin, and occurs in the descendants of cells that previously expressed renin during development [32]. Interestingly, once the stimulation perpetuates, the recruitment intensifies, and RPC may occur all along the extension of preglomerular arterioles, larger vessels, in the extraglomerular and intraglomerular mesangium, in a pattern that resembles the development of the embryonic kidney [32]. In a recent review, Gomez emphasized that recruitment does not involve migration or replication of cells, but solely concerns a phenotype switch toward renin expression by cells whose capability to produce renin is latent, and re-acquired following appropriate stimulation [33].

Nevertheless, Dzau et al. identified liver X receptor alpha (LXRα), a nuclear receptor, as an important player in the induction of renin expression in JGC. They showed that LXRα exerts its activity as a cAMP-responsive regulator, binding to a unique upstream region of the renin promoter [34]. LXRα activation additionally upregulated a set of genes (e.g., c-myc) that are involved in cellular differentiation, proliferation and migration. Accordingly, LXRα would induce JGC hypertrophy and hyperplasia, through its coordinate interaction on the renin and c-myc promoters [29].

Moreover, mesenchymal stem cells (MSC) have also been suggested to play a pivotal role in the recruitment phenomenon. Matsushita et al. demonstrated that human and murine MSC are capable of synthesizing renin following LXRα activation.

Indeed, MSC overexpressing LXRα and under continuous cAMP stimulation underwent differentiation to a RPC phenotype, which could also express α-smooth muscle actin (αSMA) [35]. Thus, on the basis of these findings, it was speculated that MSC, which are normally resident within the glomerulus, might be the origin of the RPC [35]. Wang et al. used cell lineage tracking models to further study the role of MSC in RPC recruitment. First, they isolated MSC from the adult mouse kidney and

verified the expression of typical tissue stem/progenitor cell markers, including CD44, and of metanephric mesenchymal cell markers, such as FoxD1. CD44-positive MSC-like cells differentiated into RPC following exposure to an LXRα agonist, cAMP or a phosphodiesterase inhibitor [36]. Furthermore, mice submitted to a low salt diet and captopril treatment, conditions well-established to induce JGC recruitment, exhibited an expansion of RPC in the kidney, accompanied by CD44 cells, with co-localization of MSC markers and renin. These results could not be reproduced with bone marrow MSC, suggesting that only MSC resident in the adult kidney contribute to JGC recruitment [36]. The prostaglandin E2/E-prostanoid receptor 4 pathway, also known for its involvement in tubuloglomerular feedback, played a key role in the activation of renal CD44-positive MSC during conditions of JGC recruitment [37].

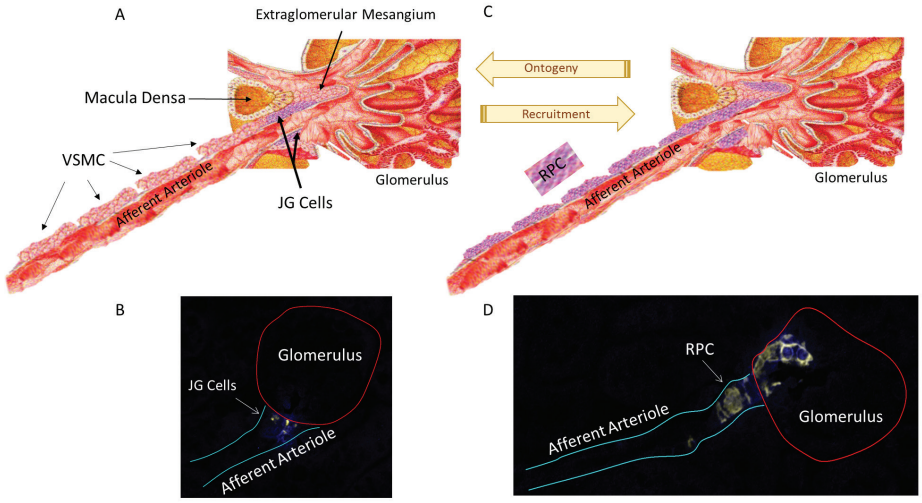

Figure 2. The 'recruitment' phenomenon, illustrated by a schematic illustration (panels A and C) and immunofluorescence and fluorescent *in situ* hybridization data from control and captopril-treated mice (panels B and D, modified from [64]). In adult kidneys, renin is produced by the juxtaglomerular (JG) cells located within the afferent arterioles at the entrance of the glomeruli. However, under chronic renin stimulation (e.g., following captopril treatment), renin lineage cells (like the vascular smooth muscle cells (VSMC)) along the afferent arterioles may convert into a renin-producing cell (RPC) phenotype in order to maintain the homeostasis.

However, the participation of adult renal MSC in JGC recruitment has been questioned. Gomez et al. reported that MSC at most have a very minor contribution in comparison with the already existing pool of arteriolar cells undergoing a phenotype switch [33]. The latter authors ascertained that JGC also express CD44, so that it is questionable whether MSC truly represent a different cell group in the recruitment process, or are just simply descendants of the renin cell lineage, capable of switching

their renin phenotype back and forth. Moreover, CD44 expression on MSC might be an *in vitro* artifact, since primary MSC from bone marrow lacked such expression, while it did occur after culturing the cells [38]. Further studies are needed to unravel the precise role of MSC in JGC recruitment.

6- cAMP Pathway and Recruitment

Renin is a cAMP-inducible gene. In all species tested, there is a functional cAMP response element on the renin promoter [12]. Among all the intracellular second messengers that control renin secretion, the cAMP signaling cascade appears to be the core pathway for the exocytosis of renin [10, 39, 40] (Figure 3). Thus, prostaglandins, kinins, and β-adrenergic agonists have a stimulatory effect on renin release, in all cases because they increase cAMP generation [41].

cAMP regulates a wide range of biological processes in cells. The binding of an extracellular signal molecule to a G-protein coupled receptor activates adenylyl cyclase (AC), an enzyme that generates cAMP from ATP, increasing its intracellular levels. This rise activates cAMP-dependent protein kinase A (PKA). PKA translocates to the nucleus to phosphorylate the gene regulatory protein CREB (cAMP responsive element binding protein). CREB recognizes a specific DNA sequence, called the cAMP response element (CRE), found in the regulatory region of many genes. Once phosphorylated, CREB recruits the coactivator CBP (CREB-binding protein), which stimulates gene transcription [42].

Interestingly, conditional deletion of G-protein subunits in RPC has a great impact on their function. Indeed, mice with protein Gsα (stimulatory subunit α) deficiency in JGC have very low plasma renin concentrations, with resulting low levels of aldosterone and arterial blood pressure. Moreover, such deletion also resulted in abnormalities in the preglomerular arterial tree [43, 44].

Elegant evidence on the regulation of renin production by cAMP was obtained by Gomez et al., who labeled cells of renin lineage with cyan fluorescent protein (CFP), and cells producing renin with yellow fluorescent protein (YFP) [45]. This yielded suitable amounts of cells which could still produce renin after several passages. CFP-labeled cells expressed VSMC markers like α-SMA and smooth muscle myosin heavy chain (SM-MHC or Myh11), but not renin. However, after stimulation with forskolin (an AC stimulator) or cAMP analogs, they began to express YFP and renin, and decreased α-SMA and Myh11 expression. This response was even bigger with more intense or longer stimuli, suggesting that the recruitment response is graded [45].

7- Role of calcium and cGMP

Calcium plays an important role in the biology of secretory cells. In general, a rise in cytosolic calcium leads to the release of their content. However, the opposite occurs in parathyroid cells and JGC, where calcium inhibits renin exocytosis. This is

known as the "calcium paradox", and it remained as a mystery for decades [41, 46].

JGC harbor 2 isoforms of AC (types V and VI), which are inhibited by cytosolic calcium. Thus, particularly in JGC, a decrease in cytosolic calcium would stimulate AC, resulting in cAMP synthesis and, consequently, renin release [47, 48]. Initially, it had been reported that calcium inhibits renin gene transcription and destabilizes renin mRNA [49]. More recently, it became clear that calcium stimulates, via the calcium sensing receptor, a calcium calmodulin-activated phosphodiesterase 1C (PDE1), an enzyme that degradates cAMP, thereby providing an additional explanation of the calcium paradox [50].

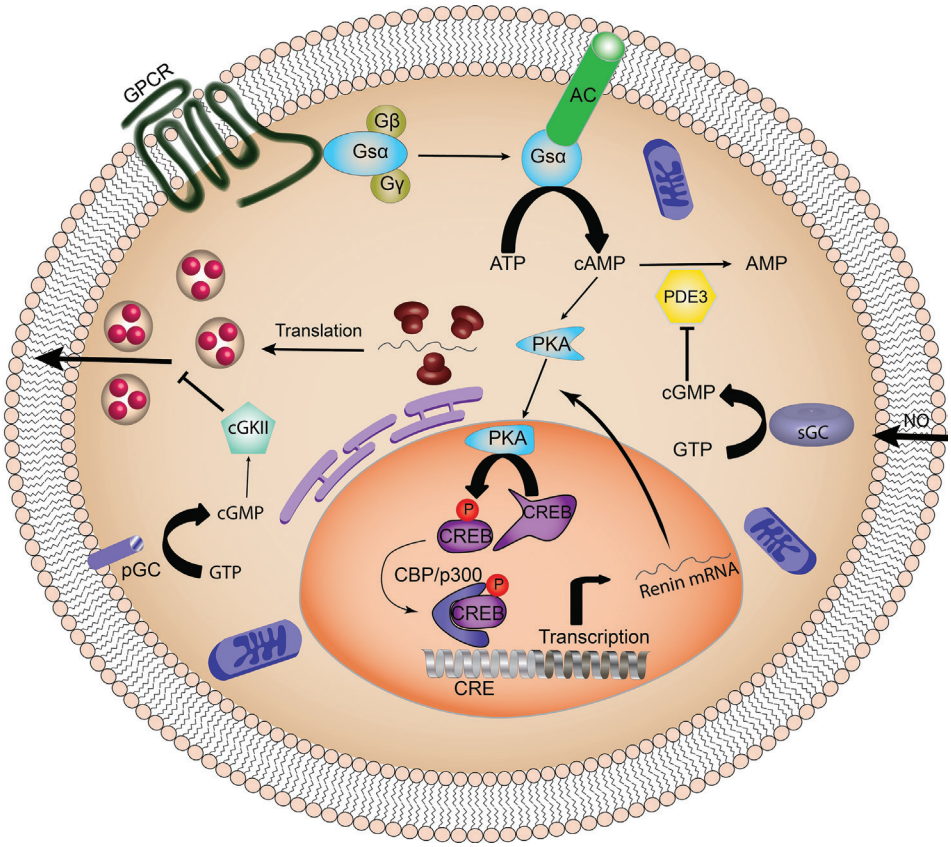

Figure 3. Simplified scheme showing how cAMP and cGMP regulate renin (modified from [33]). ATP, adenosine-triphosphate; cAMP, cyclic adenosine-monophosphate; AC, adenylate cyclase; CBP, CREB-binding protein; CREB, cAMP response element binding protein; cGMP, cyclic guanosine-monophosphate; PDE, phosphodiesterase; NO, nitric oxide; pGC, particulate guanylate cyclase; sGC, soluble guanylate cyclase; GPRC, G-protein-coupled receptor; Gsα, stimulatory G protein α-subunit; Gβ, inhibitory G protein β-subunit; Gγ, inhibitory G protein γ-subunit; PKA: protein kinase A.

The contribution of cGMP to renin release is more complex, with both stimulatory and inhibitory effects [46]. The cGMP and cAMP pathways are cross-linked. Nitric oxide (NO) activates soluble guanylate cyclase (GC) to generate cGMP, which in turn inhibits phosphodiesterase 3, a cAMP-degrading enzyme. Consequently, cAMP levels will go up, and renin release will rise [46, 51]. However, ligands that increase cGMP via activation of particulate GC (like atrial natriuretic peptide) inhibit renin exocytosis through activation of cGMP-dependent protein kinase type II [10, 46]. Interestingly, Neubauer et al. recently demonstrated that RPC recruitment is dependent on NO availability and the NO-GC signaling pathway [52].

8- Epigenetic Mechanisms and microRNA (miRNA)

Acetylation and deacetylation of histones are important epigenetics mechanisms involved in gene transcription regulation. Acetylation is mediated by histone acetyltransferase (HAT), which leads to the transfer of an acetyl functional group to histone molecules, promoting nucleosomal relaxation and transcriptional activation. Deacetylation is mediated by histone deacetylase (HDAC), and results in chromatin condensation and transcriptional repression [53].

Using the double-fluorescent reporter mouse model described above, Gomez et al. observed that chromatin remodeling contributes to the recruitment process, at the cAMP level, through histone H4 acetylation [45]. The underlying mechanism involved the CREB-recruited cofactors CBP and p300, which exhibit HAT activity and, in the CRE region, promote nucleosomal relaxation and, consequently, transcriptional activation. In support of this concept, forskolin increased histone H4 acetylation in the CRE region [45]. Studies carried out in mice with conditional deletion of CBP and p300 in RPC revealed that individual deletion of one of these cofactors did not affect renin expression, while simultaneous deletion reduced renin in adult life, and resulted in renal interstitial fibrosis [54]. CBP/p300 were also indispensable for re-expression of renin in the arteriolar VSMC of mice exposed to low sodium + captopril [55]. Taken together, RPC have a poised chromatin landscape suitable to respond properly to threats, allowing these cells to switch the renin phenotype on and off [33].

In addition to the epigenetic control mechanisms, microRNAs (miRNAs) also control JGC activity. miRNAs are endogenous small non-coding RNA molecules, containing around 18-22 nucleotides. They exert their function by targeting mRNA, inducing decay or translational repression [56]. miRNA genes are phylogenetically conserved, and are involved in many cell processes such as developmental timing, death and proliferation [57]. miRNA transcription, usually by RNA polymerase II, results in a long transcript, whose structure will be cleaved by the RNase III endonuclease Drosha and the cofactor DGCR8, yielding a miRNA precursor (pre-miRNA). This precursor is processed by the RNase III enzyme Dicer, and the product is incorporated into the RNA-induced silence complex, where gene silencing proceeds [58].

Gomez et al. established the importance of Dicer for the JGC and, even further,

for the morphologic and physiologic integrity of the kidney. They discovered that conditional deletion of Dicer in cells of the renin lineage in mice reduced the number of JGC and decreased renin gene expression, thus leading to reduced plasma renin and blood pressure levels. The animals also presented kidneys with vascular abnormalities and striped fibrosis [59]. Noteworthy, this vascular pattern was quite similar to that found in mice with ablation of the RPC, through the expression of diphtheria toxin A chain driven by the renin promoter [20]. Surprisingly, kidney vessels exhibited normal wall thickness and lumen size, in contrast to the concentric hypertrophy and thick vessel walls seen when RAS genes are deleted, including renin. Apparently, RPC are responsible for arterial wall thickening, by producing angiogenic and trophic factors [20, 21].

Furthermore, Medrano et al. identified two miRNA, miR-330 and miR-125b-5p, whose opposite actions are crucial for the recruitment phenomenon. miR-125b-5p is normally expressed in the VSMCs of the afferent arteriole, and responsible for sustaining a contractile phenotype. Yet, it is also expressed in JGC, in order to preserve their contractile function. However, under a homeostasis threat, miR-125b-5p expression diminishes in VSMC, allowing them to undergo a metaplastic transformation into a renin phenotype, and remains unaltered in JGC. miR-330 is expressed in JGC only, and inhibits contractile features, thus favoring renin production [60].

9- JGC Signature: the Myo-endocrine Profile

Although several factors have been identified that regulate renin production and secretion, RPC-specific markers were limited to renin itself, and the gene Zis transcript [14]. To overcome this problem, Brunskill at al. targeted YFP to mouse JGC and used fluorescence-activated cell sorting (FACS) to enrich tagged cells for transcriptome analysis. This approach yielded a set of 369 core genes, responsible for the JGC gene regulatory network [61]. Furthermore, this distinct array of genes that governs JGC identity is unique when compared to other cell types in the kidney. Mainly, it encompasses genes highly expressed in both smooth muscle and endocrine cells [61].

Among the genes identified in the transcriptome analysis, renin was the highest transcribed gene. The second highest one was aldo-keto reductase family 1, member B7 (Akr1b7), responsible for detoxification of steroidogenesis products. Akr1b7 is a member of aldo-keto reductase superfamily, which reduces harmful aldehydes and ketones produced from the breakdown of lipid peroxides to their respective alcohols [62]. Remarkably, despite its high expression on JGC, mice with Akr1b7-deficient kidneys had no abnormalities in renin production and secretion, while renin deletion also did not affect Akr1b7 expression [61, 63]. Therefore, Akr1b7 gene might function as a novel JGC marker, independently of renin. Other highly expressed transcripts involved genes related to the smooth muscle phenotype, like α-SMA, Myh11 and calponin (Cnn1) [61].

Interestingly, the aforementioned genetic regulatory network allows RPC to

possess either an endocrine or a contractile phenotype. Hence, this bivalent profile sustains the ability of RPC to switch phenotype, depending on the situation. Therefore, during a homeostasis challenge, renin lineage cells have the gene program to differentiate into an endocrine cell, synthesizing and releasing renin, in order to reestablish the homeostasis. Moreover, due to their position, at the glomerular hilum, JGC should also retain contractile properties, allowing them to contract or relax and, subsequently, to adjust renal blood flow and glomerular filtration rate [33, 61].

Martini et al. performed RNA transcriptome analysis on human reninomas, as an approach to further understand JGC biology [64]. Reninomas are rare, renin-producing tumors, arising from a proliferation of JGC in the kidney cortex, and are often detected because of the appearance of fulminant hypertension in young patients [65, 66]. Deep sequencing of 4 human reninomas with subsequent transcript mapping in the kidney of mice under different conditions yielded a list of genes (36 of which had never been described before) specifically expressed in RPC. When evaluating 10 of these genes on (pro)renin producing As4.1 cells, it was observed that platelet-derived growth factor beta (PDGFB) suppressed (pro)renin release and renin gene expression. Moreover, PDGFB-exposed cells displayed a phenotypic shift from myo-endocrine to inflammatory, evidenced by αSMA downregulation and interleukin-6 upregulation, and a more elongated shape (Figure 4). Here it should be mentioned that neither conditional deletion of the PDGF receptor in RPC, nor deletion of endothelial PDGFB production affected the normal development of renal RPC [67]. This may not be too surprising, given the fact that PDGFB actually is a negative regulator of renin expression, possibly coming into play only under pathological conditions. In summary, the reninoma data provide a novel role for PDGFB as a regulator of RPC.

10- Notch Signaling Pathway

The Notch signaling pathway is a highly conserved system, present in all animal species, which plays a pivotal role in local cell-cell communication, determining cell fate decisions and controlling patterns formation during ontogenesis. Its dysfunction is linked to severe developmental defects and pathologies [68]. The Notch signaling cascade starts with the binding of specific ligands to the Notch transmembrane receptor, leading to cleavage of the intracellular Notch receptor domain (NIC). NIC translocates to the nucleus, where it binds to RBPJ (recombination signal binding protein for Ig-κJ region), a transcription factor that normally represses Notch target genes by recruiting a co-repressor complex. However, once it is coupled to NIC, RBPJ recruits cofactors that activate the transcriptional machinery [69].

Interestingly, among the 369 core genes that confer the JGC identity according to the approach performed by Brunskill et al., there are members of the Notch pathway, including RBPJ [61]. In fact, RBPJ had already been related to RPC plasticity by Castellanos Rivera et al. [70]. They generated a conditional knockout (cKO) of RBPJ in renin lineage cells. This resulted in a striking reduction in the JGC number and re-

nin expression, with subsequently low plasma renin levels and a low blood pressure. Furthermore, under conditions that trigger the recruitment phenomenon, such as sodium depletion and captopril treatment, RBPJ-cKO mice were unable to exhibit renin expression along the afferent arterioles, i.e., the ability of their VSMCs to regain the renin phenotype was impaired.

Additionally, mice with RBPJ-cKO in renal cells of FoxD1 lineage displayed significant renal abnormalities, including a decline in the arteries and arterioles number, with a thinner smooth muscle cell layer and renin depletion, further upholding the concept that RBPJ is a major determinant of the transformation of FoxD1 cells progenitors into a healthy kidney vasculature [71]. Experiments in transgenic renin reporter zebrafish fully support the essential role of the Notch signaling pathway for developmental renin expression and its association with proper angiogenesis [72].

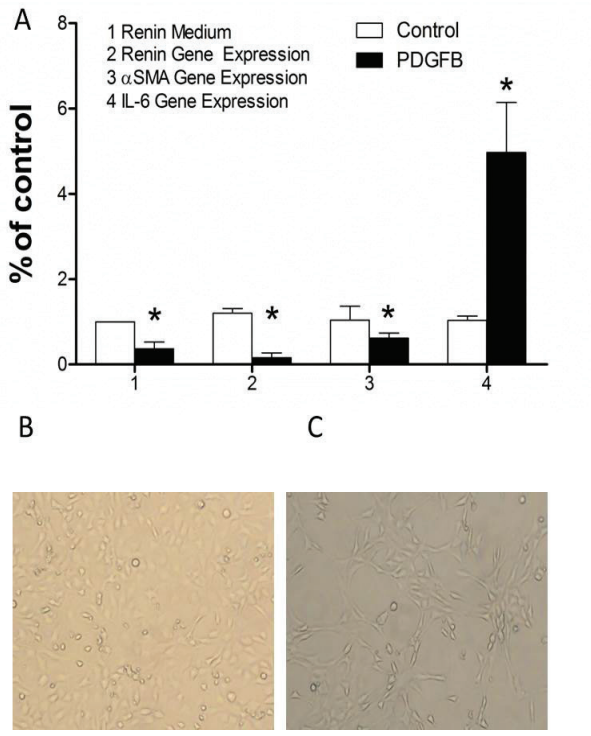

Figure 4. A, Renin levels in medium obtained from renin-producing As4.1 cells incubated for 48 hours with medium derived from HEK293 cells transfected with platelet-derived growth factor beta (PDGFB). Renin, α-smooth muscle actin (αSMA) and interleukin-6 (IL-6) gene expression are also provided. Data (mean ±SEM of n=5-11) are modified from [64], and have been expressed as a percentage of the levels in cells exposed to medium of control-transfected cells, i.e., cells treated with the transfection mix, but without cDNA. *P<0.05. B and C, As4.1 cells under control conditions and after exposure to PDGFB (10x magnification).

RBPJ also has a pivotal role in the maintenance of the JGC myo-endocrine gene program. Using a bacterial artificial chromosome reporter, it was observed that RBPJ activates the renin promoter directly [15]. A double-fluorescent mice reporter model subsequently revealed that RBPJ deletion does not affect RPC endowment. RPC were at their proper location, although unable to express renin and Akr1b7, and, surprising-

ly, also did not express the VSMC markers α-SMA, Myh11 and Cnn1. Yet, they displayed an upregulation of genes related to the immunological system, such as lipocalin-2, lysozyme-2, chemokine ligand-9 and interleukin-6 [15]. Furthermore, Belyea et al. identified renin progenitors in mouse bone marrow, and found that in the presence of RPBJ-cKO in the renin cell lineage, those progenitors gave rise to a very aggressive lymphoblastic leukemia [73].

JGC are surrounded by multiple cell types, including pericytes, endothelial cells, epithelial cells, VSMCs, mesangial cells, and macula densa cells. Direct cell-to-cell interaction will undoubtedly contribute to normal RPC functioning. It is therefore not surprising that, among the highly expressed transcripts identified by Brunskill et al. in JGC, is connexin 40 [61]. Connexins are transmembrane proteins, six of which assemble to mold a hemichannel. When 2 hemichannels of adjacent cells align, a channel is formed which allows the cytoplasma of both cells to connect. A set of these channels in parallel builds a gap junction, a structure that permits the cells to share small molecules and, therefore, to respond to extracellular signals in a coordinated way [16]°. Unexpectedly, in mice lacking connexin 40, RPC were found in the periglomerular interstitium, and not at their normal juxtaglomerular location. Besides, the recruitment phenomenon (following severe sodium depletion) did not occur at its usual location, i.e., in the wall of the upstream preglomerular arterioles [74]. Apparently therefore, cell-to-cell communication via gap junctions is essential for the correct juxtaglomerular positioning and recruitment of RPC.

11- Plasticity and Regeneration

More than 100 years have passed since renin's discovery, and the RAS now turns out to have multiple effects beyond its role in blood pressure regulation. As outlined in this review, renin cells function as pluripotent progenitors for other kidney cells types [32, 75]. Normally, a low perfusion pressure leads to an expansion of RPC. Kurt et al. investigated to what degree this relates to hypoxia per se, by deleting the von Hippel-Lindau protein in JGC, i.e., the negative regulator of the hypoxia-inducible transcriptional factor-2 (HIF-2α). Remarkably, following the upregulation of HIF-2α by this procedure, RPC started to express erythropoietin instead of renin, and the normal renin upregulation after low salt was no longer seen. In fact, JGC were reprogrammed into fibroblast-like cells, expressing collagen I and PDGF receptor β (PDGFR-ß) [76, 77]. Clearly therefore, hypoxia-inducible genes are essential for normal development and physiologic adaptation of RPC. The upregulated PDGFR-ß expression in these cells is in full agreement with the results from the human reninoma studies showing that the PDGFB- PDGFR-ß pathway downregulates renin expression [64].

Pippin et al. proposed that cells of renin lineage present alongside the preglomerular arteries participate in the glomerular regeneration after podocyte injury [78]. In support of this concept, renin cells from the juxtaglomerular apparatus were shown to migrate to the glomerular tuft, in order to replace podocytes [79]. This is quite strik-

ing, since podocytes are normally derived from a different embryonic structure, the cap mesenchyme cells.

Easier to understand is the participation of renin progenitors in glomerular mesangium regeneration, since mesangium cells, considered as specialized pericytes, are derived from FoxD1 stromal cells present at the loose mesenchyme. Indeed, in a mesangiolysis murine model, renin lineage cells repopulated the intraglomerular mesangium [80]. Interestingly, these cells stopped producing renin and expressed PDGFR-ß, i.e., the receptor linked to renin downregulation [64]. Furthermore, Stefanska et al. established that pericytes are RPC in the human kidney [81]. Traditionally, pericytes are perivascular cells that wrap around blood capillaries. They are highly plastic cells contributing to multiple processes like angiogenesis, tumorigenesis and vasculopathy progression [82]. The concept that RPC are or derive from pericytes is very interesting, since pericytes possess regenerative potential and are additionally known for the fact that they express the PDGFR-ß [81]. In fact, in zebrafish, Rider et al. observed that renin cells express this marker [83].

12- Concluding Remarks

How exactly renin cells are able to differentiate into many different phenotypes remains to be clarified. The signals allowing RPC to shift phenotype, or to migrate to an injury site are now only beginning to be unraveled. Many questions still need to be answered, most importantly, whether regeneration would eventually allow a restoration of normal function. This is of particular relevance in chronic glomerulopathies, where glomerular cells are destroyed, with subsequent overproduction of extracellular matrix, fibrosis and architectural disruption, all leading to physiological impairment. Apparently, under such severe pathological conditions, tissue regeneration by RPCs either does not occur of is insufficient. One possibility is that RPCs under pathological conditions transform to fibroblast-like cells, thus contributing to renal fibrosis themselves. Currently, RAS blockade is the cornerstone of renal and cardiovascular diseases. Simultaneously, it is well known that RAS blockers, like most anti-hypertensive drugs, induce RPCs recruitment. The long-term effect of this chronic activation is still unknown, nor do we know to what degree it contributes to (further) impairment of renal function. Detailed knowledge of this process would lead to better treatment options and more favorable outcomes. Clearly, this whole area not only illustrates the complexity of the renin cell, but also represents an exciting new field that needs to be explored in the coming years.

AIM OF THE THESIS

1) To validate a new approach to understand RPC biology, by using transcriptome analysis of human renin-producing tumors (reninoma) (Chapter 2);

2) To evaluate the occurrence of a brain RAS, and subsequently, the potential participation of RPC in this process (Chapter 3);

3) To assess whether prorenin in an 'open' conformation at the site of its release from RPC is capable of generating angiotensin locally (Chapter 4);

4) To investigate renin and prorenin reabsorption in megalin-expressing human conditionally immortalized proximal tubule epithelial cells (Chapter 5);

5) To evaluate, by performing a systematic review and meta-analysis, whether the beneficial effects of dual RAS blockade in patients with heart failure and renal dysfunction outweigh their side effect profile (Chapter 6).

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Chapter 2

TRANSCRIPTOME ANALYSIS OF HUMAN RENINOMAS AS AN APPROACH TO UNDERSTANDING JUXTAGLOMERULAR CELL BIOLOGY

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ABSTRACT
Renin, a key component in the regulation of blood pressure in mammals, is produced by the rare and highly specialized juxtaglomerular (JG) cells of the kidney. Chronic stimulation of renin release results in a recruitment of new JG cells by the apparent conversion of adjacent smooth muscle cells along the afferent arterioles. Because JG cells rapidly de-differentiate when removed from the kidney, their developmental origin and the mechanism that explains their phenotypic plasticity remain unclear. To overcome this limitation we have performed RNA expression analysis on four human renin-producing tumors. The most highly expressed genes that were common between the reninomas were subsequently used for *in situ* hybridization in kidneys of 5-day old mice, adult mice and mice treated with captopril. From the top 100 genes, 10 encoding for ligands were selected for further analysis. Medium of HEK293 cells transfected with the mouse cDNA encoding these ligands was applied to (pro)renin-synthesizing As4.1 cells. Among the ligands, only platelet-derived growth factor B (PDGFB) reduced the medium and cellular (pro)renin levels, as well as As4.1 renin gene expression. Additionally, PDGFB-exposed As4.1 cells displayed a more elongated and aligned shape with no alteration in viability. This was accompanied by a downregulated expression of α-smooth muscle actin, and an upregulated expression of interleukin-6, suggesting a phenotypic shift from myo-endocrine to inflammatory. Our results add 36 new genes to the list that characterize renin-producing cells and reveal a novel role for PDGFB as a regulator of renin-synthesizing cells.

INTRODUCTION

The aspartyl protease renin catalyzes the rate-limiting step in the renin-angiotensin system (RAS). Renin is produced almost exclusively by so-called juxtaglomerular (JG) cells that line the afferent arterioles at their point of entry into the kidney glomeruli. JG cells convert renin from its inactive precursor (prorenin) by proteolysis and store the resulting active renin in lysosome-like dense cytoplasmic granules whose content can be rapidly released in response to such stimuli as a sudden drop in the perfusion pressure of the afferent arteriole or beta-adrenergic stimulation. During kidney development, renin-producing cells are more broadly distributed along the lumen of the nascent arterioles [1] and gradually become restricted to the glomerular poles after birth. Prolonged exposure to stimuli, such as blood pressure-lowering medication, a low-salt diet or ischemia results in an apparent recruitment of new renin-producing cells along the afferent arterioles [2], which has been proposed to represent a partial return to the developmental pattern of JG cell distribution. Altogether, renin-producing JG cells display many unique biological features and because of their central role in modulating blood pressure and fluid balance, there has been long-standing interest in obtaining a better understanding of their origins and biology.

Efforts to characterize JG cells have historically been hampered by two factors: First, JG cells rapidly de-differentiate when placed in culture, losing their secretory

granules and, concomitantly, their ability to proteolytically activate prorenin. As a result, attempts to immortalize both mouse and human renin-producing cells have resulted in the establishment of cell lines that have lost the ability to secrete renin [3-4]. Second, JG cells make up only approximately 1/1000 of the cell mass of the kidney, making it difficult to isolate sufficient quantities of pure cells for further characterization. As an alternative approach, Brunskill et al. [5] targeted yellow fluorescent protein to mouse JG cells and used fluorescence-activated cell sorting (FACS) to enrich tagged cells for expression analysis. The resulting study proposed a 369 gene "signature" that characterizes mouse JG cells, although few of these have been verified in subsequent studies.

In an effort to extend these studies and to determine if the genes identified in the mouse also characterize human renin-producing cells, we have catalogued the gene expression pattern of biopsies derived from 4 human renin-producing tumors, or reninomas. Reninomas are rare, benign tumors arising from a proliferation of JG cells in the kidney cortex [6-7] and are often detected due to the appearance of fulminant hypertension in relatively young patients (mean age under 30 years) with no obvious explanatory risk factors. RNA sequencing (RNA-Seq) revealed a set of 54 highly expressed genes that are common to all four tumors and another 30 expressed in 3 of the 4 biopsies tested. *In situ* hybridization of mouse kidney sections subsequently confirmed JG cell expression of 44 of these genes, the vast majority of which have not previously been reported to be expressed in JG cells. Finally, hypothesizing that the highly expressed reninoma genes affect renin-synthesizing capacity, we selected 10 ligands (based on known relevance for blood pressure and kidney disease) and studied their effects on (pro)renin release by As4.1 cells. These cells are derived from a transgene-induced mouse kidney tumor, and do not store renin. They may thus be considered as de-differentiated JG cells which have lost their capacity to secrete lysosomes. Consequently, As4.1 cells might serve as a model to study the effect of reninoma-specific ligands on JG cell plasticity. Results revealed an unexpected suppressant role for platelet-derived growth factor B (PDGFB).

METHODS

Human and mouse studies

Total RNA was isolated using Trizol (Invitrogen) from four reninomas surgically obtained from four anonymous patients (Paris1, Paris2, Montreal, Rotterdam), and underwent RNA-sequencing analysis using the HiSeq2000 (Illumina). The top 100 most up-regulated genes presented in all four, and at least in three out of four reninomas were submitted to immunofluorescence and fluorescent *in situ* hybridization (iFISH) in order to analyze their expression in the juxtaglomerular apparatus of kidney mice under different situations (5 day old, 10-12 week old and 10-12 week old treated with

captopril for 7 days). For further details, see the Methods section in the online-only Data Supplement.

Cell culture studies

Human Embryonic Kidney (HEK) 293 cells were transfected with plasmids encoding ligands selected from the transcriptome analysis on four reninomas. The conditioned medium derived from these cells was used to study the effect of these ligands on (pro)renin-synthesizing As4.1 cells. For further details, see the Methods section in the online-only Data Supplement.

Statistical analysis

Results are expressed as mean±SEM. Data were analyzed for normal distribution using a Shapiro-Wilk's test ($P>0.05$). Differences were tested using one-way or two-way ANOVA, followed by Holm-Sidak's or Dunnett's multiple comparison test. $P<0.05$ was considered significant.

RESULTS

Deep sequencing of RNA (RNA-Seq) was performed on three biopsies of a first reninoma from Paris (Par1B1-B3), one biopsy from a reninoma from Montréal (Mon), two biopsies from a reninoma from Rotterdam (RotB1, B2), and a second reninoma from Paris (Par2) along with a biopsy from adjacent supposedly normal tissue from the same patient (Par2N) (Table S1). We obtained from 45-100 million reads per sample (Table 1) with comparable overall sample quality (Figure S1). Remarkably, the Fragments Per Kilobase of transcript per Million (FPKM) mapped reads values for renin were quite similar in the four tumor samples and, in each tumor, renin was expressed at 15-41 times the level of the next most abundant transcript, confirming the diagnosis of *bona fide* reninomas (Table 2).

Results obtained from the three independently analyzed biopsies of the Paris1 reninoma demonstrated an inter-sample Spearman correlation of >0.98 (Figure S2, red outlines) and similar results were obtained with the two biopsies of the reninoma from Rotterdam (Figure S2, green outlines) suggesting that the sample preparation and results obtained were reliable and reproducible. In the one case in which we had biopsies of both tumor and adjacent tissue from a single patient (Paris 2), there was a 217-fold increase in the number of renin reads in the reninoma versus the "normal" sample (Table 2) although the samples overall were very highly correlated (0.92 for all genes; see Figure S2, blue outlines). By limiting the gene set analyzed in all the samples to those that were expressed at least 4-fold higher in the reninoma versus the "normal" biopsies for Paris 2, we reduced the gene set to just over 1000 (Table S2) and this resulted in the removal of low reads (which are commonly unreliable) and many "housekeeping" genes from the analysis. Using this limited gene set, we identified the

100 most-expressed genes in each biopsy (Table S3). Re-calculation of the Spearman correlation using these highly expressed genes revealed a higher discrimination between samples (Figure S3). Although the repeat biopsies continued to exhibit a correlation >0.98, the correlation between the reninomas varied between a low of 0.46 (Par1 versus Par2) and a high of 0.74 (RotB1 and Par2). Surprisingly, the highest correlation is between the Par2 and Par2N samples (0.81, Figure S3), raising the possibility that the supposedly normal sample from this patient contains some reninoma. A Venn diagram reveals that 54 of the 100 most expressed genes were common to all 4 reninomas (Table S3 and Figure S4) and another 30 genes were highly expressed in 3 out of 4 of the tumors analyzed. Functional annotation clustering analysis (DAVID) did not reveal any statistically significant correlation for cellular function using the genes most commonly expressed in the reninomas (data not shown).

Table 1. Quality control of sequenced reads

Sample	Number of reads before trimming	Number of reads after trimming	% reads post-filtering	Exonic alignment rate
Par1B1	88058080	83611860	94.95	0.811
Par1B2	88713782	84187709	94.90	0.803
Par1B3	74683086	71782557	96.12	0.813
Mon	76131363	73212125	96.17	0.760
Rot1B1	117331030	109192033	93.06	0.753
Rot1B2	95468148	88616034	92.82	0.766
Par2	55053372	47536436	86.35	0.766
Par2N	117480551	101148769	86.10	0.647

Table 2. Ratio of the number of renin reads (FPKM) obtained in the various biopsies versus those for the next most abundant transcript. Also shown is the ratio of renin reads obtained between the putative tumor and normal adjacent tissue (Par2N) biopsies for the Par2 samples. Data are drawn from Table S1.

Sample	Par1 (ave.)	Mon	Rot (ave.)	Par2	Par2N	Par2/Par2N
Ren FPKM	74372	110034	110062	117805	541	217
2nd transcript (FPKM)	GNAS (2218)	GNAS (2652)	KISS1 (6830)	KISS1 (8110)		
Ratio	**33.53**	**41.49**	**16.11**	**14.53**		

To test if these reninoma-enriched genes were also expressed in JG cells, we performed iFISH for 71 of the above 84 common reninoma transcripts (excluding, among others, genes that have no mouse equivalent) in kidney sections from C57bl/6 mice using the equivalent mouse probes (Table 3 and Figure S5). As expected, combined *in situ* hybridization and immunofluorescence for renin reveals perfect co-localization in the JG cells of adult mice (Figure 1A-1D). Furthermore, treatment of the mice

with captopril for one week resulted in the expected recruitment of JG cells along the afferent arterioles (Figure 1E), while co-localization was also observed throughout the maturing arterioles of 5 day-old mice (Figure 1F). These results confirm the validity of this approach to assess the co-localization of the transcripts from the reninoma in the mouse JG cells.

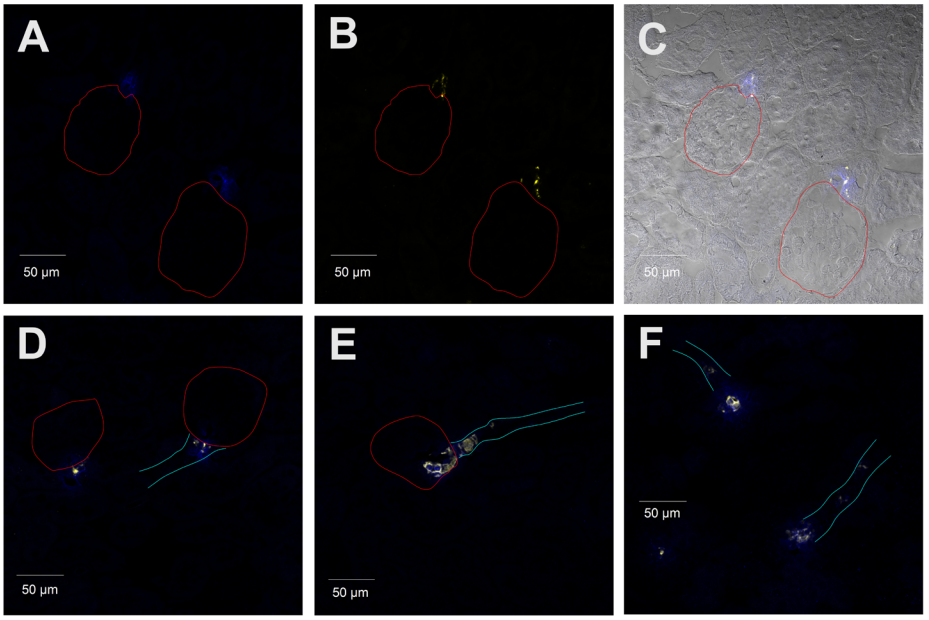

Figure 1. iFISH for renin in mouse kidney. Where possible, glomeruli are outlined in red and vessels in light blue. (A) Immunofluorescence for renin in adult mouse kidney is shown in blue; (B) In situ hybridization for renin in the same section is shown in yellow; (C) merged image from panels a and b, including phase contrast microscopy showing that renin is expressed at the base of the glomeruli as expected; (D) iFISH overlay for renin in kidneys from adult; (E) adult mouse treated for one week with captopril, and (F) 5 day-old mouse. Original magnification X 20. Scale bar=50µm.

In order to obtain a semi-quantitative comparison of gene expression levels in renin-producing cells, all confocal microscopy images were taken using the same parameters and a ranking from 0 (for no expression observed) to 4 (highly expressed) was assigned for each gene under each condition tested (Table 3). Among the 71 genes tested, 44 genes are expressed in both reninomas and mouse JG cells. Twenty-four of these were detected in all three conditions (5 day-old, adult and adult treated with captopril kidneys) while the remainder showed preferential expression in one or more of the conditions (Figure S5 and Table 3). Functional annotation clustering analysis using the 44 genes expressed in both the reninomas and mouse JG cells identified "developmental processes" as the most likely gene ontology (data not shown).

Table 3. Summary of the iFISH analysis on mouse kidney for the genes most highly expressed in the reninomas. The genes found to express in renin-producing cells (RPC) are listed in their order of expression in the reninomas from highest to lowest (see Table S1) and their relative expression in each sample is estimated on a scale of 0 (no detectable expression) to 4 (highest expression), based upon the data in Figure S5. *Described as part of a 369 gene list that makes up a renin-producing cell signature, according to Brunskill et al.5; #Reported as reninoma marker. Of the genes confirmed by iFISH, 36 have not been reported before in relationship to renin-producing cells.

Gene symbol	Gene ID	Expression in mouse renin-producing cells (iFISH)			Notes
		Developing Kidney	Adult Kidney	Adult Kidney + Captopril	
PRESENT IN ALL 4 RENINOMAS					
REN*#	Renin	4	2	4	
KISS1	KiSS-1 metastasis-suppressor	1	0	2	
GNAS	GNAS complex locus	4	1	4	
A2M	alpha-2-macroglobulin	3	0	0	
PTP4A3*	protein tyrosine phosphatase type IVA, member 3	1	0	4	
SPARCL1	SPARC-like 1 (hevin)	4	4	4	
C4A	complement component 4A (Rodgers blood group)	0	1	2	
VIM#	vimentin	4	2	4	also in mesangial cells and arterioles
MYL9	myosin, light chain 9, regulatory	4	3	3	strongest in developing arterioles
NOTCH3	Notch homolog 3 (Drosophila)	1	1	2	
MFGE8	milk fat globule-EGF factor 8 protein	2	2	4	
PDGFRβ	platelet-derived growth factor receptor, beta polypeptide	3	2	2	

SPARC	secreted protein, acidic, cysteine-rich (osteonectin)	2	2	0	mostly in cells adjacent to JG
JAG1	jagged 1 (Alagille syndrome)	0	1	1	mostly in arterioles adjacent to JG
SERPINI1*	serpin peptidase inhibitor, clade I (neuroserpin), member 1	0	0	1	
TBC1D1	TBC1 (tre-2/USP6, BUB2, cdc16) domain family, member 1	0	0	1	
CXCL12	chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	3	0	1	mostly in cells adjacent to RPC in developing arterioles
TPM2	tropomyosin 2 (beta)	1	0	1	
SLIT3	slit homolog 3 (Drosophila)	3	0	1	only in some RPC 5 day and recruited JG capto (faint)?
ITGA7	integrin, alpha 7	0	0	2	
GJA4	gap junction protein, alpha 4, 37kDa	1	0	2	
DBNDD2	dysbindin (dystrobrevin binding protein 1) domain containing 2	3	2	2	
FLNA	filamin A, alpha (actin binding protein 280)	3	2	4	
ISYNA1	inositol-3-phosphate synthase 1	1	1	2	
CALD1	caldesmon 1	4	2	4	also mesangial cells?
ADCY3	adenylate cyclase 3	1	1	0	
MXRA8	matrix-remodelling associated 8	1	1	2	

OAZ2	ornithine decarboxylase antizyme 2	2	2	3	
TIMP1	TIMP metallopeptidase inhibitor 1	0	0	1	
TINAGL1	tubulointerstitial nephritis antigen-like 1	1	2	4	
LBH	limb bud and heart development homolog (mouse)	1	1	4	
NRARP	NOTCH-regulated ankyrin repeat protein	0	1	1	
IGF2	insulin-like growth factor 2 (somatomedin A)	0	0	0	
IFITM3	interferon induced transmembrane protein 3 (1-8U)	0	0	0	
SFRP4	secreted frizzled-related protein 4	0	0	0	
CSRP2	cysteine and glycine-rich protein 2	0	0	0	
IFITM1	interferon induced transmembrane protein 1 (9-27)	0	0	0	
COL6A2	collagen, type VI, alpha 2	0	0	0	
CPE	carboxypeptidase E	0	0	0	
LGALS1	lectin, galactoside-binding, soluble, 1	0	0	0	
TGM2	transglutaminase 2 (C polypeptide, protein-glutamine-gamma-glutamyltransferase)	0	0	0	

CBLN4	cerebellin 4 precursor	0	0	0	
SRGN	serglycin	0	0	0	
TBX2	T-box 2	0	0	0	
COL6A1	collagen, type VI, alpha 1	0	0	0	in extraglomerular cells surrounding JG
NT5DC2	5′-nucleotidase domain containing 2	0	0	0	weak expression in cells adjacent to JG
TPM1	tropomyosin 1 (alpha)	0	0	0	
GPR124	G protein-coupled receptor 124	0	0	0	
HSPB6	heat shock protein, alpha-crystallin-related, B6	0	0	0	
CD34#	CD34 molecule	0	0	0	
HCFC1R1	host cell factor C1 regulator 1 (XPO1 dependent)	0	0	0	
PLEKHH3	pleckstrin homology domain containing, family H (with MyTH4 domain) member 3	0	0	0	
PRESENT IN 3 OUT OF 4 RENINOMAS					
MDK	midkine (neurite growth-promoting factor 2)	1	1	4	
CPA3	carboxypeptidase A3 (mast cell)	1	2	0	
RGS5*	regulator of G-protein signaling 5	4	4	2	
ACTA2	actin, alpha 2, smooth muscle, aorta	4	0	2	mostly in VSMC in adult
CD248	CD248 molecule, endosialin	3	2	4	

IFITM2*	interferon induced transmembrane protein 2 (1-8D)	0	0	1	
TUBA1A	tubulin, alpha 1a	2	3	4	
BGN	biglycan	1	2	4	
ATP1B2*	ATPase, Na+/K+ transporting, beta 2 polypeptide	2	2	2	
MCAM	melanoma cell adhesion molecule	2	2	3	
TAGLN	transgelin	3	2	3	also in adjacent arterioles
CRIP1*	cysteine-rich protein 1 (intestinal)	0	1	4	
MEF2C	myocyte enhancer factor 2C	0	0	0	
TGFBI	transforming growth factor, beta-induced, 68kDa	0	0	0	
TPSB2	tryptase alpha/beta 1; tryptase beta 2	0	0	0	
NPPC	natriuretic peptide precursor C	0	0	0	
GPC1	glypican 1	0	0	0	
FKBP10	FK506 binding protein 10, 65 kDa	0	0	0	
FXYD1*	FXYD domain containing ion transport regulator 1	0	0	0	

From the top 100 highly expressed genes, focusing on blood pressure regulation and renal disease, we selected 10 genes coding for ligands (FSTL1, IGF2, LGALS1, MDK, MFGE8, NPPC, PDGFB, SPARC, SPARCL1, WFDC2) in order to investigate their potential role in JG recruitment. The corresponding mouse cDNAs were cloned with a c-myc epitope and transfected into HEK293 cells to obtain high concentrations of these ligands in the supernatant of the culture medium. WB experiments confirmed the presence of the proteins of each of the transfected genes in the HEK293 cell me-

dium at 48 hours after transfection (Figure S6). Since all ligands were tagged with a c-myc epitope, the same primary antibody could be used.

After adding the 48-hour conditioned HEK293 medium (containing the highest level of the desired protein) to (pro)renin-producing As4.1 cells for 2 days, we studied As4.1 cell morphology and quantified (pro)renin in the medium. Only PDGFB altered As4.1 cell morphology, in that the cells after exposure to this ligand displayed a more elongated densely packed and aligned shape (Figure S7). Moreover, PDGFB also induced parallel decreases in the medium and cellular (pro)renin levels, by 64±5% and 53±10%, respectively (n=11), although only the former was significant (P=0.03; Figure 2A). Under control conditions, the medium contained predominantly (>95%) prorenin. In contrast, the cell lysate contained renin only, at levels corresponding to <1% of the total amount of renin+prorenin in the medium. qPCR experiments revealed that the reduction in (pro)renin also occurred at the expression level, decreasing its level by 84±5% (P=0.005, n=5; Figure 2A). Furthermore, when applying commercially obtained PDGFB directly to As4.1 cells, it lowered (pro)renin concentration-dependently (Figure 2B), and from these data, it could be estimated that the PDGFB level in the conditioned HEK293 medium was >100 ng/mL. MTT assays confirmed that the effect of PDGFB was not due to a reduction in cell viability (Figure 2B). Moreover, PDGFB did not affect total protein content, suggesting that its effect was also not due to a decrease in cell number (data not shown).

Finally, we evaluated the effect of PDGFB-containing HEK293 medium on the expression of markers that illustrate the occurrence of a smooth muscle, endocrine and/or immunological phenotype, i.e., aldo-keto reductase family 1, member B7 (Akr1b7), α-smooth muscle actin (Acta2), interleukin-6 (IL-6), and Recombination signal binding protein for immunoglobulin kappa j (Rbpj). Figure 2C illustrates that PDGFB upregulated IL-6 (P<0.0001, n=4), and downregulated Acta2 (P=0.01, n=6) in As4.1 cells. Non-significant increases in Akr1b7 (P=0.06, n=4) and Rbpj (P=0.06, n=5) expression were also observed.

DISCUSSION

The aim of this study was to gain insight into human JG cell biology. Given their rapid de-differentiation in culture, such knowledge is currently lacking. Instead, information on renin synthesis is largely derived from renin-producing tumor-derived cell lines, like As4.1 (mouse), Calu-6 (human) and human mastocytoma cells (HMC-1). In an attempt to catalogue the genes expressed in JG cells, Brunskill et al. [5] used a fluorescent reporter mouse model, in which they have used cell sorting to enrich for gene-tagged renin-expressing cells in the mouse kidney. Comparison of gene expression arrays from the sorted cells versus the whole kidney led to the identification of 369 core genes whose expression was proposed to constitute a "gene signature" for the mouse JG cell. In the current study, we have used direct RNA sequencing (RNA-Seq) of human reninomas to independently quantify gene expression levels in each

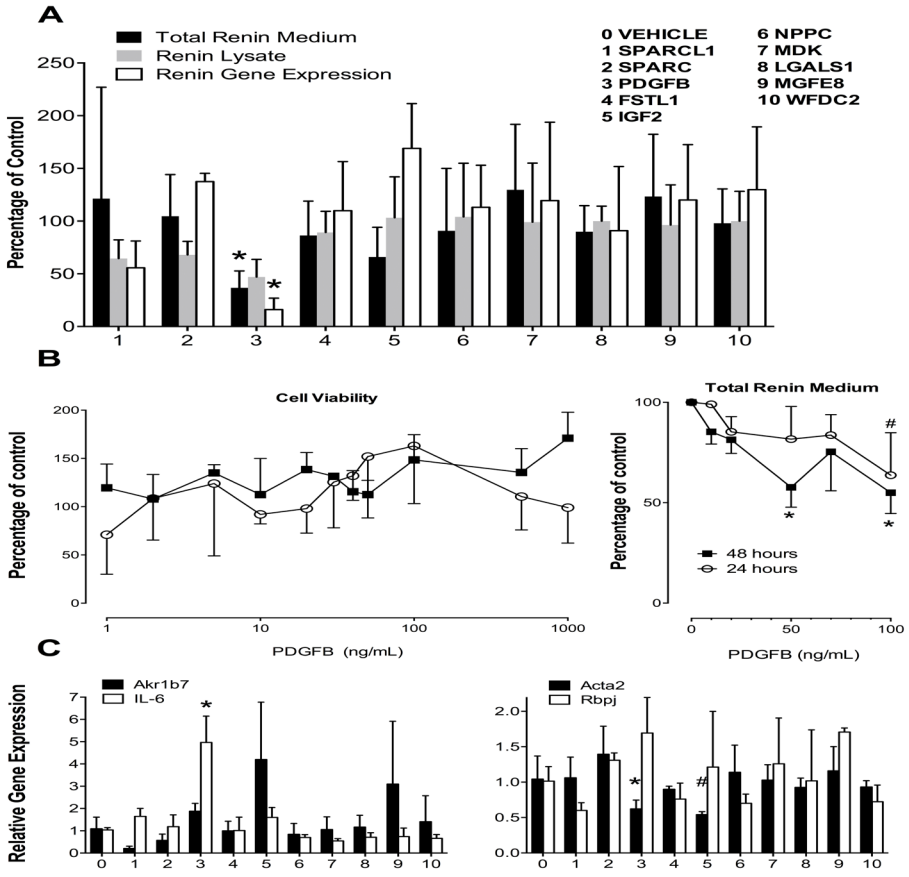

Figure 2. A) Total renin (i.e., renin + prorenin) levels in the medium, and renin levels in the cell lysate obtained from As4.1 cells incubated for 48 hours with medium obtained from HEK293 cells transfected with 10 genes expressing ligands that were upregulated in the 4 reninomas. Renin gene expression is also provided. Data are mean ±SEM of 5-11 experiments and have been expressed as a percentage of the levels in cells exposed to medium of control-transfected cells, i.e., cells treated with the transfection mix, but without cDNA. *P<0.01. B) Cell viability and total renin levels in the medium of As4.1 cells exposed to PDGFB for 24 or 48 hours. Data are mean ±SEM of 3 experiments and have been expressed as a percentage of the levels in cells not exposed to PDGFB ('vehicle'=0). *P<0.01, #P<0.05 vs. 100%. C) Aldo-keto reductase family 1, member B7 (Akr1b7), α-smooth muscle actin (Acta2), interleukin-6 (IL-6), and recombination signal binding protein for immunoglobulin kappa j (Rbpj) expression in As4.1 cells incubated for 48 hours with medium obtained from HEK293 cells transfected with 10 genes expressing ligands that were upregulated in the 4 reninomas. Data are mean ±SEM of 4-6 experiments and have been expressed as fold change versus the levels in cells exposed to medium of control-transfected cells, i.e., cells treated with the transfection mix, but without cDNA ('vehicle'). *P<0.01, #P<0.05 vs. 1.0.

of the biopsies we obtained, and to test for correlation of the gene expression patterns between mouse and human renin-expressing cells. This approach has the additional advantage that a control tissue to which expression can be compared was not required. This was an important consideration since our samples were derived from frozen pathology specimens, and only one of them had a corresponding biopsy of adjacent, presumed non-tumor, tissue. The high reproducibility and sequence "depth" achieved confirm that this approach is valid, robust and applicable to archival samples and the comparison of the reninoma transcriptomes allowed us to derive a gene expression "fingerprint" for the tumors. In addition, the high expression of renin, CD34, and vimentin, all of which have been previously reported as markers of reninomas, [7-8] confirms the diagnosis of bona fide reninoma in all of the tumors.

At least 3 of the 4 reninomas expressed genes at relatively high levels that were also reported to be highly expressed in mouse renin-expressing cells [5] including, in addition to renin, *RGS5, CRIP1*, and *ATP1B2*. Conversely, a number of the most highly expressed transcripts previously identified in the mouse JG cell preparations were not detected in the 100 most-expressed genes in the reninomas including *Syne2, Plac9, Myh11, Jph2, Myo18, Akr1b7* and *Mgp*. Interestingly, *Akr1b7*, a member of the alpha-keto reductase gene family was previously found to be highly enriched in mouse JG cell preparations and to serve as a specific marker for JG cells in the mouse kidney [5]. Even though *Akr1b7* does not have a homologue in humans, its closest human analogues, *AKR1B10* and *AKR1B15*, are not expressed at detectable levels in the reninomas (data not shown). Since more recent results have shown that targeted deletion of *Akr1b7* in mouse JG cells does not affect renin production [9] it is possible that the expression of *Akr1b7* in mouse JG cells is unrelated to renin synthesis.

Indeed, of the extended list of 369 core genes previously reported to be enriched in the mouse renin cell preparations [5] only 8 were among the 84 genes expressed in at least 3 of the 4 reninomas (Table 3 and Table S3). Although the exact reason for these differences is unclear, they could be due to several factors. First, some of the highly-expressed transcripts in the reninomas might be related to the tumoral transformation of the renin-producing cells. For example, the expression of neither CD34, a commonly reported marker of reninomas and myelo-lymphopoetic precursors, nor insulin-like growth factor 2 (IGF2) was detected in the mouse JG cells. IGF2 also did not affect (pro)renin release by As4.1 cells. Alternatively, the differences could be due to the fact that in both the current study and that of Brunskill et al. [5], the gene expression patterns were obtained from impure populations of renin-producing cells and could reflect expression of a gene in a contaminating non-JG cell. Finally, they could reflect true differences between mouse and humans.

To overcome some of these limitations, we used iFISH to test whether the commonly expressed genes in the reninomas were also expressed in mouse JG cells (Figure S5). Even though the sensitivity of this analysis is limited by the level of expression of the candidate genes, it nevertheless confirmed that 44 of the gene transcripts expressed in the reninomas were also detected in mouse JG cells (Table 3) and thereby significantly extends the list of potential markers for this unique cell type.

Previous studies have reported that JG cells express contractile proteins, are related to pericytes [5] and, like pericytes, are derived from FoxD1-expressing lineages [10]. Simultaneously, renal CD44(+) mesenchymal stem cell-like cells, whose differentiation is triggered by LXR stimulation [11] have been suggested to differentiate into JG-like renin-expressing cells [12]. The current analysis confirmed the expression of a number of contractile proteins in both reninomas and JG cells, including myosin light chain 9 (MYL9), tropomyosin 2 (TPM2), transgelin (TAGLN), myocyte enhancer factor 2C (MEF2C), caldesmon (CALD1) and very low levels of ACTA2, as well as some pericyte markers including RGS5 and osteonectin (SPARC). We also confirmed the expression of vimentin (VIM), a marker of mesenchyme-derived cells, in both the reninomas and JG cells, but did not detect expression of either LXR receptor or *CD44* in the tumors.

Our data also call into question the model that "recruited" JG cells (e.g., after captopril treatment) represent a return to the embryonic stage of renin-producing cells. The *ACTA2* gene codes for a-smooth muscle actin, a marker for smooth muscle cells. Consistent with what has been previously reported [13] there is a demarcation in *Acta2* expression with the expected higher expression in smooth muscle cells of the afferent arteriole and very low expression in adjacent JG cells of adult mice (Figure 3A). This demarcation persists after recruitment of new JG cells following captopril treatment (Figure 3B). In contrast, there appear to be 2 populations of renin-expressing cells in the developing kidney: one population that exists in clusters similar to the JG apparatus in adult kidney (Figure 3C, arrow) and which express only very low levels of *ACTA2* and others which line the vessels of the developing kidney and exhibit significant overlap of renin staining and *ACTA2* expression (Figure 3C, light blue outline). Other genes whose expression was detected almost exclusively in renin-producing cells of the developing kidney include *A2m, Slit3* and *Cxcl12*. Conversely, a number of genes appeared to be preferentially expressed in the JG cells of adult control and captopril-treated mice including *Ptp4a3, C4a, Tinagl1, Lbh, Mdk, Bgn* and *Crip1*. Taken together, these results suggest that JG recruitment does not simply involve a return to the embryonic renin-producing cell, but may rather reflect differentiation of smooth muscle to either an intermediate or alternative phenotype.

KISS1, which codes for a metastasis suppressor and is the second to third most abundant transcript in each of the four of the reninomas (Table 3), also presents a major difference in expression in the reninomas and mouse kidney. The *KISS1* gene is located immediately adjacent to the renin gene on chromosome 1 in both mice and humans (http://www.ncbi.nlm.nih.gov/gene/3814). When large chromosomal fragments containing both genes are used to generate transgenic mice, coordinate expression of renin and *KISS1* is detected in brain, kidney, lung and placenta [14]. While we detected some expression of *Kiss1* in the JG cells of captopril-treated mice (Figure S5), we did not detect its expression in adult mouse JG cells, raising the possibility that the neighboring *Kiss1* gene is expressed only in conditions of very high renin gene expression. Whether it explains the fact that intra-renal reninomas rarely metastasize is currently unknown.

Also among the most highly expressed genes in all 4 reninomas is *DBNDD2*

(Table S3), a paralog of the gene *DTNBP1*, which is a component of the BLOC-1 complex that is required for normal biogenesis of lysosome-related organelles (LRO), such as platelet dense granules and melanosomes. Thus, *DBNDD2* might be an interesting candidate to explain the high content of LROs found in the JG cells.

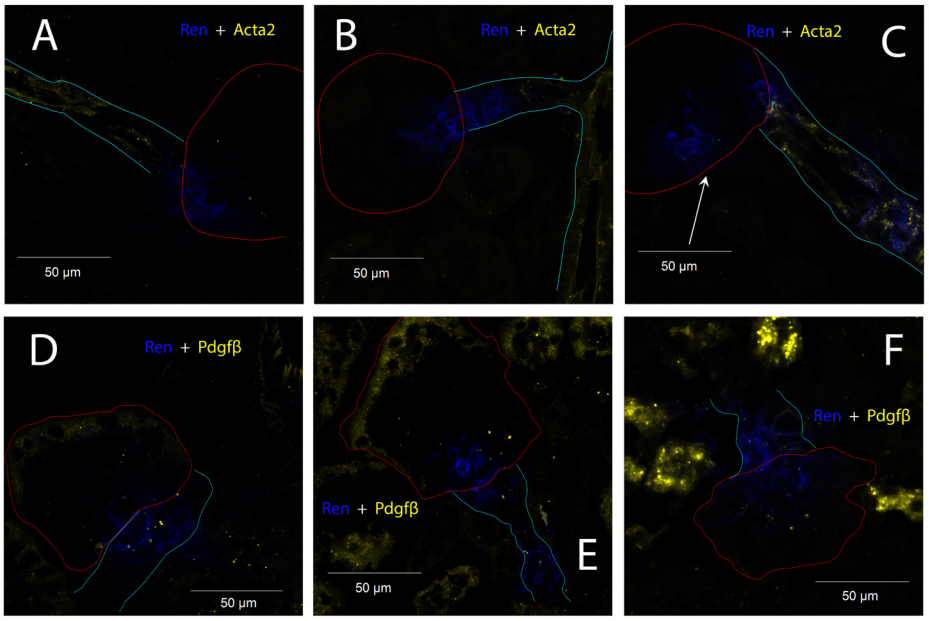

Figure 3. iFISH showing renin immunofluorescence in blue and in situ hybridization for Acta2 or Pdgfrβ in yellow in kidneys from adult (A, D), captopril-treated (B, E), 5 day-old (C, F) mice. Note the existence of 2 types of renin-producing cells in the 5 day-old mouse: those that co-express Acta2 (outlined in the vessel in light blue) and those that don't (arrow). Original magnification X 40. Scale bar=50µm.

A number of ligands and receptors were also found to be highly expressed in the reninomas and in mouse renin-producing cells, some of which might play a role in either the maintenance of the JG cell phenotype or in JG cell recruitment. Of particular interest is the high level of expression of the Notch3 receptor and PDGF receptor ß (*PDGFR*ß) in the reninomas and JG cells. Indeed, RBPJ, the main effector involved in Notch receptor signaling, activates the myo-endocrine program of the JG cell, and conditional deletion of *Rbpj* in renin-producing cells results in a dramatic decrease in the number of JG cells seen in adult mice [15]. Yet, in humans mutations in *NOTCH3* have been identified as the underlying cause of cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), with no known effects on renin. In the current study we observed that PDGFB suppressed (pro)renin synthesis in As4.1 cells, and altered their morphology. The concomitant suppression

of Acta2 and upregulation of IL-6 suggests an As4.1 phenotype change from myo-endocrine to inflammatory. IL-6 upregulation by PDGFB is well-known, and occurs in a RBPJ-independent manner [16]. No significant alterations were observed in Akr1b7 and Rbpj, nor did PDGFB affect cell viability. Classically, endothelium-derived PDGFB recruits pericytes and promotes VSMC proliferation [17]. Importantly, pericytes in the human kidney were recently reported to produce renin [18]. Hypoxia transforms renin-producing cells into erythropoietin-producing cells, [19] and erythropoietin-expressing cells are assumed to be derived from capillary pericytes [20]. Finally, following severe mesangial injury, renin-positive precursor cells moved into intraglomerular sites and differentiated into (renin-negative), PDGFRß-expressing mesangial cells [21]. As shown in Figure 3 (panels D-F), *PDGFR*ß modestly overlapped with renin in kidneys of adult, young, and captopril-treated adult mice, in addition to its well-known expression in interstitial cells. Moreover, Rider et al. observed that renin cells in the zebrafish kidney express both Acta2 and Pdgfrβ [22]. Given the well-known role of endothelial PDGFB as a facilitator of mesangial cell proliferation and migration, an additional effect of PDGFB might now be suppression of renin expression. Here it should be mentioned that neither conditional deletion of the PDGFRß in renin-producing cells nor deletion of endothelial PDGFB production affected the normal development of renal renin-expressing cells [23]. This may not be too surprising, given the fact that PDGFB actually is a negative regulator of renin expression, possibly coming into play only under pathological conditions.

Besides the high level of *PDGFR*ß expression in the reninomas and the JG cells, both also express the genes for SPARC, SPARCL1 (SPARC-like 1 or hevin) and MFGE8 (lactadherin) at relatively high levels. It has been reported that SPARC can both prevent the binding of PDGF to its receptor [24] and potentiate its profibrotic effects [23], while lactadherin potentiates PDGFRß signaling [25]. This raises the possibility that a more complex interplay between SPARC, SPARCL1, MFGE8 and PDGF regulates either the recruitment or other physiological responses of JG cells in their natural setting. In agreement with this concept, the current study revealed no effect of either SPARC, SPARCL1 or MGE8 alone on (pro)renin synthesis by As4.1 cells. Additionally, ligands that are known to be involved in blood pressure regulation (NPPC, or natriuretic peptide precursor C), diabetic nephropathy (LGALS1 or galectin-1) [26], nephrogenesis (MDK or midkine) [27], renal fibrosis (WFDC2, or WAP four-disulfide core domain protein 2) [28], and renal injury prevention (FSTL1, or follistatin-related protein 1) [29] also did not directly affect (pro)renin synthesis by As4.1 cells.

Somewhat surprisingly, no proteases were included in the 100 most expressed genes in the reninomas (Table S3) where we might have expected to find one or more candidate prorenin processing enzymes (PPE) necessary for proteolytic activation of the renin secreted by these tumors. However, examination of the gene lists before subtraction of the genes expressed in the Par2N "normal" biopsy (Table S1) reveals that the rank of Cathepsin B (*CTSB*) expression varies from the 8th (Par1B1) to the 78th (Par2) position in the reninomas, although these expression levels did not appear to correlate with the apparent renin expression levels in each sample (not shown). However, be-

cause *CTSB* was only expressed 1.1-fold more in the Par2 reninoma than in the Par2N control kidney biopsy, it was eliminated from our gene list. Nevertheless, because CTSB has been co-localized with renin in JG cells secretory granules [30] and shown to accurately process human prorenin in vitro [31], it is quite possible that it fulfills the role of the PPE in the reninomas, even though it is a ubiquitously distributed protease. This analysis also reveals one of the potential shortcomings of our approach since by focusing on genes whose expression was consistently enriched in tumor biopsies we may have eliminated consideration of genes that play an important functional role in the biology of the JG cell, but whose expression is more widely distributed.

The present study is the first to use a reninoma transcriptome analysis as an approach to understand human JG cell biology. It has yielded multiple new candidates that might play a role in the developmental plasticity of these cells and, among them, raises the PDGFB-PDGFRβ signaling pathway as a promising novel pathway that controls renin-expressing cells.

PERSPECTIVES

By comparing the transcriptomes of 4 reninomas and subsequently mapping the expression patterns of the most highly expressed genes in mouse kidney during development, adulthood and following renin-producing cell recruitment in response ACE inhibition, we have confirmed the expression of 36 new genes (Table 3) in this unique cell type. As a result, we propose the PDGFB-PDGFRβ signaling pathway as a promising novel pathway that controls the developmental plasticity of JG cells.

ACKNOWLEDGMENTS

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Chapter 3

THE BRAIN RENIN-ANGIOTENSIN SYSTEM: DOES IT EXIST?

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ABSTRACT
Due to the presence of the blood-brain barrier, brain RAS activity should depend on local (pro)renin synthesis. Indeed, an intracellular form of renin has been described in the brain, but whether it displays angiotensin I-generating activity (AGA) is unknown. Here, we quantified brain (pro)renin, before and after buffer perfusion of the brain, in wild-type mice, renin knockout mice, DOCA-salt-treated mice, and angiotensin II-infused mice. Brain regions were homogenized and incubated with excess angiotensinogen to detect AGA, before and after prorenin activation, using a renin inhibitor to correct for non-renin-mediated AGA. Renin-dependent AGA was readily detectable in brain regions, the highest AGA being present in brainstem (>thalamus=cerebellum=striatum=midbrain>hippocampuscortex). Brain AGA increased marginally after prorenin activation, suggesting that brain prorenin is low. Buffer perfusion reduced AGA in all brain areas by >60%. Plasma renin (per mL) was 40-800x higher than brain renin (per g). Renin was undetectable in plasma and brain of renin knockout mice. DOCA-salt and angiotensin II suppressed plasma renin and brain renin in parallel, without upregulating brain prorenin. Finally, angiotensin I was undetectable in brains of spontaneously hypertensive rats, while their brain/plasma angiotensin II concentration ratio decreased by 80% after AT_1 receptor blockade. In conclusion, brain renin levels (per g) correspond with the amount of renin present in 1-20 μL plasma. Brain renin disappears after buffer perfusion, and varies in association with plasma renin. This indicates that brain renin represents trapped plasma renin. Brain angiotensin II represents angiotensin II taken up from blood rather than locally synthesized angiotensin II.

INTRODUCTION

Since the discovery of renin in the brain nearly 50 years ago [1], numerous studies have proposed that a so-called brain renin-angiotensin system (RAS) exists. Given the presence of the blood-brain barrier, brain RAS activity should depend on the local synthesis of renin or prorenin (together denoted as (pro)renin) in the brain rather than uptake from blood. In support of this concept, an intracellular, non-secreted form of renin (icREN) has been shown to occur exclusively in the brain. This renin isoform is derived from an alternative transcript of the renin gene, lacking the signal peptide and part of the prosegment [2-3]. To what degree this truncated prorenin truly generates angiotensin (Ang) I remains elusive. Lee-Kirsch et al. detected low Ang I-generating activity (AGA) levels in cell lysates of AtT20 cells transfected with icREN during incubation with excess angiotensinogen, but failed to demonstrate to what degree this AGA was renin-mediated (e.g., by making use of a renin inhibitor). Peters et al. showed increased AGA in cardiac homogenates of transgenic rats overexpressing icREN and were able to block this with the renin inhibitor CH732 [4]. Yet, unexpectedly, the AGA increase was observed only after prosegment removal with trypsin, in disagreement with the fact that truncated prorenin does not require prosegment removal to display activity [5]. Moreover, icREN overexpression in the heart, if anything, resulted in effects that were unrelated to angiotensin formation [6].

DOCA-salt treatment is widely believed to stimulate brain RAS activity. Confusingly, it lowers icREN expression, but increases the expression of the classical, secreted form of renin in brain tissue (sREN) [7], possibly because, icREN, via an unknown mechanism, inhibits sREN expression [8]. Li et al. proposed that DOCA-salt selectively increases brain prorenin, which, in the absence of a prosegment-cleaving enzyme in the brain, requires interaction with the (pro)renin receptor to allow Ang I generation locally [9-10]. The underlying assumption of this concept is that prorenin binding to the (pro)renin receptor results in a conformational change in the prorenin molecule, allowing it to display enzymatic activity without prosegment cleavage [11]. Yet, the low (nanomolar) affinity of the (pro)renin receptor implies that very high prorenin levels are required for receptor binding [11], for which there currently is no evidence [10].

Given these uncertainties, in the present study we set out to re-evaluate the occurrence of (pro)renin in the brain. We quantified brain (pro)renin in a wide variety of brain regions, derived from control mice, mice exposed to DOCA-salt or Ang II, and renin-deficient mice. Under all conditions, a comparison was made with plasma (pro) renin, and the renin inhibitor aliskiren was applied in the assay to evaluate whether AGA was truly renin-mediated. Mice were studied given the fact that their (pro)renin levels are several orders of magnitude higher than those in humans or rats, thereby facilitating the detection of renin-dependent AGA, even in areas with low (pro)renin levels. To obtain a more complete understanding of the brain RAS, we also quantified brain angiotensinogen, and we studied the changes in brain angiotensin generation making use of brainstem tissue obtained from control spontaneously hypertensive rats (SHR) and SHR treated with the Ang II type 1 (AT_1) receptor blocker (ARB) olmesartan or the ACE inhibitor lisinopril for 4 weeks.

METHODS

Mouse and rat studies

Renin, prorenin and angiotensinogen were measured in plasma and brain regions (cerebellum, brainstem, cortex, hippocampus, midbrain, striatum and thalamus) obtained from wild-type (WT) mice, mice treated with deoxycorticosterone acetate (DOCA)-salt or angiotensin II, and renin-deficient mice ($Ren1c^{-/-}$), either without and with transcardial perfusion with PBS to wash away blood from the brain vasculature. Renin expression in brain tissue was also explored by using Ingenuity Pathway Analysis (IPA), and angiotensinogen synthesis was additionally studied in rat primary cortical astrocytes. Angiotensins were measured in plasma and brainstem tissue obtained from SHR treated with vehicle, lisinopril or olmesartan. For further details, see the Methods section in the online-only Data Supplement.

Statistical analysis

Data are expressed as mean±SEM. Univariate linear associations between plasma and brain renin levels were assessed by calculation of Pearson's coefficient of correlation. Differences between groups were evaluated by Student's t-test or ANOVA, and corrected for multiple testing by post-hoc Bonferroni analysis when needed. $P<0.05$ was considered significant.

RESULTS

Aliskiren inhibits angiotensin I-generating activity (AGA) in the mouse brain

Aliskiren identically inhibited AGA in mouse plasma (n=2), mouse kidney homogenate (n=2) and mouse brain homogenate (n=3) (Figure 1). Its IC_{50} was in the nanomolar range, as has been reported before for mouse renin [12-13]. These data suggest that AGA in mouse brain homogenates is due to renin. All subsequent AGA measurements were performed both in the absence and presence of 10 μmol/L aliskiren in order to correct for non-renin (i.e., non-aliskiren-inhibitable) AGA.

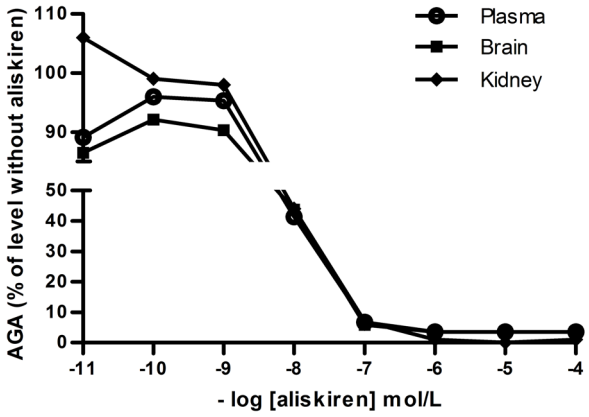

Figure 1. Concentration-dependent inhibition of angiotensin I-generating activity (AGA) by aliskiren in mouse plasma (n=2), mouse kidney homogenate (n=2) and mouse brain homogenate (n=3, representing pooled brainstem, cortex and midbrain regions, respectively, from 3-4 mice each).

Buffer perfusion reduces mouse brain renin by >60%

Renin-dependent (i.e., aliskiren-inhibitable) AGA was readily detectable in brain regions, the highest AGA being present in brainstem (>thalamus =cerebellum =striatum =midbrain >hippocampus =cortex) (Figure 2, n=5/group). AGA increased in each individual brain region after prorenin activation, but only when analyzing all brain regions together by multivariate ANOVA did this increase reach significance ($P<0.05$). Applying the prorenin activation procedure to 3 mouse brain homogenates (cortex,

midbrain and brainstem, respectively) to which recombinant human prorenin had been added yielded values in a renin IRMA (211±12 pg/mL) that were similar to those when activating the same amount of recombinant human prorenin in buffer with aliskiren [14] (169±6 pg/mL). This confirms that our prorenin activation procedure was appropriate. PBS perfusion of the mouse brain reduced AGA in all brain areas by >60% (Figure 2; P<0.01), and diminished the percentage of AGA that could be blocked by aliskiren (Table S2). These data suggest that blood removal predominantly washes away renin, but not non-renin enzymes that are also capable of reacting with angiotensinogen. Plasma renin (expressed per mL plasma) was 40-800x higher than brain renin (expressed per g tissue), and, as expected, identical in mice that were exposed to buffer perfusion. Plasma prorenin levels were of the same order of magnitude as plasma renin levels, as demonstrated earlier in mice [15].

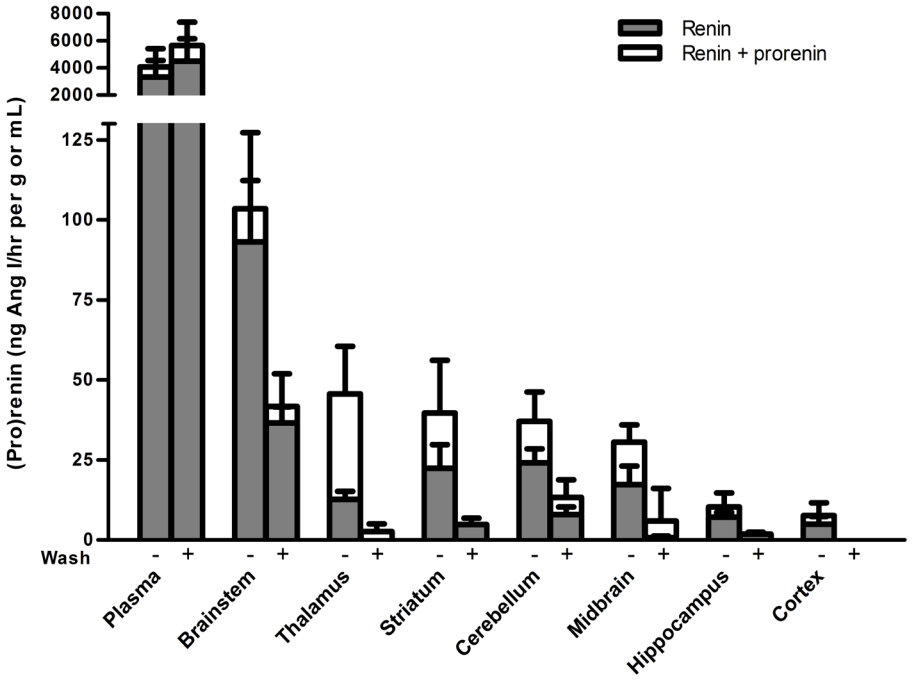

Figure 2. Renin and total renin (=renin + prorenin) levels in plasma and brain regions of mice before and after buffer perfusion (wash) of the brain. Data are mean±SEM of n=5. Multivariate ANOVA showed that total renin levels were higher than renin levels (P<0.05), and that buffer perfusion reduced renin by >60% in all regions (P<0.01).

Comparable reductions in brain and plasma (pro)renin after DOCA-salt treatment, angiotensin II infusion and renin-deficiency

DOCA-salt (n=6) and Ang II (n=7) suppressed plasma renin versus WT (n=6), and parallel decreases were observed for brainstem, midbrain and cortex renin (Figure 3), although significance was not reached in all cases. Nevertheless, brain renin levels (expressed per g tissue) correlated significantly with plasma renin levels (expressed per mL plasma) in all 3 brain regions (Figure 4). The different slopes may reflect the different blood content of each brain region. Plasma prorenin levels were comparable to plasma renin levels, and prorenin activation in brain regions non-significantly increased brain AGA. Plasma renin and prorenin were undetectable in Ren1c$^{-/-}$ mice (n=4), and renin (i.e., aliskiren-inhibitable AGA) was also undetectable in the 3 brain regions obtained from Ren1c$^{-/-}$ mice. However, low levels of aliskiren-inhibitable AGA were present in brainstem (1 out of 4) midbrain (3 out of 4) and cortex (2 out of 4) after prorenin activation in the Ren1c$^{-/-}$ mice. Since this cannot represent prorenin, these data imply that our prorenin activation procedure occasionally activated a renin-like enzyme, capable of reacting with angiotensinogen, the activity of which can be blocked by 10 μmol/L aliskiren.

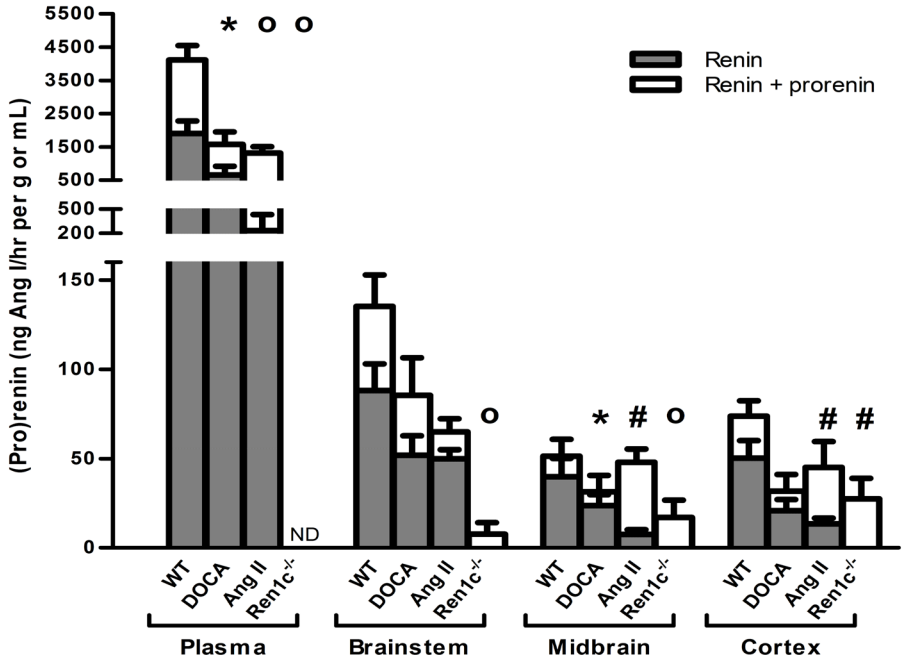

Figure 3. Renin and total renin (=renin + prorenin) levels in plasma and brain regions of untreated mice (WT), mice treated with DOCA-salt, mice infused with Ang II, and Ren1c$^{-/-}$ mice. Data are mean±SEM of n=4-7. Differences in renin levels were assessed by one-way ANOVA, followed by correction for multiple testing by post-hoc Bonferroni analysis. *P<0.05, #P<0.01, ºP<0.001 vs. WT.

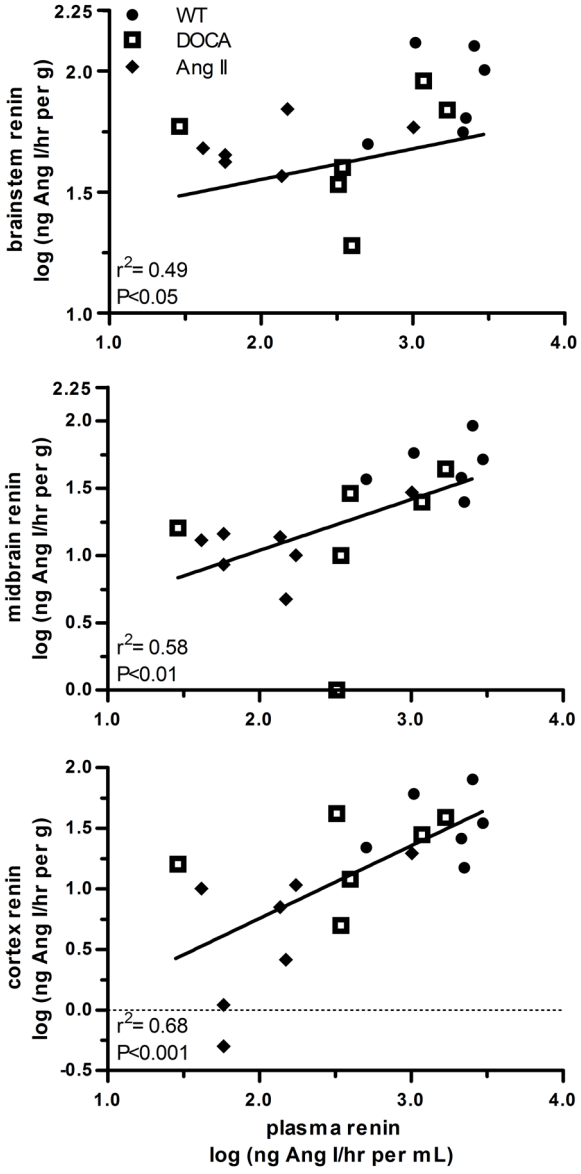

Figure 4. Relationship between renin in plasma and renin in 3 different brain regions in untreated mice (WT, n=6), mice treated with DOCA-salt (n=6) and mice infused with Ang II (n=7).

Renin expression in the brain

Renin (secreted + intracellular), sREN or icREN mRNA expression levels were un-

detectable in all brain regions in WT, DOCA-salt-treated and Ang II-infused mice. Primer specificity for renin (secreted + intracellular) and sREN was validated by measuring renal renin expression in Ren1c$^{-/-}$ mice (Figure S1B). In the IPA tissue expression data sets, renin expression was found in cerebellum, hypothalamus and pituitary, but only in 3 out of 9 different datasets examined (data not shown).

Despite angiotensinogen expression, angiotensinogen protein is undetectable in mouse brain and rat astrocytes

Mouse plasma contained detectable levels of angiotensinogen (28±5 pmol/mL). Angiotensinogen mRNA expression was observed in different brain regions (Figure 5), at C_t values of 23 (cerebellum), 22 (thalamus), 25 (hippocampus) and 27 (striatum) versus ≈18 in the liver. Brain expression levels changed inconsistently after DOCA-salt, Ang II and renin deficiency: an increase was observed in the thalamus after DOCA-salt ($P<0.05$), while decreases occurred in the cerebellum after DOCA-salt ($P<0.05$), and in the hippocampus after Ang II ($P<0.05$). Yet, angiotensinogen protein was undetectable (<1 pmol/g) in mouse cortex and brainstem (n=4 of each). Angiotensinogen was also undetectable in the medium of cultured rat astrocytes (<0.3 pmol/mL), cultured for 24, 48, 72 or 96 hours (n=4 for each condition), or the accompanying cell lysates (<0.3 pmol/mg protein).

Angiotensins in the SHR brain with and without RAS blockade

Ang I, Ang-(1-7) and Ang-(2-8) were below detection limit in brain tissue of untreated SHR (n=6), while Ang II could be detected in the rat brain at levels corresponding with ≈25% of the Ang II levels in blood plasma (Table S3 and Figure 6). Ang I and Ang-(2-8), but not Ang-(1-7), were detectable in plasma in untreated SHR. Brain Ang-(1-7) and Ang-(2-8) remained undetectable after olmesartan (n=6) or lisinopril (n=4), while Ang I became detectable in the rat brain after both types of RAS blockade ($P<0.001$ for both). Since plasma Ang I increased ≈20-fold after olmesartan and lisinopril ($P<0.001$ for both), it could be calculated that, during both types of RAS blockade, brain Ang I levels corresponded with ≈1% of the Ang I levels in plasma. Olmesartan increased brain Ang II ≈5-fold ($P<0.001$) and plasma Ang II ≈25-fold ($P<0.001$), so that after AT_1 receptor blockade the brain/plasma ratio of Ang II decreased by ≈80% ($P<0.05$). Lisinopril decreased plasma Ang II by >90% ($P<0.001$), and diminished brain Ang II to undetectable levels ($P<0.001$). Lisinopril also decreased plasma Ang-(2-8) to undetectable levels, and greatly increased plasma Ang-(1-7), while olmesartan increased both plasma Ang-(2-8) and plasma Ang-(1-7). Taken together, given that brain Ang I levels correspond with ≈1% of the circulating Ang I levels, 10 μL plasma per g brain tissue is sufficient to explain the entire brain Ang I content. Brain Ang II levels,

relative to plasma Ang II levels, are higher, suggesting either local synthesis or an active uptake mechanism. The massive decrease in the Ang II brain/plasma ratio after olmesartan supports the latter.

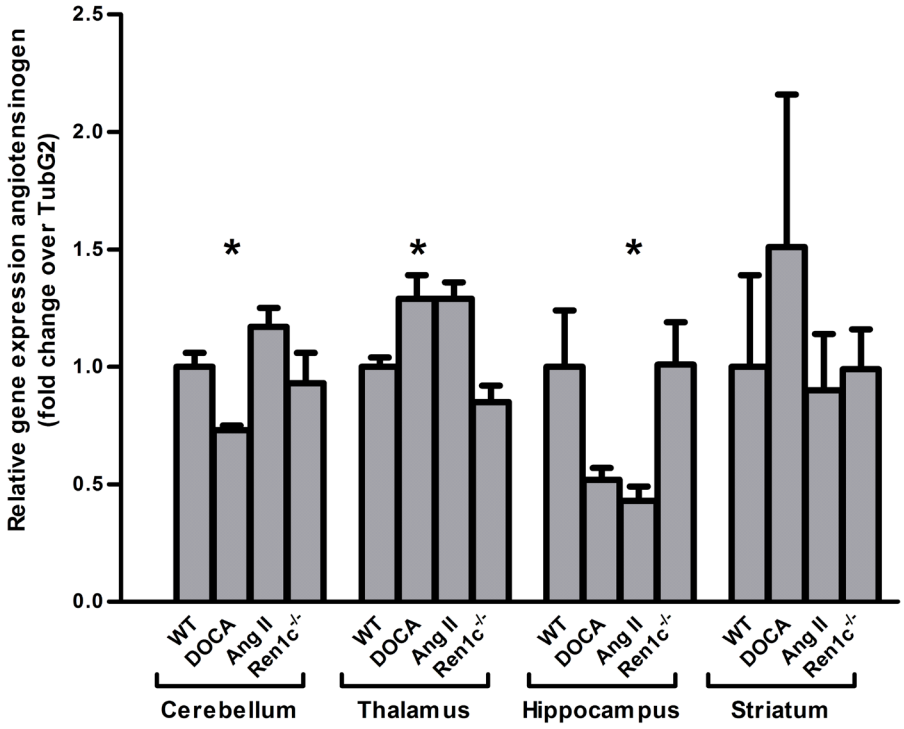

Figure 5. Angiotensinogen mRNA expression in different brain regions in untreated mice (WT), mice treated with DOCA-salt, mice infused with Ang II, and Ren1c$^{-/-}$ mice. Data, presented as fold change over TubG2 relative to WT levels, are mean±SEM of n=3-6. Differences were assessed by one-way ANOVA, followed by correction for multiple testing by post-hoc Bonferroni analysis. *P<0.05 vs. WT.

DISCUSSION

The present study confirms that renin-dependent AGA can be detected in virtually every region of the mouse brain. Yet, as compared to plasma, brain renin levels were very low, corresponding with the amount of renin in 1-25 μL blood plasma per g brain tissue (0.1-2.5 % (v/v)). This volume mimics the amount of blood plasma in various brain regions determined with tritiated inulin or Evans blue dye [16-17]. Moreover, perfusing the brain with PBS prior to the collection of the various regions, reduced brain renin uniformly by >60%. Had local renin synthesis occurred in one or more spe-

cific brain regions, the washout percentage should have been much lower in these regions, similar to the fact that in the kidney one cannot wash away stored renin [18-19], while this does happen in non-renin producing organs like the heart [20]. Furthermore, DOCA-salt, like Ang II, reduced circulating renin, and, contrary to our expectations, did not increase brain prorenin. In fact, if anything, both DOCA-salt and Ang II lowered brain renin in parallel with plasma renin. Aliskiren-inhibitable AGA was entirely absent in the brain of Ren1c$^{-/-}$ mice, supporting the validity of our brain renin measurement. Taken together, our data do not support the presence of kidney-independent (pro)renin synthesis in the brain, nor the concept that this occurs particularly in the DOCA-salt model. In fact, brain renin levels are so low that the accumulation of renin at brain tissue sites outside the blood compartment seems unlikely. This greatly differs from other organs (e.g., the heart), where renin diffuses freely into the interstitium and/or binds to a receptor, thereby reaching tissue levels that are, on a gram basis, at least as high as the renin levels in blood plasma (on a mL basis) [20-22]. Clearly, the presence of the blood-brain barrier prevents such distribution.

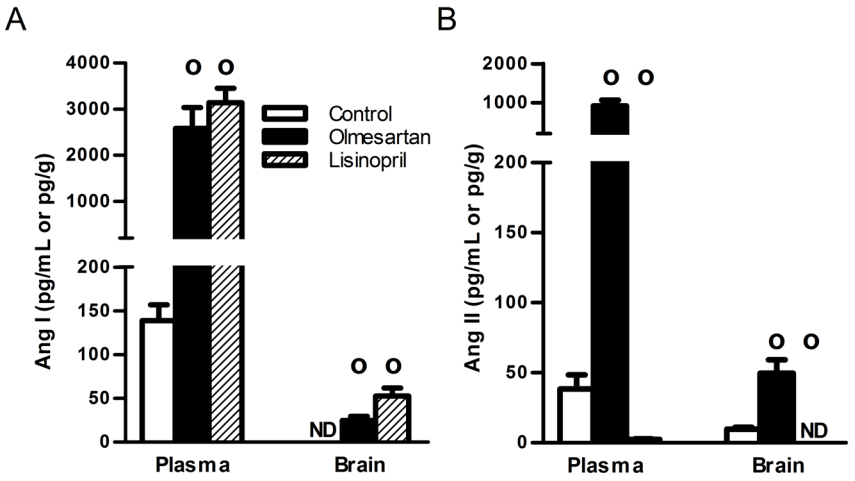

Figure 6. Ang I and II levels in plasma and brain of SHR treated with vehicle (control), olmesartan or lisinopril. ND, not detectable. Data are mean±SEM of n=4-6. Differences were assessed by one-way ANOVA, followed by correction for multiple testing by post-hoc Bonferroni analysis. °P<0.001 vs. control.

Prorenin activation resulted in modest AGA increases in all brain regions, and significance for this increase was only obtained by analyzing all regions together. Applying recombinant human prorenin to brain homogenates prior to the prorenin activation procedure (on the basis of acid activation [22]) confirmed that this procedure resulted in complete prorenin activation. However, small rises in aliskiren-inhibitable

AGA were also observed in brain homogenates from Ren1c$^{-/-}$ mice after their exposure to acid. Since Ren1c$^{-/-}$ mouse brain tissue cannot contain prorenin, this implies that the brain contains a non-renin proenzyme, which is activated by acid exposure, and which is capable of cleaving Ang I from angiotensinogen in an aliskiren-inhibitable manner. A possible candidate is pro-cathepsin D. Indeed, renin inhibitors, at high micromolar concentrations, do inhibit cathepsin D [23]. Our difficulty to demonstrate prorenin in the brain is reminiscent of earlier studies in organs not synthesizing prorenin themselves, like the heart [22]. Obviously, blood plasma contains prorenin, and thus some prorenin should be detected in the blood-containing homogenates derived from such tissues. Yet only under conditions where circulating prorenin levels were greatly elevated, like in heart failure in humans, did we reliably detect prorenin in cardiac tissue [24]. In mice, in contrast to humans, circulating prorenin levels are relatively low (versus renin), making it even more difficult to show a rise in AGA on top of already low renin-mediated AGA. A further complicating factor is that tissue homogenization per se may result in (partial) prorenin activation. In summary, given the presence of prorenin in blood plasma, brain homogenates should minimally contain the amount of prorenin present in a few μL of blood. The rises in AGA after prorenin activation are consistent with this view, but should still be interpreted with caution given the fact that non-renin enzymes also came into play after acid activation. Brain-selective prorenin rises, e.g. after DOCA-salt, were not observed. This implies that prorenin-(pro)renin receptor interaction is unlikely to occur in the mouse brain, particularly after DOCA-salt (which lowers brain (pro)renin), although it may obviously occur after intracerebroventricular infusion of pharmacological prorenin doses into the brain [10].

Most, if not all, studies on brain renin relied on the detection of renin mRNA in the brain, either under normal conditions, or after deleting/overexpressing sREN or icREN. Deleting sREN in neurons or glia did not affect blood pressure, heart rate, water intake, or metabolic rate [25], while preservation of icREN did not compensate for the consequences of whole body sREN-deficiency (hypotension, renal defects, lethality) [26]. Surprisingly, brain-selective deletion of icREN even caused neurogenic hypertension, possibly because icREN inhibits sREN [8]. These data seem to argue against icREN as an Ang I-generating enzyme. Yet, overexpressing either human icREN or sREN in astrocytes, if combined with human angiotensinogen, resulted in Ang II-dependent hypertension and an increase in drinking volume [27]. Since icREN under the latter conditions was not detectable in cerebrospinal fluid (CSF), it was concluded that this phenomenon involved intracellular Ang II formation. We attempted to detect renin mRNA, using either specific assays for sREN or icREN, or a non-specific assay that detects both sREN and icREN. Under no condition were we able to show renin (secreted + intracellular), sREN or icREN gene expression in any of the different regions of the brain: the expression level was below the detection threshold of the RT-PCR assay, even with the use of the highly sensitive Taqman probes. The specificity of our renin primers was validated by making use of the kidneys of Ren1c$^{-/-}$ mice. Of course, poor renin expression in the brain has been noted before [28-30]. Because of the technical limitations inherent in any RT-PCR assay, we could not load more than 100 ng of total

RNA. Our results therefore indicate that, if renin is expressed in the brain, its expression is $>2^{18}$-fold lower than that in the kidney (no signal after 40 cycles, with renin detection in the kidney at C_t=22). The IPA expression data sets confirm this view. Yet, Kubo et al. observed a blood pressure drop after intraventricular renin antisense injection in SHR [31]. In their hands only one of three tested antisense oligonucleotides acted hypotensive, and this response was accompanied by a 20% drop in renin mRNA (detected after 45 cycles of RT-PCR). These authors did not measure renin levels in brain or plasma, and were unable to rule out antisense leakage to the kidney. Therefore, these data cannot be taken as definitive proof for the existence of an independent brain RAS.

The mouse RAS differs from the human RAS, in that the circulating renin levels in mice are up to 1000-fold higher (on a ng Ang I/ml.hr basis) than in humans. As a consequence, circulating angiotensinogen levels in mice are far below K_m range, as confirmed in the present study. Nevertheless, despite these differences, mouse angiotensin levels in blood and tissue are comparable to those in humans, rats and pigs [32-35]. We attempted to measure angiotensinogen in the mouse brain, both at the mRNA and protein level. Although we did observe angiotensinogen mRNA expression in different regions of the brain, in full agreement with previous work [36-37], expression was up to 500-fold lower than in the liver. Under no condition were we able to detect angiotensinogen protein in the brain. Given the detection limit of our assay (1 pmol/g), this implies that brain angiotensinogen, if present, occurs at levels (per g tissue) that are <3% of the levels in plasma (per mL plasma). Such low levels have been reported before in the rat brain, as well as in human and rat CSF [16,38-40], and thus our data entirely agree with the literature. Clearly, mice, given their low angiotensinogen levels, are not the optimal species to study brain angiotensinogen. As astrocytes are assumed to be the source of brain angiotensinogen [41-42], we additionally studied angiotensinogen synthesis by rat primary cortical astrocytes, but again failed to detect any angiotensinogen. Nevertheless, data from Schink et al. [40] do support the functional presence of angiotensinogen in the rat brain. These authors artificially elevated renin in the brain by either intracerebroventricular renin infusion (in Sprague-Dawley rats) or by making use of transgenic hypertensive rats overexpressing mouse Ren2. The responses to both approaches (drinking and blood pressure reduction, respectively) were greatly diminished after lowering brain angiotensinogen by brain-selective expression of an antisense RNA against angiotensinogen mRNA.

Finally, given our observation that brain renin is confined to the plasma compartment, while brain angiotensinogen is extremely low (if not also confined to the plasma compartment), an urging question is what degree local angiotensin generation truly occurs in the brain. We therefore collected brainstem tissue (i.e., the brain region with the highest renin level) from SHR under control conditions and during RAS blockade with olmesartan or lisinopril. Rats rather than mice were used here, because at identical angiotensin levels in both species, the larger rat brainstem would allow a more reliable quantification of angiotensins. Without treatment, brain Ang I was undetectable, while Ang II occurred at levels that were ≈25% of the levels in plasma (per gram tissue weight). This contrasts with other organs where Ang II is usually much

higher than in plasma, while Ang I is easily detectable [43-46]. RAS blockade induced the usual rise in Ang I levels in plasma, and now brain Ang I became detectable, however, at only 1% (v/v) of its plasma levels. It seems reasonable to assume that also in the untreated animals brain Ang I levels were in the 1% range of plasma Ang I, and therefore too low to be detected with our assays. If so, this implies that under all conditions, brain Ang I at most represented the amount of Ang I that is inherently present in brain tissue because it contains a small amount (≈1%) of blood [16]. Lisinopril decreased brain Ang II to undetectable levels, while olmesartan reduced the brain/plasma Ang II ratio by >80%. The latter finding suggests that, normally, circulating Ang II accumulates in brain tissue via binding to AT_1 receptors. Such uptake occurs in multiple organs [47], and facilitates the intracellular accumulation of Ang II [48]. Without receptors (i.e., in AT receptor-deficient mice), tissue Ang II levels drop dramatically [49], suggesting that tissue Ang II levels do not originate intracellularly. If Ang II binding to AT_1 receptors is the only source of Ang II in the brain, one would expect angiotensin metabolites that do not (or only with low affinity) bind to this receptor to be undetectable in the brain. This is indeed what we observed for both Ang-(2-8) and Ang-(1-7). An olmesartan-induced reduction in brain Ang II levels was also observed in Dahl-sensitive hypertensive rats, albeit in the absence of an effect on blood pressure [50]. Clearly therefore, the changes observed in brain Ang II are blood pressure-independent.

PERSPECTIVES

The absence of renin-dependent AGA in the brain outside the blood compartment implies that angiotensin generation in the brain, if occurring, does not involve renin. Brain prorenin levels, if anything, were even lower than brain renin levels, and therefore, like renin, at most represented the amount of prorenin expected in brain tissue based upon its blood content. Selective brain prorenin upregulation, e.g. after DOCA-salt, could not be observed, arguing against the concept that DOCA-salt-induced neurogenic hypertension involves prorenin-(pro)renin receptor interaction. Finally, the absence of Ang I in brain tissue outside the blood compartment (which contrasts sharply with the presence of Ang I in every other organ of the body) strongly suggests that there is no local Ang I generation in the brain. Apparently therefore, non-renin enzymes do not compensate for the absence of renin, assuming at least that brain angiotensinogen levels are of sufficient magnitude to allow independent Ang I generation at all. Only ex vivo, after prohormone-activating procedures, did we occasionally obtain evidence for a very modest contribution of such non-renin enzymes, but the in-vivo relevance of these findings is questionable. Brain Ang II therefore appears to originate in the blood compartment. Of course, circulating Ang II will bind to brain AT receptors that are outside the blood-brain barrier (e.g., in the circumventricular organ). Yet, it may also gain access to brain areas behind this barrier, e.g. under conditions where blood-brain barrier permeability is compromised, like in (DOCA-salt) hypertension [51-52]. In fact, Ang II itself may be responsible for disturbing the blood-brain

barrier, thus facilitating its own access to critical brain areas like the hypothalamus and brainstem. From this perspective, the brain 'RAS' in reality represents circulating Ang II that accumulates in brain nuclei, possibly after it has (partially) broken down the blood-brain barrier. This Ang II subsequently activates sympatho-neurohumoral outflow, for instance by upregulating reactive oxygen species [52-53]. This is entirely different from other organs like heart, kidney and vascular wall, where renin and angiotensinogen diffuse freely into the interstitial space, allowing local production of Ang I to occur [20,54-55]. Blocking AT_1 receptors or enhancing Ang II degradation (e.g., by ACE2) will prevent the effects of circulating Ang II in the brain, thereby explaining the success of intracerebroventricular application of losartan or brain-selective ACE2 overexpression in DOCA-salt hypertension [56].

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Chapter 4

DO PRORENIN-SYNTHESIZING CELLS RELEASE ACTIVE, 'OPEN' PRORENIN?

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ABSTRACT
The function of prorenin, the inactive precursor of renin, remains unclear after many decades of research. The discovery of a (pro)renin receptor suggested that prorenin, by binding to this receptor, would become active, i.e., obtain an 'open' conformation. However, the receptor only interacted with prorenin at levels that were many orders of magnitude above its normal levels, making such interaction in vivo unlikely. Prorenin occurs in 2 conformations, an open, active form, and a closed, inactive form. Under physiological conditions (pH 7.4, 37°C), virtually all prorenin occurs in the closed conformation. This study investigated to what degree prorenin-synthesizing cells release prorenin in an open conformation. Renin- and prorenin-synthesizing human mast cells, and prorenin-synthesizing HEK293 cells (transfected with the mammalian expression vector pRhR1100, containing human prorenin) and human decidua cells were incubated with the renin inhibitor VTP-27999. This inhibitor will trap open prorenin, since after VTP-27999 binding, prorenin can no longer return to its closed conformation, thus allowing its detection in a renin immunoradiometric assay. No evidence for the release of open prorenin was found. Moreover, incubating decidua cells with angiotensinogen yielded low angiotensin levels, corresponding with the activity of ≈1% of prorenin in the medium, i.e. the amount of open prorenin expected based upon the equilibrium between open and closed prorenin under physiological conditions. Our study does not reveal evidence for the release of open, active prorenin by prorenin-synthesizing cells, at least under cell culture conditions. This argues against prorenin activity at the site of its release.

INTRODUCTION

Renin belongs to the A1 family of aspartic proteases. Its 3D structure consists of two β-sheet domains, the N- and C-domain. Renin has an inactive precursor, prorenin, in which the catalytic binding site is covered by the N-terminal part of mature renin that, in turn, is covered by the prosegment [1]. After removal of the prosegment, this N-terminal part becomes part of a six-stranded β-sheet on the back of the mature renin molecule, previously occupied by the prosegment. This requires a conformational change that fully exposes the active cleft [1].

Prosegment unfolding occurs in a pH- and temperature-dependent manner, and, if not followed by cleavage, results in two prorenin conformations (Figure 1): a 'closed', inactive form, and an 'open' form that displays full enzymatic activity [2, 3]. In addition, an intermediate form exists where the prosegment has moved away from the cleft, but where the renin part still has to undergo the afore-mentioned conformational changes. Under physiological conditions (pH 7.4, 37°C), <2% of prorenin is in the open conformation. The renin inhibitor aliskiren binds to prorenin in the open conformation as well as to the intermediate form of prorenin [2, 3]. Binding to the intermediate form induces prorenin unfolding. Due to the tight binding of the renin inhibitor, the refolding step (i.e., the return to the closed conformation) is now no longer possi-

ble, and thus, the equilibrium between the closed and open conformation will shift in favor of the open conformation. Eventually, all prorenin molecules may be open ('non-proteolytic activation'), allowing its recognition by the active site-directed antibodies used in renin immunoradiometric assays (IRMAs), despite the fact that the prosegment is still present and aliskiren is bound to the active site [4, 5].

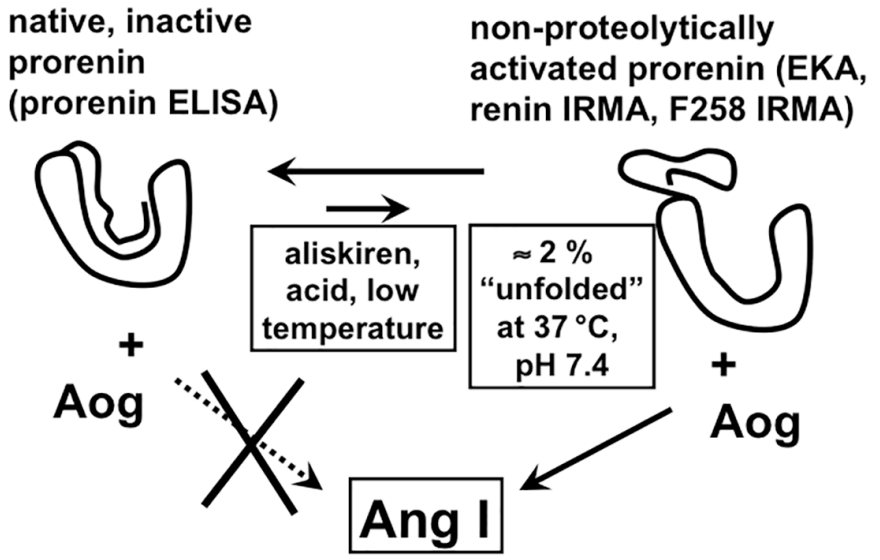

Figure 1. Model displaying the closed and open prorenin conformations, as well as the assays allowing the detection of these prorenin variants. Normally, <2% of prorenin is in the open conformation. Aliskiren, acid and low temperature shift the equilibrium to the right, allowing the detection of prorenin in a renin IRMA, on the basis of its prosegment (F258 IRMA), or by enzyme-kinetic assay (EKA). Only a direct prorenin ELISA allows the detection of intact, closed prorenin.

Unexpectedly, VTP-27999, a new, active site-directed renin inhibitor with an IC_{50} (0.3 nmol/L) that is comparable to that of aliskiren, did not induce prorenin unfolding [6]. Yet, it blocked the aliskiren-induced unfolding of prorenin. This suggest that VTP-27999 does bind to the intermediate form of prorenin, but that such binding has no conformational consequences. Furthermore, VTP-27999 increased renin immunoreactivity. This may relate to the observation, based on crystallization studies, that there are 2 renin conformations [7]. Aliskiren and VTP-27999 bind to both conformations, but only in the case of VTP-27999, the conformations of the two monomers are nearly identical [6]. These conformational differences are likely to affect the affinity of the active site-directed antibodies applied in renin IRMAs. As a consequence, the renin IRMA yields higher levels in the presence of VTP-27999 than without this inhibitor.

A long-standing question concerns the function of prorenin. As mentioned above, it displays limited activity in plasma. Nguyen et al. proposed that it binds to

the so-called (pro)renin receptor at nanomolar affinity [8]. This resulted in a conformational change, allowing prorenin to display full activity. Theoretically, this would result in angiotensin generation, at least in (pro)renin receptor-expressing tissues, by receptor-bound prorenin. Initially, this finding was received with great enthousiasm, since it finally provided a role for prorenin: although synthesized in only a limited number of organs, given the wide occurrence of the (pro)renin receptor, prorenin might now contribute to angiotensin generation in virtually every organ of the body. However, although in-vitro studies applying high prorenin concentrations confirmed this concept, the prorenin concentrations in vivo are many orders below its K_d for the (pro) renin receptor, thereby making this possibility unlikely [9, 10]. As a consequence, this concept is now being abandoned, and we need alternative hypotheses to explain the function (if any) of prorenin. One possibility is that prorenin acts exclusively at its site of synthesis. For this to occur, prorenin-synthesizing cells should release prorenin in an open conformation, allowing it to display local activity in the immediate vicinity of the cells, before it returns to its inactive, closed conformation, e.g., when reaching blood plasma. If true, renin inhibitors capable of interfering with open prorenin might have an advantage over other RAS blockers, since they can specifically suppress angiotensin generation in prorenin-synthesizing tissues. Possibly, novel inhibitors need to be developed to optimally exert this effect.

To test this concept in the present study, we made use of human prorenin-synthesizing cells. For comparison, renin-releasing cells were also evaluated. We used 3 approaches. First, we incubated the cells with VTP-27999. This inhibitor, like aliskiren, would bind to prorenin if it is released in an open conformation, thus preventing its return to the closed conformation, allowing its detection in a renin IRMA. VTP-27999, unlike aliskiren, would not additionally convert all closed prorenin molecules into the open conformation, and thus this renin inhibitor is ideal to quantify the percentage of prorenin molecules released in the open conformation. We applied renin-, prorenin-, and prosegment-specific assays (available for human (pro)renin only) to verify this concept. Second, we studied angiotensin I generation during prorenin release by adding angiotensinogen to the medium, the underlying assumption being that only open prorenin would be able to display enzymatic activity. Finally, we verified to what degree prorenin in human plasma displays angiotensin I-generating activity.

METHODS

Human samples

All studies were approved by the Medical Ethical Review Board, and performed in accordance with the Declaration of Helsinki and Good Clinical Practice. Informed consent was obtained from each subject. Blood samples were obtained from 6 healthy controls (4 men, 2 women; age 23-45 years, mean 31 years). Blood was collected in polystyrene tubes containing 6.25 mmol/L EDTA (final concentration). The samples were centrifuged at room temperature at 3000 g for 10 minutes, and plasma was either

used immediately or stored at -20°C. Placenta's were harvested from 7 women after delivery (age 26-41, mean 34 years). Renin- and prorenin-synthesizing human mast cells (HMC-1) were a gift from Dr. J.H. Butterfield (Mayo Clinic, Rochester, MN, USA) [11].

Cell culture studies

Cell culture studies were performed at 37°C in a humidified 5% CO_2 incubator (Sanyo, Panasonic Biomedical Sales Europe B.V., Etten-Leur, The Netherlands).

HMC-1 cells were cultured in 75 cm^2 culture flasks using Iscove's modified Dulbecco's medium (Lonza, Verviers, Belgium), supplemented with 10%, iron-supplemented bovine calf serum (Hyclone, Thermo Fisher Scientific, Breda, The Netherlands), 1% Penicillin Streptomycin (PS; Invitrogen, Bleiswijk, The Netherlands) and 1.2 mmol/L α-thio-glycerol (Sigma, Zwijndrecht, The Netherlands).

HEK293 cells were transfected with the mammalian expression vector pRhR1100, containing human prorenin [12], a gift from Dr. Reudelhuber (IRCM, Montréal, Canada). In short, cells were transfected with Lipofectamine2000 (Invitrogen), according to the manufacturers protocol, and put on selection using 0.6 mg/mL G418 (Invitrogen). The clone with the highest expression of prorenin was isolated, and used for further experiments. Cells were cultured in DMEM-F12 (GIBCO, Breda, The Netherlands) medium supplemented with 10% fetal calf serum (FCS; Lonza), 1% PS, and 0.6 mg/mL G418.

Decidua cells were harvested from the maternal side of the placenta according to Markoff et al. [13]. In short, tissue was chopped and washed twice with DPBS (GIBCO), alternated by centrifugation steps of 10 minutes at 700 rpm. The pellet was resuspended in 5 mL RPMI-1640 (GIBCO) per gram tissue, supplemented with 1% FCS, 0.1% collagenase and 0.1% hyaluronidase (Sigma) in a tissue culture flask, and put on an orbital shaker (150 rpm) for two hours at 37°C. This suspension was filtered using a sieve with a mesh of 40 μm. The filtered solution was centrifuged for 10 minutes at 700 rpm, after which the pellet was washed with RPMI-1640, and centrifuged again. The pellet was then exposed to 20 mL RBC lysis solution (5PRIME, Hilden, Germany) for 10 minutes, followed by another 10 minutes of centrifugation. This was followed by another two wash steps with RPMI. Next, cells were cultured in RPMI-1640 containing 10% FCS, 12.5 mmol/L HEPES (GIBCO), 50 μg/mL gentamycin (Sigma) and 5 μg/mL fungizone (Sigma). Medium was changed every 24 hours in the first 72 hours.

For experiments verifying the release of open prorenin, cells were seeded in 6- or 12-well plates at a concentration of $0.5*10^6$ cells/mL in fresh medium in the presence or absence of VTP-27999 (0.1-1000 nmol/L). After 2 to 5 days, medium was collected, and spun down 5 minutes at 2500 rpm to remove remaining cells. Supernatant was stored at -20°C.

To study prorenin-induced angiotensin generation, decidua cells were incubated for 24 hours in the presence of 150 nmol/L human angiotensinogen (Sigma). Cells incubated without angiotensinogen served as control. Medium samples (75 μL) for the determination of renin and prorenin were collected at 4, 8 and 24 hr. Medium samples (500 μL) for the determination of angiotensin I were collected at 24 hours with 25 μL

inhibitor stock solution (containing 0.1 mmol/L aliskiren, 200 mmol/L EDTA and 0.2 mmol/L lisinopril), frozen in liquid N_2, and stored at -70°C. The first-order rate constant for angiotensin I elimination was determined by incubating the cells with 100 nmol/L angiotensin I for maximally 4 hrs in the presence or absence of 100 nmol/L captopril. Medium samples (50 μL) for the determination of angiotensin I were collected at 0, 0.5, 1 and 4 hr, mixed with 2.5 μL inhibitor stock solution, and stored as described above.

Biochemical assays

Renin and prorenin were determined either indirectly, by enzyme-kinetic assay (i.e., quantifying angiotensin I-generating activity) or directly, by IRMA (i.e., quantifying renin or prorenin immunoreactively).

Enzyme-kinetic assays were applied to human plasma samples only, to verify prorenin activity. The sample was incubated with excess sheep angiotensinogen in the presence of angiotensinase inhibitors, either directly after its removal from the patient, after freezing and thawing, or after keeping it for 18 hours at 37°C. Imidazole buffer (final concentration 0.1 mol/L) was added to the incubation mixture to keep pH at 7.4.

Immunoreactive renin was measured with the Renin III (Cisbio, France) IRMA. This assay, which makes use of a monoclonal antibody (4G1) [14] directed against renin's active site, also recognizes intact, open prorenin [2, 15]. This implies that intact prorenin can be measured with this assay after incubating it with acid (pH=3.3) or after exposing it for 48 hours at 4°C to 10 μmol/L aliskiren, since both procedures induce the conversion of all prorenin molecules into the open conformation [4].

Intact, closed prorenin was measured with an enzyme-linked immunosorbent assay (ELISA) that recognizes residues 32-39 of the prosegment (Molecular Innovations, Novi, MI, USA) [3]. This prorenin assay was performed according to the instructions of the manufacturer, making use of human recombinant prorenin to construct the standard curve.

In a select set of samples, intact, open prorenin was measured on the basis of its prosegment, replacing the ^{125}I-labeled active site-directed monoclonal antibody of the Cisbio kit by a prosegment-directed ^{125}I-labeled monoclonal antibody (F258-37-B1) in the IRMA ('F258 IRMA'). F258-37-B1 is directed against the C-terminal part (p20-p43) of the propeptide and does not react (<0.1%) with renin. F258-37-B1 also does not react (<0.1%) with intact, closed prorenin [16]. However, it does react with prorenin after the converting all prorenin molecules into the open conformation by incubation with acid. Thus, the acid-induced non-proteolytic conformational change, causing the propeptide to move to the surface of the molecule, allows the recognition of prorenin by both the active site-directed antibody of the Cisbio kit, and the prosegment-directed antibody of the prorenin IRMA.

Angiotensin I and II levels in the medium were measured by radioimmunoassay after SepPak extraction and reversed-phase high performance liquid chromatography separation [17].

Data analysis

Results are shown as mean±SEM. Differences were tested using one-way ANOVA, followed by Dunnett's multiple comparison test; $P<0.05$ was considered significant. Angiotensin I–generating activities obtained in the enzyme-kinetic assay were converted to renin concentrations based on the fact that 1 ng of angiotensin I per milliliter per hour corresponds with 2.6 pg of human renin per milliliter [18].

RESULTS

Plasma prorenin displays enzymatic activity

When incubating freshly obtained plasma samples from 6 healthy controls with excess angiotensinogen at 37°C, angiotensin I generation was linear over time (Figure 2, left panel). Results obtained in the same samples after they had been frozen and thawed were identical. However, when performing the assay after the samples had been kept for 18 hours at 37°C (to inactivate open prorenin) the degree of angiotensin I generation was lower ($P<0.05$). This was not due to (pro)renin degradation, since total renin levels (determined by immunoreactive assay) did not change over the 18 hour-period (Figure 2, right panel). The average decrease in angiotensin I-generating activity after 18 hours (≈1 ng angiotensin I/mL.hr) corresponded with ≈3% of the immunoreactive prorenin levels. This confirms that both blood handling/centrifugation (at room temperature) and freezing/thawing induce a conformational change in prorenin, allowing a small percentage of the prorenin molecules to display enzymatic activity in the enzyme-kinetic assay. Prolonged incubation at 37°C reversed this phenomenon.

VTP-27999 increases renin immunoreactivity, but does not unravel the release of 'open' prorenin

Open prorenin release was studied in cells that synthesize both renin and prorenin (HMC-1 cells) and in cells that exclusively synthesize prorenin (human prorenin-expressing HEK293 cells and decidua cells).

In medium samples obtained from HMC-1 cells incubated without inhibitor, renin was measured by renin IRMA, both before and after activating prorenin with aliskiren (i.e., converting all closed prorenin molecules to open prorenin molecules). Aliskiren exposure increased renin immunoreactivity ≈3-fold (Figure 3, left panel; n=7), illustrating that the cells predominantly released prorenin. Total renin measurements after aliskiren exposure did not change significantly following incubation of the cells with increasing concentrations of VTP-27999, nor did prorenin immunoreactivity (determined by prorenin ELISA) change in the presence of this inhibitor. This implies that VTP-27999 did not affect (pro)renin release per se. Yet, renin immunoreactivity (expressed as a % of the total renin levels without VTP-27999) almost tripled following

incubation of the cells with VTP-27999 (Figure 3 left panel; n=6). Given the absence of changes in the release of (pro)renin, these data suggest that VTP-27999 affects the renin assay itself (i.e., yields higher renin levels for a given amount of renin).

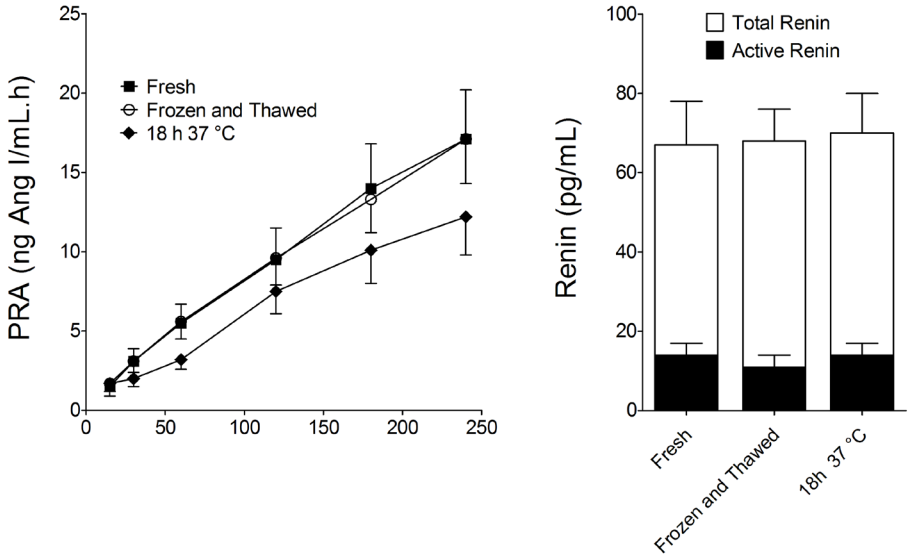

Figure 2. Left, angiotensin I generation in human plasma during its incubation with excess sheep angiotensinogen, either immediately after collecting the plasma samples (fresh), after keeping the samples for 18 hours at 37°C, or after freezing and thawing the samples. Right, renin and total renin levels measured by renin IRMA after 4 hours of incubation. Data are mean±SEM of n=6.

Aliskiren increased renin immunoreactivity in the medium of control (i.e., incubated without inhibitor) HEK293 and decidua cells >10-fold (Figure 3, middle and right panels; n=5). This is in agreement with the fact that these cells exclusively release prorenin, and that, at pH=7.4 and 37°C, only a few percent of prorenin is in the open conformation (i.e., can be recognized in a renin IRMA). Total renin measurements after aliskiren exposure did not change following incubation of the cells with increasing concentrations of VTP-27999, nor did prorenin immunoreactivity change in the presence of this inhibitor. Thus, as in HMC-1 cells, VTP-27999 did not affect prorenin release in either HEK293 cells or decidua cells. Yet, VTP-27999 doubled renin immunoreactivity in both cell types.

The latter increase in renin immunoreactivity might be suggestive for the release of open prorenin. To verify this further, we first repeated the studies in (pro)renin-containing medium (obtained from control cells) incubated with VTP-27999 in the absence of cells. Results were identical (Figure 4; n=5-7). Second, we exposed (pro)renin-containing medium of the 3 cell types to acid, to induce the open (renin-like) conformation in all prorenin molecules. Under this condition, the prosegment is still present, but folded away from the active site (allowing prorenin detection both by renin

- and prosegment IRMA). Next, pH was returned to pH=7.4 in the absence or presence of either 10 μmol/L aliskiren or VTP-27999. Without renin inhibitor, prorenin will rapidly return to its closed conformation, no longer allowing its detection by renin - or prosegment IRMA. But with renin inhibitor, prorenin will be trapped in its open conformation, allowing its detection in both IRMAs. Results show that both renin inhibitors greatly increased renin and prosegment immunoreactivity (Figure 5, n=3 for all conditions), confirming their capacity to trap prorenin in its open conformation. Importantly, the increases in renin immunoreactivity after VTP-27999 were ≈20-fold, i.e. double those seen after aliskiren. In contrast, the increases in prosegment immunoreactivity were identical for both inhibitors. This implies that VTP-27999 increases renin immunoreactivity, but not prosegment immunoreactivity.

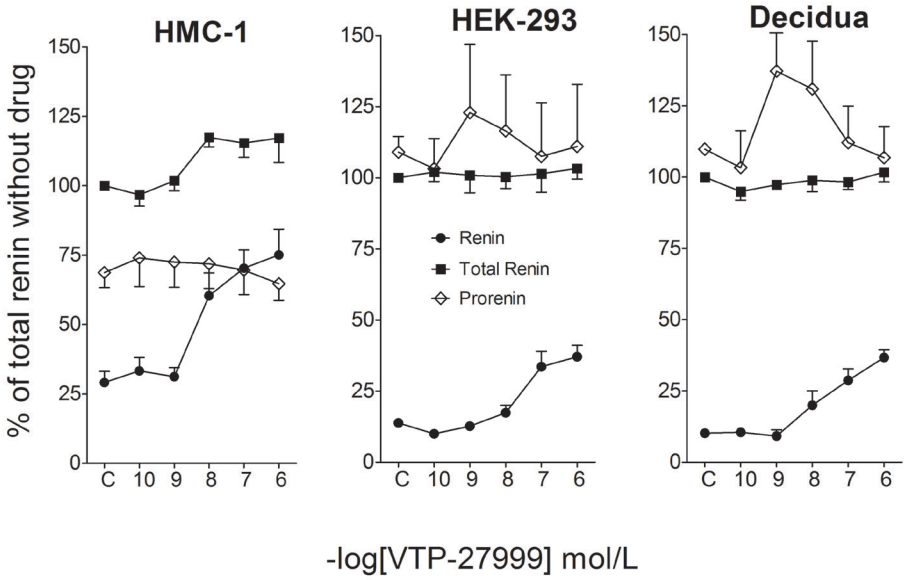

Figure 3. Renin, total renin (measured following after aliskiren pretreatment) and prorenin in the medium of renin+prorenin-secreting HMC-1 cells or exclusively prorenin-secreting HEK293 and decidua cells, cultured in the presence of increasing concentrations VTP-27999. Renin and total renin were measured by renin IRMA, prorenin was measured by direct prorenin ELISA. Data are mean±SEM of n=5-7, and have been expressed as a percentage of the total renin levels without VTP-27999.

Taken together, these data show that incubation of all 3 cell types with VTP-27999 yields higher renin immunoreactivity levels in the medium. Yet, this is due to the fact that VTP-27999, for a given amount of renin or open prorenin, increases renin immunoreactivity in the renin IRMA 2-3 fold. It is not due to the release of open prorenin, since a) results were identical in the absence of cells, and b) renin immunoreactivity rises in the medium of prorenin-releasing cells were far below the 20-fold that would have been expected had all prorenin been released in the open conformation.

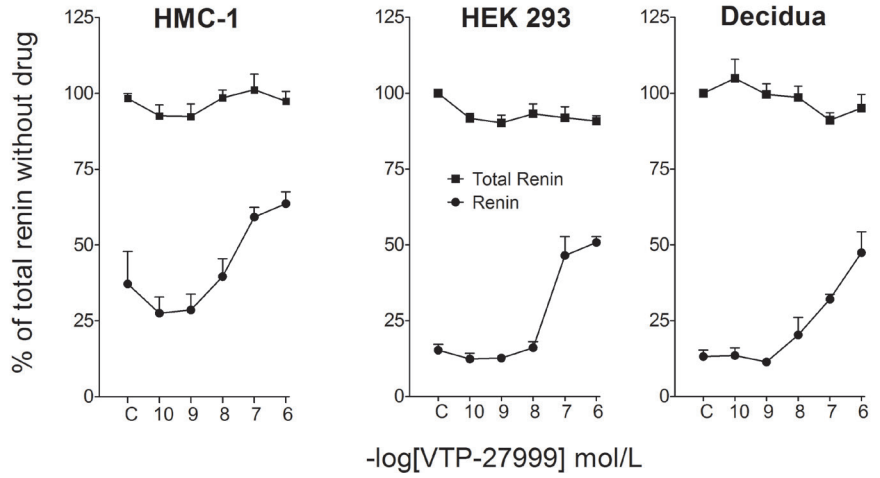

Figure 4. Renin and total renin (measured following after aliskiren pretreatment) in medium obtained from renin+prorenin-secreting HMC-1 cells or exclusively prorenin-secreting HEK293 and decidua cells, incubated without cells in the presence of increasing concentrations VTP-27999. Renin and total renin were measured by renin IRMA. Data are mean±SEM of n=5-7, and have been expressed as a percentage of the total renin levels without VTP-27999.

Angiotensin generation in decidua cells

The medium prorenin levels of decidua cells continuously rose during a 24 hour-incubation (Figure 6, left panel; n=3-7). Angiotensin I, when added to these cells, disappeared with a half life ($t_{1/2}$) of 0.45±0.04 hr (n=7). In the presence of captopril, the angiotensin I half life increased to 1.41±0.13 hr (P<0.01), implying that 66±4% of the angiotensin I metabolism was due to conversion by ACE (Figure 7, left panel). Importantly, the angiotensin I disappearance was identical when repeating these studies in (serum-containing) medium in the absence of cells (n=5), suggesting that the majority of the angiotensin I metabolism involved serum-derived ACE (Figure 7, right panel). In other words, we found no evidence for the presence of decidual ACE. Consequently, when investigating angiotensin generation after adding angiotensinogen, we only focused on angiotensin I, and not angiotensin II. Angiotensin I levels in the medium at 24 hours after adding angiotensinogen were 4-5 times higher than the (background) levels measured in the absence of angiotensinogen (Figure 6, right panel; n=7). Normally, 2.6 pg/mL renin corresponds with 1 ng angiotensin I/ml.hr (v_{max}), at least when activity is determined at angiotensinogen levels above K_m (=1200 nmol/L). However, our experiments were performed at angiotensinogen levels 8 times below K_m (150 nmol/L). This implies that angiotensin I generation in our experimental set-up occurred at a rate

v corresponding with v = 150/(150+1200) x v_{max}. Given the angiotensin I half life of 0.45 hr, the expected steady-state level of angiotensin I level can be calculated as v x $t_{1/2}$/ ln2. Had all prorenin detected at 24 hours been active, this steady-state angiotensin I level would have been 12109±4488 fmol/mL. In reality, the steady-state angiotensin I levels at that time point amounted to only 1.3±0.7% of this level. This suggest that ≈1% of prorenin displayed enzymatic activity under our experimental conditions. This is comparable to the percentage of 'active' prorenin in human plasma, and thus opposes the concept that prorenin-synthesizing cells release open prorenin.

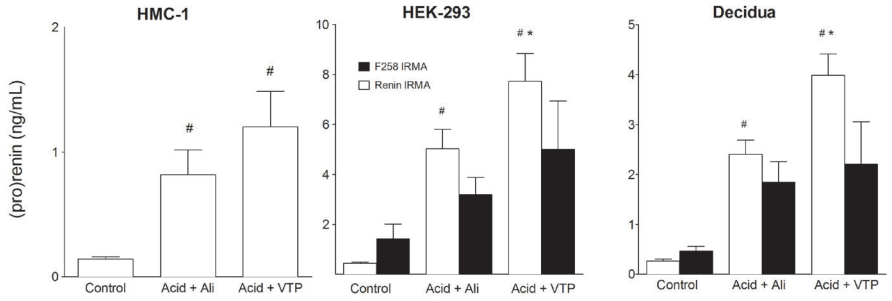

Figure 5. (Pro)renin immunoreactivity, measured by renin IRMA (open bars) or prosegment (F258) IRMA (closed bars) in the acid-pretreated medium of HMC-1 cells, HEK293 cells or decidua cells, followed by a 1-hour incubation with buffer (control), 10 µmol/L aliskiren (acid+ali), or 10 µmol/L VTP-27999 (acid+VTP). # P<0.05 vs. control. *P<0.05 vs. aliskiren. Data are mean±SEM of n=3.

DISCUSSION

The current study, making use of human prorenin-releasing cells, does not provide evidence for the concept that such release occurs in an 'open' conformation, subsequently allowing prorenin to display (full) activity at the site of its release. Theoretically, given the low pH conditions under which (pro)renin synthesis/release occurs [19], this might have happened, given that a low pH shifts the equilibrium between the open and closed conformation of prorenin into the direction of the former. Our data in acid-pretreated medium samples (containing 100% open prorenin [20]) confirm that the renin inhibitor VTP-27999 would have kept prorenin in this open conformation, allowing its detection in a renin assay, or an assay detecting the prosegment.

The renin inhibitor aliskiren would have done the same. However, this inhibitor, unlike VTP-27999, also affects the equilibrium between the open and closed conformation by inducing a conformational change in prorenin [4]. Therefore, aliskiren, if applied at sufficiently high concentrations, would have converted all closed prorenin molecules into the open conformation. This would have resulted in an overestimation of the number of open prorenin molecules, and thus aliskiren cannot be used to quantify the release of open prorenin.

When applying VTP-27999 to cells that release renin (HMC-1 cells), we observed that this inhibitor increased renin immunoreactivity 2-3-fold. This has been observed before in human plasma [6], and is related to the existence of 2 renin conformations [7], and the different affinity of the active site-directed antibody for these 2 conformations. The immunoreactivity increase also occurred when applying VTP-27999 to acid-activated prorenin, i.e., prorenin that has the open conformation. This confirms that the 3D structure of open prorenin resembles that of renin, despite the fact that the prosegment is still present [2]. Since VTP-27999 did not affect the outcome of the prosegment assay (F258 IRMA), it can be concluded that the binding of VTP-2799 to renin's active site selectively affected the affinity of the active site-directed antibody.

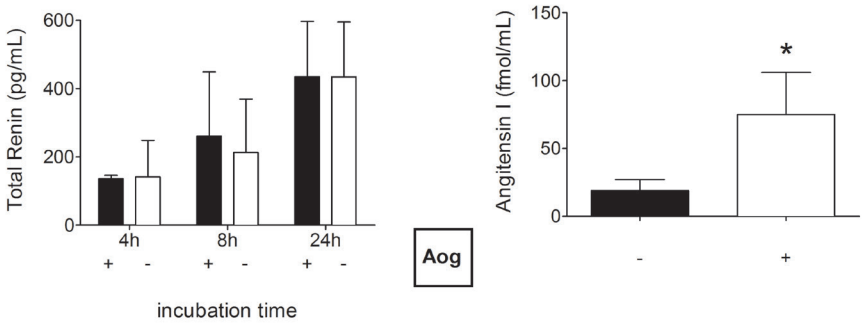

Figure 6. Left, total renin levels in the medium of human decidua cells after 4, 8 and 24 hours (mean±SEM of n=3-7) in the absence (open bars) or presence (closed bars) of 150 nmol/L human angiotensinogen. Levels were measured by renin IRMA after prorenin activation with aliskiren. Right, angiotensin I levels in the medium of human decidua cells after 24 hours (mean±SEM of n=7) in the absence (open bars) or presence (closed bars) of 150 nmol/L human angiotensinogen.

Under physiological conditions, only a small percentage of prorenin is in the open conformation [2]. This was also the case in the medium of our HEK293 and decidua cells (i.e., cells that exclusively released prorenin). Therefore VTP-27999 should have minimally bound to this percentage of the prorenin molecules, and, given its effect on the affinity of the active site-directed antibody, it might subsequently marginally have increased renin immunoreactivity in the renin IRMA. This is indeed what happened: no substantial rises in renin immunoreactivity were observed after VTP-27999, although a >20-fold rise would have been feasible (Figure 5) in case all prorenin molecules would have been released in an open conformation. Moreover, the rises observed in prorenin-containing medium incubated with VTP-27999 in the absence of cells were identical to those observed with cells. These data therefore strongly argue against the release of open prorenin.

In addition, our angiotensin measurements after applying human angiotensinogen to the medium of human decidua cells also do not support the release of open, active prorenin. At most, the generated levels of angiotensin I in the medium of these cells support the idea that ≈1% of prorenin displayed activity, i.e., is in the open

conformation. Such low percentages are in the range occurring in human blood plasma, and simply represent the open/closed equilibrium at pH=7.4 and 37°C, i.e., our cell culture conditions [20-22]. Lowering temperature will increase this percentage, as confirmed by our measurements in human plasma, and only keeping the samples for a prolonged time at 37°C allowed prorenin to refold into its closed, inactive conformation. Clearly, depending on the assay conditions, prorenin may be detected as renin, and given its much higher levels than those of renin, this should be taken into account when quantifying renin in patients with low renin levels [23]. Decidua cells neither released angiotensinogen themselves, nor generated ACE. Yet, these components are present in the 10% FCS added to the medium [24], and obviously this should be corrected for when studying angiotensin generation by decidua cells.

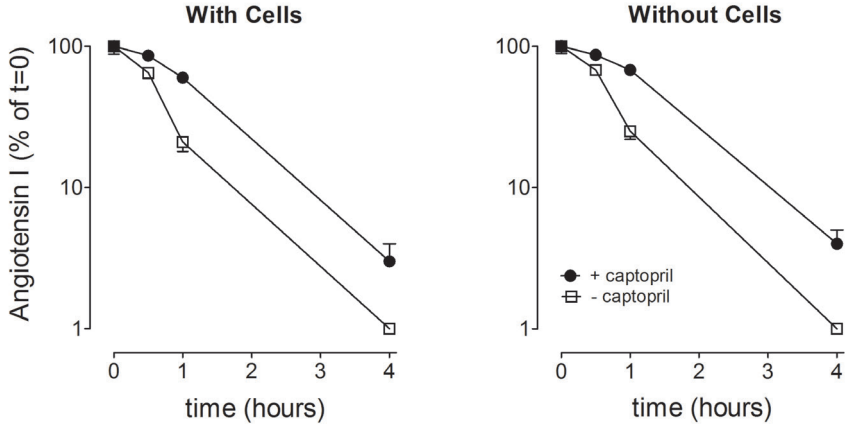

Figure 7. Left, angiotensin I levels in the medium of human decidua cells in the presence (closed circles) or absence of 100 nmol/L captopril (open squares) after adding 100 nmol/L angiotensin I at t=0. Right, angiotensin I levels in medium alone (i.e., without cells), in the presence or absence of 100 nmol/L captopril after adding 100 nmol/L angiotensin I at t=0. Data are mean±SEM of n=5-7, and have been expressed of the levels of angiotensin I at t=0.

In summary, the function of prorenin, if any, remains elusive. The discovery of the (pro)renin receptor [8] caused a lot of excitement, but recent studies have revealed that its in-vivo function in reality is largely, if not entirely, prorenin-independent [25-27]. All studies investigating prorenin binding to this receptor applied highly non-physiological concentrations, which have no relevance in vivo [10, 28]. Our current studies do not reveal evidence for the release of open, active prorenin by prorenin-synthesizing cells. This argues against prorenin activity at the site of its release, at least when applying cell culture conditions, but does not exclude that there are sites where prorenin may display activity. At such sites, either a prorenin-activating enzyme should be present, or conditions favouring prorenin unfolding, e.g. a low pH or a novel, high-affinity prorenin-binding receptor. Finally, to minimize the contribution of prorenin in renin assays, samples should not be exposed to low temperatures

and preferably be incubated immediately. However, since this is not feasible in daily clinical practice, the best is to centrifuge them at room temperature (i.e., not at 4°C), to freeze and thaw the samples only once, and to incubate them as long as possible at 37°C in the assay.

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Chapter 5

RENIN AND PRORENIN UPTAKE BY HUMAN PROXIMAL TUBULE CELLS: ROLE OF MEGALIN?

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ABSTRACT
Renin is filtered by the glomerulus, and subsequently reabsorbed in the proximal tubule in a megalin-dependent manner, as evidenced by a 20-40-fold rise in urinary renin in patients with Dent disease or Lowe syndrome, i.e. disorders characterized by defective proximal tubular reabsorption. Remarkably, the reabsorption of filtered prorenin in healthy subjects appears to be complete. To further investigate megalin-mediated renin and prorenin reabsorption, human conditionally immortalized proximal tubule epithelial cells (ciPTEC) were incubated at 37°C or 4°C with recombinant human renin or prorenin (1000 U/L), in the absence or presence of the mannose 6-phosphate (M6P) receptor blocker M6P or the recombinant megalin ligand receptor-associated protein bound to glutathione-S-transferase (RAP-GST). Cell lysate (pro)renin levels were measured by immunoradiometric assay both before and after prorenin activation. At 37°C, cellular renin and prorenin accumulation leveled off after 6-8 hours, and the majority of prorenin was efficiently converted to renin. Incubating ciPTEC at 4°C diminished the cellular accumulation of renin and prorenin by ≈90%, and at this temperature prorenin remained in its inactive conformation. Both M6P and RAP-GST blocked renin and prorenin uptake by >80% at 37°C, without affecting prorenin activation. Blocking effects at 4°C were more modest. Importantly, M6P blocked the uptake of fluorescently labeled albumin (IC_{50} ≈7 mmol/L) to the same degree as the megalin inhibitor RAP-GST (IC_{50} ≈140 μg/mL). Since albumin is internalized in a megalin-dependent manner, these data indicate that M6P, on top of blocking M6P receptors, also interferes with the megalin-dependent uptake process. In conclusion, ciPTEC bind and internalize renin and prorenin in a time- and temperature-dependent manner. This process involves megalin and/or M6P receptors, and results in prorenin activation at 37°C.

INTRODUCTION

The kidney is a major player in the maintenance of the body fluid-electrolyte homeostasis. This is achieved by complex mechanisms, in which 3 major processes occur: glomerular filtration, tubular secretion, and tubular reabsorption [1]. Kidney proximal tubular epithelial cells (PTEC) are responsible for 60-70% of the reabsorption of water, NaCl, $NaHCO_3$, and for the majority of the reabsorption of nutrients in the glomerular ultrafiltrate [2]. Fluid and electrolyte reabsorbtion by the proximal tubule (PT) is the result of transcellular and paracellular fluxes, with the participation of both active transport and passive paracellular electrochemical diffusion [3]. The uptake of proteins filtered by the glomerulus in the PT largely relies on the endocytic receptors megalin and cubilin [4]. For instance, albumin as well as the renin-angiotensin system (RAS) components renin and angiotensinogen are reabsorbed in the PT by endocytotic uptake in a megalin-dependent manner [5].

Megalin is a large glycosylated receptor of 600 kDa that belongs to the low-density lipoprotein receptor family, encoded by the *LRP2* gene. More than 40 ligands have been identified that bind to megalin by its large extracellular domain. It works in con-

cert with cubilin, a 460-kDa glycosylated extracellular protein, forming a multireceptor complex with the bound ligands, driving their internalization and subsequent endocytosis [4, 6, 7]. Both megalin and cubilin are abundantly coexpressed in the PT apical (brush-border) membrane, and the physiological and pathological roles of these multiligand receptors have been detected in animal models and human allelic syndromes [6]. For instance, the loss-of-function mutations in either the *CLCN5* gene, responsible for Dent-1 disease, or the *OCRL* gene, responsible for Dent-2 disease and Lowe syndrome, lead to dysfunctional receptor-mediated endocytosis and lysosomal function, in which the expression or trafficking of receptors (including megalin and cubilin) is impaired [8]. This results in low-molecular weight (LMW) proteinuria, hypercalciuria with nephrolithiasis, nephrocalcinosis, and other variable proximal tubular defects [8]. Interestingly, in patients with Dent or Lowe syndrome, both renin and angiotensinogen rise 20-40 fold in urine, supporting impaired reabsorption [9]. Moreover, in such patients prorenin becomes detectable in urine, while normally this precursor of renin is entirely undetectable in urine, even in patients with elevated plasma prorenin levels, like diabetics, or in transgenic animals with prorenin levels that are >1000-fold above normal [9].

To investigate megalin-mediated renin and prorenin reabsorption in further detail, we used human conditionally immortalized proximal tubules cells (ciPTEC), a cell line that is highly suitable to study the effect of ligands on PTEC function, since, unlike other cell lines, it expresses multiple influx and efflux transporters, in addition to the whole endocytosis machinery [10, 11]. Cells were incubated with recombinant human renin or prorenin at 37°C (allowing binding and internalization) or 4°C (allowing binding only), in the presence or absence of the megalin inhibitor recombinant receptor-associated protein bound to glutathione-S-transferase (RAP-GST). Because renin and prorenin are also known to be internalized via mannose 6-phosphate (M6P) receptors, binding and uptake were additionally studied in the presence of the M6P receptor antagonist M6P. Finally, we evaluated to what degree M6P interferes with megalin-mediated processes, making use of the fact that ciPTEC internalize bovine serum albumin (BSA) exclusively via megalin [10].

METHODS

Cell Culture

The ciPTEC line was derived from a healthy donor [10]. Cells were cultured in phenolred-free DMEM/F12 (Invitrogen, Breda, the Netherlands) supplemented with 10% (v/v) FCS (MP Biomedicals, Uden, The Netherlands), containing insulin (5 µg/mL), transferrin (5 µg/mL), selenium (5 ng/mL), hydrocortisone (36 ng/mL), epithelial growth factor (10 ng/mL), and tri-iodothyronine (40 pg/mL). The cells were seeded at a density of 55000 cells per cm^2 in 96- or 24-wells plates (Costar, Corning, New York, USA). They were cultured for 24 hours at 33°C and matured for 7 days at 37°C, in 5% CO_2.

Prorenin and Renin Binding and Uptake

To study prorenin and renin binding and internalization by ciPTECs, cells were incubated at 4°C or 37°C with 1000 U/L recombinant human prorenin and renin (a gift from Actelion Pharmaceuticals, Alschwill, Switzerland) diluted in phosphate buffer. Incubations lasted up to 24 hours, and were performed in 24-well plates in the absence or presence of M6P (10 mmol/L) and/or recombinant RAP-GST (100 µg/L). After incubation, the medium was removed, and the cells were washed three times with ice-cold Hank's Balanced Salt Solution (HBSS). Then, the cells were lysed with ice-cold 0.2% triton X-100, and centrifuged for 15 minutes at 4°C at 14000 rpm. Supernatants were collected and stored at -20°C. Immunoreactive renin in the supernatants was measured with the Renin III (Cisbio, Gif-sur-Yvette, France) immunoradiometric assay. Prorenin was also measured with this assay, after its conversion to the renin conformation (allowing its detection in the assay) by incubating it with aliskiren [12, 13]. Prorenin measurements were performed both before and after aliskiren incubation, with levels being detected before aliskiren exposure representing prorenin that had been activated by the cells, and levels after aliskiren exposure representing the total amount of prorenin.

Albumin Uptake

To study whether M6P interferes with megalin-mediated uptake, cells were incubated in a 96-well plate setup at 37°C with 10 µg/mL fluorescently labelled BSA (BSA Alexa Fluor™ conjugate, Invitrogen , Carlsbad, USA), in the absence or presence of M6P (1 µmol-100 mmol/L) or RAP-GST 1.5-200 µg/mL) for 4 hours [10]. During the last 10 minutes, the Hoechst33342 stain (Life Technologies, Carlsbad, USA) was added to dye the nucleus. Then, the medium was removed, and the wells were washed with HBSS at room temperature. Florescence readings were performed using a CV7000S high-content imager (Yokogawa, Tokyo, Japan).

Data Analysis

Results are shown as mean±SEM. Differences were tested using two-way ANOVA, followed by Bonferroni multiple comparison test. $P<0.05$ was considered significant. Data below detection limit were assumed to equal the detection limit.

RESULTS

Prorenin and Renin Binding and Uptake

Incubating ciPTEC with either prorenin (Figure 1) or renin (Figure 2) at 37°C resulted in a time-dependent increase in their cellular levels, a plateau being reached after 6-8 hours. The majority of cellular prorenin (up to 90%) was recognized as renin,

suggestive for prorenin activation upon binding and/or internalization. In contrast, following incubation at 4°C, the majority of prorenin was in its inactive form, and the steady-state levels of both renin and prorenin were ≈5-10-fold lower than those at 37°C (Figures 1 and 2). At 37°C, M6P reduced cellular prorenin ($P<0.05$) and renin ($P<0.01$) accumulation by >80%, without altering the degree of prorenin activation. At 4°C, the blocking effect of M6P was much more modest, significance being reached in the case of renin only ($P<0.05$).

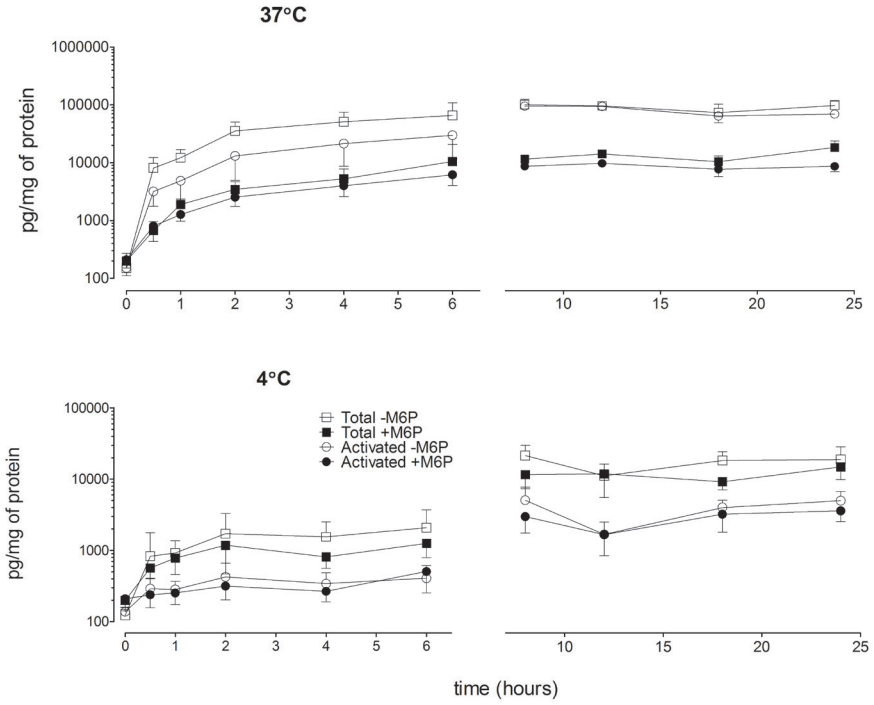

Figure 1. Time-dependent increase in the cellular levels of total and activated prorenin after incubation with 1000 U/L of prorenin in the absence or presence of 10 mmol/L M6P at 37°C or 4°C. Data (mean±SEM of 3-5 experiments) are expressed per mg of protein. Please note the difference in scale for the 37°C or 4°C data.

At 37°C, RAP-GST reduced cellular prorenin and renin accumulation after 8 hours by >80%, both in the absence and presence of M6P (Figure 3). At 4°C, its blocking effects were more modest, particularly in the presence of M6P.

Albumin Uptake

Both M6P and RAP-GST blocked the uptake of labelled BSA at 37°C in a concen-

tration-dependent manner, with IC_{50}'s of ≈ 7 mmol/L and 140 µg/mL (corresponding with 21 µmol/L), respectively (Figure 4).

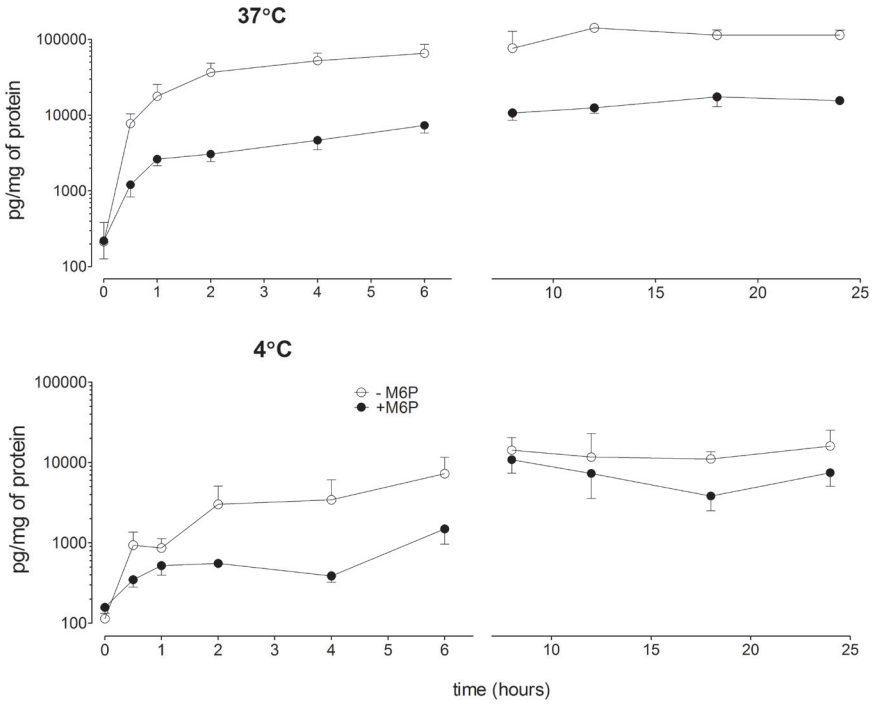

Figure 2. Time-dependent increase in the cellular levels of renin after incubation with 1000 U/L of renin in the absence or presence of 10 mmol/L M6P at 37°C or 4°C. Data (mean±SEM of 3-5 experiments) are expressed per mg of protein

DISCUSSION

The aim of this study was to investigate megalin-mediated renin and prorenin uptake by ciPTEC. PT physiology involves complex mechanisms for substance transporting. Until now, information about such transport arose from cell lines expressing at most a few of the many transporters physiologically present in the PT, while megalin endocytosis has been investigated predominantly in non-human cells [11]. ciPTEC express most of these transporters and efflux pumps, and possess the complete endocytosis machinery, thus making these cells a more appropriate model to study PT physiology [11].

The RAS is essential to maintain fluid-electrolyte homeostasis and blood pressure regulation. Moreover, the RAS also participates in the pathophysiology of cardiovascular and renal diseases, and consequently, its blockade is currently the crux in the

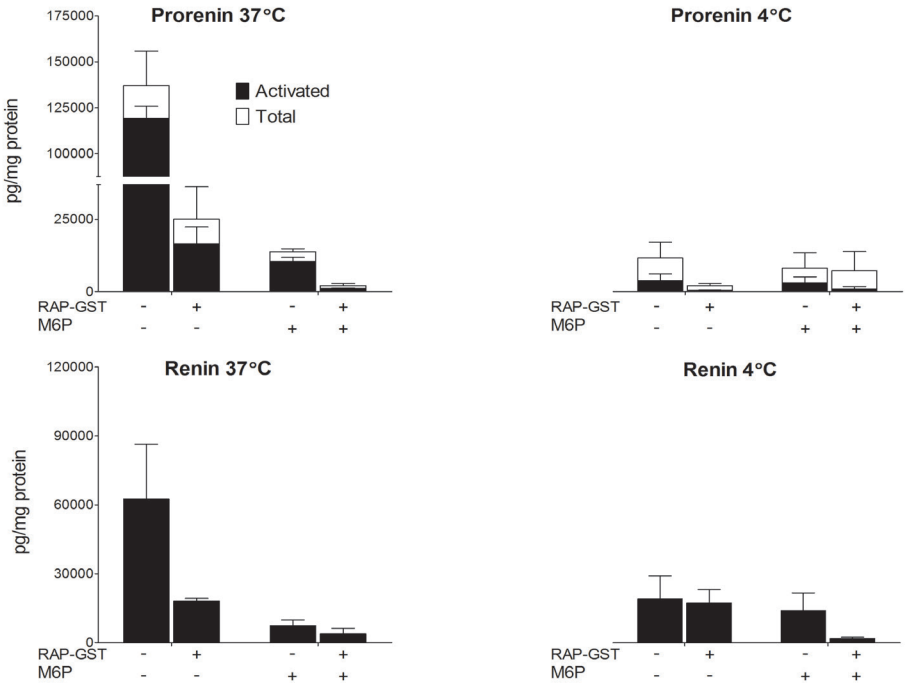

Figure 3. Cellular levels of prorenin (activated and total) and renin in the presence or absence of 10 mmol/L M6P and/or 100 µg/mL RAP-GST in ciPTEC exposed to prorenin or renin (both 1000 U/L) at 37°C or 4°C. Data (mean±SEM of 3 experiments) are expressed per mg of protein

treatment of these pathologies [14, 15]. Recently, it has been suggested that urinary levels of RAS components, specifically renin and angiotensinogen, correlate to intrarenal RAS activity, thus making it possible to monitor hypertension worsening and kidney function deterioration by simply measuring renin and/or angiotensinogen in urine [16, 17]. Yet, confusingly, although multiple studies suggest that angiotensinogen is synthesized in the PT (supporting the existence of an independent intrarenal RAS), renal angiotensin II generation turned out to depend exclusively on liver-derived angiotensinogen [18], i.e., it was not affected by renal angiotensinogen KO, both under normal and pathological conditions. If so, a renal angiotensinogen uptake process might be a prerequisite for renal angiotensin production, e.g. involving megalin-mediated endocytosis [5]. Curiously, prorenin, which is undetectable in urine, has been detected by Roksnoer et al. in urine of patients with Dent disease and Lowe syndrome, two disorders that are characterized by mutations that impair receptor-mediated endocytosis in the PT [9]. This suggests that, normally, prorenin is completely reabsorbed from urine via the megalin pathway. This is not the case for renin, since renin can be detected in urine at levels corresponding to 6-7% of its plasma levels [17]. Nevertheless, renin levels rose 20-40 fold in urine of patients with Dent disease and Lowe syn-

drome, in parallel with the levels of angiotensinogen. This finding, together with the close correlation between angiotensinogen and albumin levels in urine [9, 17, 19], argues against tubular secretion of renin and angiotensinogen, and rather suggests that these urinary components are derived from blood via glomerular filtration.

In many studies with different cell types such as rat vascular smooth muscle cells, cardiomyocytes, fibroblasts, and human endothelial cells, it has been demonstrated that besides megalin, M6P receptors also bind and internalize renin and prorenin [20-22]. Moreover, M6P receptor-mediated prorenin uptake resulted in prorenin activation, most likely as part of an intracellular clearance process, evidenced by the fact that we were unable to link this to intracellular angiotensin generation [21, 23]. In the current study, the M6P-induced decrease in prorenin and renin uptake by ciPTEC suggests that these cells also display M6P receptor-mediated uptake of renin and prorenin. Not surprisingly, the effect of M6P was best seen at 37°C, since at this temperature (but not at 4°C), M6P receptors cycle continuously between the cell surface and the intracellular compartment, thus allowing substantial (pro)renin accumulation in the cells. In contrast, at 4°C, renin and prorenin can only bind to cell surface M6P receptors, without being internalized [24], nor can prorenin activation occur. Thus, at 4°C, cellular renin/prorenin accumulation is very limited, making it more difficult to show blockade of this effect by M6P.

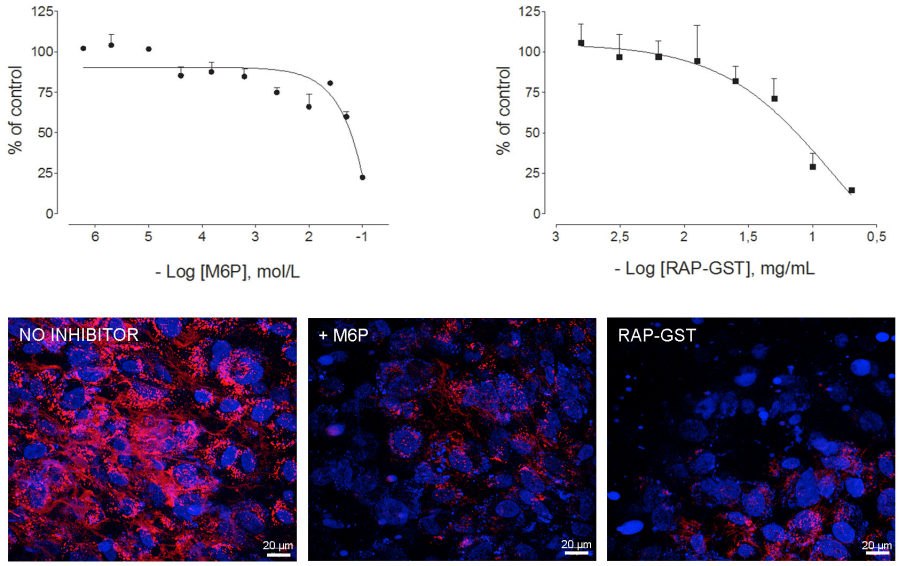

Figure 4. Inhibition of BSA uptake in ciPTEC by M6P and RAP-GST (top panels), and fluorescence staining representing BSA accumulation (bottom panels). Data (mean of 2-4 experiments) have been expressed as a percentage of control (cells incubated with BSA in the absence of inhibitor)

Two different M6PRs have been identified: the cation-independent type (or

insulin-like growth factor II [IGFII] receptor) and the cation-dependent type. The IGFII/M6P receptor harbours two M6P binding sites, and is therefore able to bind to M6P-containing proteins, like renin and prorenin [20]. Interestingly, this receptor was also described as a ligand for the megalin receptor [25]. To what degree M6P itself acts as a ligand for the megalin receptor is unknown. Our results now show that the megalin inhibitor RAP-GST exerted identical effects as M6P (up to 90% inhibition of renin/prorenin uptake at 37°C, and a comparable, but much smaller effect at 4°C), and that both antagonists still exerted effects when added on top of each other. These data are suggestive for an interaction between megalin and M6P receptors and/or imply that M6P additionally blocks megalin-mediated pathways. To evaluate the latter, we studied the effect of M6P on the uptake of BSA, a widely accepted megalin ligand [7]. Importantly, M6P not only blocked BSA uptake to the same degree as RAP-GST, but it also decreased RAP-GST endocytosis (data not shown). This therefore strongly suggests that M6P is a megalin receptor blocker.

In summary, ciPTEC bind and internalize renin and prorenin in a time- and temperature-dependent manner. This process involves megalin and/or M6P receptors, and results in prorenin activation at 37°C. Both M6P and RAP-GST block this phenomenon to the same degree, most likely because M6P additionally blocks megalin-mediated uptake.

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Chapter 6

EFFECTS OF DUAL BLOCKADE OF THE RENIN-ANGIOTENSIN SYSTEM IN PATIENTS WITH HEART FAILURE AND RENAL DYSFUNCTION: SYSTEMATIC REVIEW AND META-ANALYSIS

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Submitted

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ABSTRACT

Renin-angiotensin system (RAS) blockade improves morbid-mortality in heart failure (HF) patients. Although the combination of angiotensin-converting enzyme inhibitor (ACEi) with angiotensin II receptor blocker (ARB) or aliskiren blocks RAS more efficiently, the effect of dual RAS inhibition in HF patients is still controversial, and it has been associated with worsening of renal function. Since renal dysfunction (RD) in HF patients is frequent, investigating the effects of dual blockade of RAS in patients with HF and RD is crucial. We performed a systematic review and meta-analysis of randomized clinical trials in HF patients with RD treated with dual therapy to analyse death, cardiovascular (CV) death, HF hospitalization and adverse events. Cochrane, Embase, LILACS, PubMed and Web of Science were searched. Of 4,794 screened records, 4 studies for quantitative analyses were included (13,641 patients). The meta-analysis demonstrated that dual RAS inhibition reduced the Hazard Ratio for death to 0.92 (p=0.07) and significantly diminished CV death or HF hospitalization (p=0.0006) in patients with and without RD. Moreover, dual RAS blockade significantly increased the risk for renal impairment (29%), hyperkalemia (32%), hypotension (28%) and discontinuation of treatment due to these adverse events (3.14% versus 1.91% p=0.00001). This meta-analysis demonstrated that dual RAS blockade reduces the risk of CV death and HF hospitalization in patients with and without RD. Although the combination therapy increased the frequency of adverse events, few patients had to discontinue treatment. Therefore, dual RAS inhibition should not be categorically contraindicated, and cost-benefit ratio should be evaluated.

INTRODUCTION

Heart failure (HF) is considered a public health burden affecting about 26 million people worldwide [1]. In adults of emergent nations, the prevalence and incidence achieves 1–2% and 5–10 per 1,000 persons per year respectively [2]. In the USA and Europe, HF is a concern cause of morbid-mortality, and despite the evidence-based treatment improves survival of HF patients, death and hospitalization rate remains high [3-4]. Recently, the European Society of Cardiology Heart Failure Long-Term Registry (ESC-HF-LT-R) involving 12,440 patients, showed an incidence of 14.5% for mortality or HF hospitalization within 1 year in chronic heart failure patients [5].

HF is commonly associated with other comorbidities, such as chronic kidney disease (CKD) [6-7]. For this reason, the prevalence of CKD in HF patients ranges from 32 to 51% [7-11]. Also, the presence of CKD was a predictor of poor prognosis in HF, and the mortality was inversely proportional to the value of the estimated glomerular filtration rate (eGFR) [11].

The blockade of the Renin-Angiotensin System (RAS) with ACEi, ARBs or aliskiren has been widely used to treat HF, and for more than 20 years, it has improved the mortality and morbidity of these patients, decreasing the cardiovascular mortality and hospitalization in HF by 26% [12-13]. Notwithstanding, it is interesting to note that due to the presence of alternative RAS pathways, the blockade of RAS is not absolute

when using a single drug [14-15]. In order to overcome this problem, several studies investigated whether the combination of RAS inhibitors can improve HF outcome [16-20]. However, the potential benefit of the combination of these drugs to RAS inhibition remains to be elucidated [21].

So far, only two systematic reviews have examined the effect of dual versus single drug RAS inhibition in morbidity and mortality in HF patients [22-23]. Upon analysing 8 studies that included a total of 18,061 patients, Kuenzli et al. concluded that the combination of ACEi and ARBs reduced hospitalizations for HF, but not for all-cause hospitalizations, albeit the cardiovascular mortality has not been analysed. In another meta-analysis, it was observed that the dual blockade in HF patients did not improve either all-cause or cardiovascular mortality, although it did reduce HF hospitalizations. Both of them registered that dual therapy was associated with hyperkalemia, hypotension and renal impairment, suggesting that the risk-benefit ratio does not justify the association. In spite of that, this meta-analysis exhibits some limitations, since the efficacy and safety parameters were examined in heterogeneous cohorts of patients [24], and also they did not evaluate the effects of dual blockade in HF and CKD patients.

Here, in this systematic review and meta-analysis, we compared the effect of dual combination therapy (ARBs and ACEi or aliskiren and ACEi or ARBs) in HF patients with and without renal dysfunction (RD) in all-causes of death, CV death or HF hospitalization and adverse events.

Methods

This systematic review adheres to the Cochrane Collaboration methodology and was conducted following the Preferred Reporting Items for Systematic Reviews and Meta-Analysis PRISMA Checklist [25]. We registered this study on PROSPERO (Registration# CRD42015029351).

Information sources and search strategy

A specific search was developed for each bibliography database (Supplementary Table S1): the Cochrane Library CENTRAL, Embase, LILACS (Latin American and Caribbean Health Sciences Literature), PubMed and Web of Science. The selection was supplemented including two clinical trial registry databases: ICTRP (WHO International Clinical Trials Registry Platform) and NCT (number of clinical trials, deposit in the clinicaltrials.gov site), two databases of grey literature: Google Scholar and Open Grey, as well as manual search through the reference lists of the articles, included in the first phase. The search included articles published until March 16, 2017.

Study selection

We included randomized clinical trials that investigated the dual blockade of RAS in HF patients and RD, defined as having an eGFR less than 60 mL/min/1.73m^2. The selection was carried out in two stages by two reviewers in an independent man-

ner. In phase 1, in order to select articles that apparently met the selection criteria for inclusion, two authors (A.R.S. and A.G.M.) reviewed all titles and abstracts of the references found. In phase 2, the same authors read the full text of all articles included in phase 1. The studies that did not meet the inclusion criteria were excluded with proper justification. In the absence of consensus between the two authors, the third reviewer (F.A.R.N.) acted as a mediator, to obtain the final decision.

Risk of bias and data collection

The Cochrane Collaboration's tool for Assessing risk of bias for randomized studies was applied. Information was accessed from previously published studies on protocols in order to accurately judge each domain proposed by the tool. In the absence of any data, the authors attempted to contact those responsible for the study. The author of this study contacted 19 authors for further information. Three authors responded, and one provided numerical data. All data collected were checked by the second author (A.G.M.) and reviewed by the third reviewer (F.A.R.N.).

Summary measures

All analyses were performed using the RevMan 5.3 software [26]. For time-to-event outcomes (death, CV death or HF hospitalization,) the log hazard ratio and its standard error were calculated by the "generic inverse variance method" using fixed-effect model. The meta-analysis for adverse event outcome and the relative risk (RR) were assigned considering a 95% confidence interval, conducted by the Cochran-Mantel-Haenszel method, using random-effect model.

RESULTS

Study selection and Study characteristics

We identified 6,447 references in the databases (Figure 1). The excluded articles were identified with the justifications properly described (Supplementary Table S2 and S3). Only 5 articles (ARIANA-CHF-RD 2015, ASTRONAUT 2013, ATMOSPHERE 2016, Val-HeFT 2009 and Val-HeFT 2013) were included for the review, while 4 of them were selected for the quantitative analysis. The ARIANA-CHF-RD [19] study was not included in the quantitative analysis because it did not assess the death, CV death or HF hospitalization outcomes. In addition, it was not possible to combine it with other studies for the adverse event outcomes, since the study only conducted analysis on patients with RD, and the analysis of the adverse events was performed for all participants independent of renal function, due to lack of data in this group. All studies selected are randomized double blind clinical trials, published in English.

Table 1 shows the main characteristics of the studies included. The meta-analy-

sis was performed with the ASTRONAUT [20], ATMOSPHERE [27] and Val-HeFT [28-29] studies. Some studies the outcomes results did not have the group data with and without renal disease.

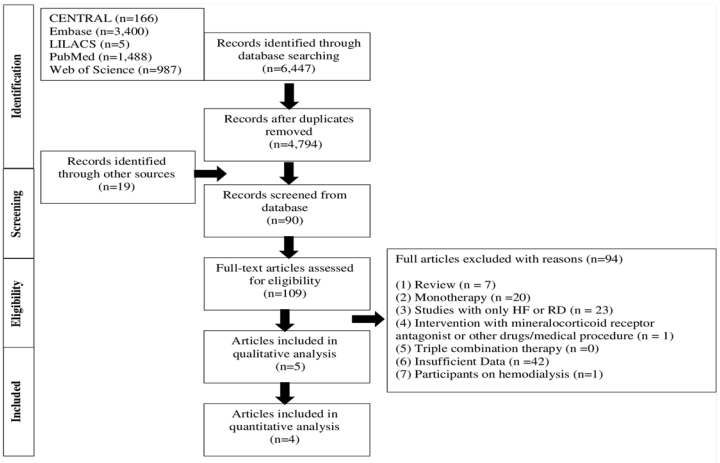

Figure 1 - Flow Diagram of literature search and selection criteria adapted from PRISMA.

Risk of bias

A graph and summary of study quality are presented in Figure 2. The Cochrane Collaboration's tool for assessing the risk of bias was applied (Appendix 1) for each study included, as "high," "low" or "unclear". To evaluate the quality of evidence and strength of recommendations, it was used the Grading of Recommendations, Assessment, Development and Evaluations (GRADE). (Supplementary Table S4).

Death

Initially, we performed a meta-analysis for death involving overall-population from The Val-HeFT, ATMOSPHERE and ASTRONAUT studies. This analysis pointed to a tendency to reduce death of patients using of the combined therapy, however without statistical significance (p = 0.07; Figure 3 – A). Next, we compared the outcome of death for patients with RD (eGFR < 60 mL/min/1.73m^2) versus without RD (≥ 60 mL/min/1.73m^2) in Val-HeFT and ATMOSPHERE studies, since ASTRONAUT study did not publish data separately for these groups. This analysis did not show a statistical difference between the group of patients with or without RD and the total effect was not significant HR 0.94 (0.86-1.02) p=0.16 (Figure 3 -B).

Table 1. Characteristics of included studies. Name and year of the clinical studies; Number of patients enrolled in the study (N); Outcomes evaluated in each study; Follow-up time in months; Percentage of patients with renal dysfunction at baseline; Monotherapy (also referred to placebo) the type of drug classes and the percentage of patients in use. Therapeutic combination referring to dual-blockade with different classes; Percentage of patients using beta-blockers, mineralocorticoid receptor antagonists; Exclusion criterion by renal function for the selection of patients in the studies. Glomerular filtration rate (GFR); Estimated glomerular filtration rate (eGFR); Cardiovascular death (CV) and heart failure (HF) hospitalization; Early worsening of renal function (EWRF); Angiotensin converting enzyme inhibitor (ACEi); Angiotensin II receptor blocker (ARB).

Study name/ Year	N°	Outcomes	Follow-up (months)	Patients with renal dysfunction (%)	Monotherapy Placebo (%)	Combination Therapy (%)	Use of Beta-Blockers (%)		Use of Mineralocorticoid receptor antagonists (%)		Exclusion Criteria (eGFR/creatinine)
							Combination Therapy	Monotherapy	Combination Therapy	Monotherapy	
ARI-ANA-CHF-RD, 2015	39	Renal Blood Flow GFR Adverse Events	6.1	39 (100)	ACEi or ARB (100)	Aliskiren + ACEi or ARB (100)	92.0	86.0	52.0	28.6	< 30 mL/min/ 1.73 m2
ASTRONAUT, 2013	1615	CV death or HF rehospitalization Adverse Events	11.3	629 (38.9)	ACEi or ARB (83.6)	Aliskiren + ACEi or ARB (84.9)	81.7	83.4	55.4	58.6	< 40 mL/min/ 1.73 m2
ATMOSPHERE, 2016	7016	Death, CV death or HF First hospitalization Adverse Events	36.6	1214 (13.7)	Enalapril or equivalent (100)*	Aliskiren + Enalapril (100)	92.0	91.9	36.6	37.8	< 40 mL/min/ 1.73 m2
Val-HeFT, 2009	5010	Death First morbid event	23	2916 (58)	ACEi (92.8)	Valsartan + ACEi (92.6)	34.5	35.3	5.0	4.9	> 2.5 mg/dL
Val-HeFT, 2013	5010	Death, CV death or HF hospitalization EWRF	23	2346 (46.8)	ACEi (92.8)	Valsartan + ACEi (92.6)	34.5	35.3	5.0	4.9	> 2.5 mg/dL

Figure 2. Risk of bias graph and summary: review authors' judgements about each risk of bias item presented as percentages across all included studies

Cardiovascular death (CV) or heart failure (HF) hospitalization

A meta-analysis of Val-HeFT (2013), ASTRONAUT (2013) and ATMOSPHERE (2016) demonstrated that dual blockade reduces the risk for CV death or HF hospitalization in 11% (p= 0.0006) (Figure 3 – C). Interesting, the benefit of dual blockade was similar between the subgroup of patients with RD and without RD. The risk for CV death or HF hospitalization in the subgroup of patients with RD and without RD was diminished by 14% and 9%, respectively (test for subgroup differences – p=0.44). The heterogeneity tests of the meta-analysis exhibited a $Chi^2 = 7.43$ df = 5 (P = 0.19) and I^2 = 33% suggesting an adequate homogeneity between the studies.

Adverse Events and Discontinuation of therapy

Due to the lack of data reported for groups with and without RD, meta-analysis of the adverse events was performed for the overall-population included. The outcomes of hyperkalemia, hypotension, and renal impairment were assessed in ATMOSPHERE [27] and ASTRONAUT [20] studies, but not for the Val-HeFT study, since the data was not published in the latter study. To analyse the therapy discontinuation the data of Val-HeFT, 2001 study were included [16].

Dual RAS blockade therapy increased the risk of adverse events by 30% (29% to renal impairment; 32% to hyperkalemia and 28% to hypotension) (Figure 4 - A). Treatment discontinuation by adverse events was described by 3.14% with RAS dual blockade and 1.91% in monotherapy. The risk for patients using dual RAS blockade increased in 1.69 (p < 0.00001): 1.93 to renal impairment, 1.94 to hyperkalemia and 1.63 to hypotension (Figure 4 - B).

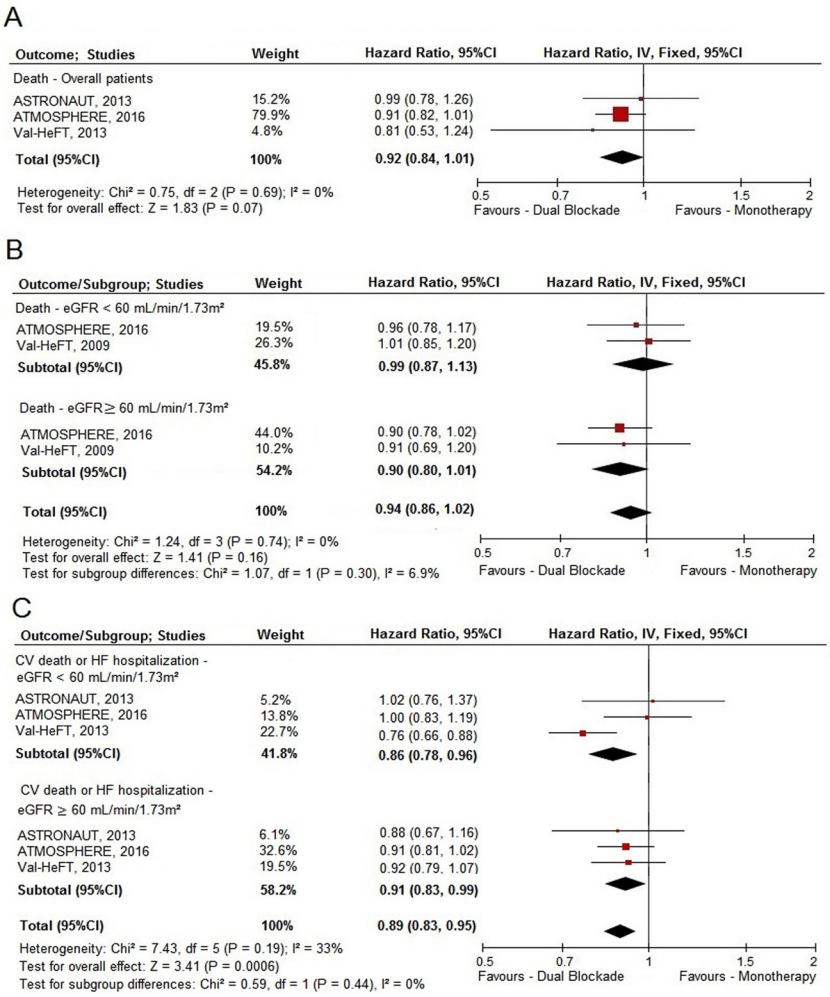

Figure 3 . (A) Meta-analysis of death with overall patients independent of renal function. **(B)** Meta-analysis of death with the subgroups according to the estimated glomerular filtration rate: renal dysfunction (eGFR <60ml/min/1.73 m^2) versus without renal dysfunction (eGFR <60ml/min/1.73 m^2. **(C)** Meta-analysis of cardiovascular (CV) death or heart failure (HF) hospitalization with overall patients and the subgroups according to eGFR: renal dysfunction (eGFR <60ml/min/1.73 m^2) versus without renal dysfunction (eGFR <60ml/min/1.73 m^2. The name and year of the studies are presented followed by weight of each study and the Hazard Ratio (HR) with 95% confidence interval (CI)

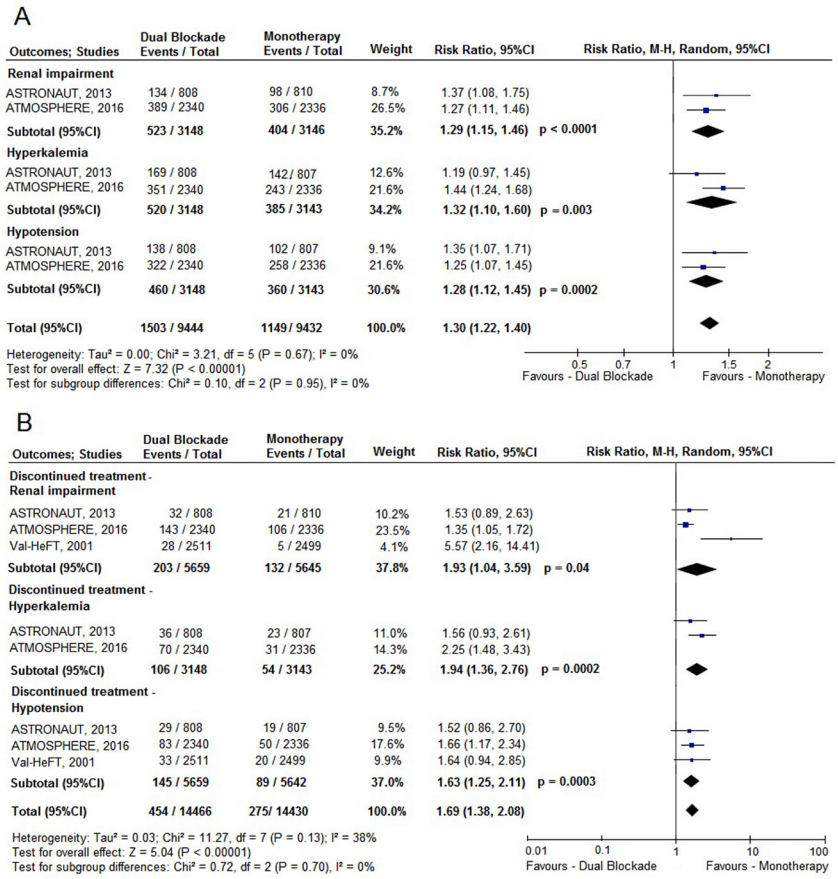

Figure 4. (A) Meta-analysis of adverse events and **(B)** discontinuation of treatment by renal impairment, hyperkalemia, and hypotension involving with overall patients independent of renal function. The graph shows the name and year of the studies followed by the number of events and the total number of participants.

DISCUSSION

Summary of evidence

The importance of the RAS blockade in the treatment of HF is unquestionable. RAS inhibition can be achieved by isolated use of ACEi, ARBs, or aliskiren. Additionally, the combination therapy with two or more of these drugs is an interesting strategy to inhibit the RAS more significantly, since it overcomes alternative pathways for angiotensin II generation [14-15]. Nevertheless, this combination increases the risk of some

adverse effects such as renal impairment [22,23-30], and brings some concerns whether the risk-benefit ratio justifies the combination therapy. Although 30-40% of heart failure patients have renal dysfunction, to our knowledge, the impact of RAS inhibition with combined therapy was not investigated in patients with both disease (HF and RD). Here we described the first systematic review and meta-analysis to examine the effect of dual RAS blockade in patients with heart failure and renal disease.

Only five articles were included for the review [19,20,27-29], while 4 of them were selected for the quantitative analysis. One study exhibited the results with dual blockade using the combination ARBs and ACEi, and two other with aliskiren and ACEi/ARBs.

The meta-analysis displayed that the dual blockade did not reduced the overall-population death outcome, although there was a beneficial tendency in favor of drug therapy combination (p=0.07) (Figure 3- A). This result is similar to previously meta-analysis [22-23], however we must consider that these former meta-analyses did not include the results of the ATMOSPHERE [27] and ASTRONAUT [20] studies, in which the dual RAS blockade occurred with the addition of aliskiren to the therapy.

Next, we compared the role of dual RAS inhibition in the subgroups with and without renal dysfunction, and the meta-analysis exhibited a similar result that was observed in overall population, since death outcome was not statistically significant, between these groups (p=0.16) (Figure 3 - B). It was not possible to include the ASTRONAUT [20] study in this analysis, because the results were not published for the subgroup with and without RD for that outcome

To further investigate whether dual RAS blockade can display a positive impact in HF patients, we examined the CV death or HF hospitalization. Our results demonstrated that RAS dual-blockade significantly reduced the risk of CV death or HF hospitalization by 11% (p= .0006). Interesting, the favorable effect of combination RAS inhibitors was observed in patients with and without RD, since it reduced the hazard ratio in 14% and 9% respectively (Figure 3- C). As a result, the benefit effect of RAS dual-blockade treatment did not differ due to eGFR.

Our meta-analysis enlightens the controversies of the effect of dual RAS blockade in the treatment of HF patients. Val-HeFT study displayed a benefit in favor of the combination therapy [16]. On contrary, the ASTRONAULT and ATMOSPHERE studies did not support the same finding [20,27]. The explanation for theses discrepancies is not clear. An element that could influence would be the reduced percentage of patients in use of beta-blockers (34.5%) and mineralocorticoid receptor antagonists (5%) in the Val-HeFT study (Table 1), when compared to ASTRONAUT (81.7% and 55.4 % respectively) and ATMOSPHERE (92.0% and 36.6%, respectively) [16,20,27,29].

The effect of RAS dual blockade in reducing CV death or HF hospitalization in heart failure patients in our meta-analysis cannot be attributed to the sample size weighting differences in the aforementioned studies. The sample size weighting of Val-HeFT (22.7%) study in the subgroup with RD was larger than ASTRONAUT [20] (5.2%) and ATMOSPHERE [27] (13.8%), but in the subgroup without RD, Val-HeFT (19.5%) was smaller than ATMOSPHERE (32.6%) (Figure 3-C).

Interestingly, another randomized study with dual-blockade using ARB (candesartan) and ACEIs (CHARM-added) in patients with HF registered benefit for CV death or HF hospitalization, HR 0.85 (0.75 - 0.96; 95% CI and p 0.01) [15]. In the group candesartan combined with the ACE inhibitor, 55% were in the use of beta-blocker and 17.4% in the mineralocorticoid receptor antagonists at baseline, and 33% of the patients had an eGFR <60mL/min/1.73m^2. Unfortunately, the results of the subgroups with and without RD were neither published nor available for analysis [18].

The observation of the favorable results of the Val-HeFT (31) and CHARM-added [18] studies for the use of RAS dual-blockade with BRA and ACEi, in contrast to the results of the ASTRONAUT [20] and ATMOSPHERE [27] studies, in which aliskiren was also added, may suggest pharmacological differences in the RAS inhibition pathway. Both ARB and ACEi elevate the levels of angiotensin 1-7 (the product of angiotensin I and II degradation) [31-32], whereas aliskiren, by the upstream blocking of the RAS, does not increase, and may even decrease the concentration of this peptide [33]. Since angiotensin 1-7 antagonizes the deleterious actions of angiotensin II, favoring a vasodilator and anti-proliferative action, displaying cardioprotection in animal models, it is conceivable that combined use of ARB and ACEi justifies the favorable results of the Val-HeFT [29] and CHARM-Added [18] studies but not in ASTRONAUT [20] and ATMOSPHERE [21] studies.

In order to analyse this possibility, we performed a multiple treatment meta-analysis comparing studies that use the dual-blockade with ARB combined with ACEi (CHARM-added [18], VALIANT [17] and Val-HeFT) [29] versus studies using aliskiren added (ASPIRE [34], ASTRONAUT [20] and ATMOSPHERE [27]). The results of the CHARM-added, VALIANT and ASPIRE studies were now included for this analysis. The results (Figure 5) demonstrated a 9% (p=0.010) risk reduction for CV death or HF hospitalization in patients treated with dual-blockade. Nonetheless, this meta-analysis did not observe a difference between the group of patients treated with ABR plus ACEI vs. aliskiren with ACEi/ARB, as the test for subgroup difference effect on the treatment was not significant. It is important to mention that this analysis was not the main objective of the present study.

The results for all adverse events assessed were also not favorable to the use of the dual-blockade treatment (Figure 4). The meta-analysis for adverse events (Figures 4-A) exhibited that there was a 29% increased risk for renal impairment, 32% for hyperkalemia, and 28% for hypotension in patients using dual-blockade therapy when compared to monotherapy. The analysis of treatment discontinuation due to adverse effects (Figure 4-B) demonstrated that the combination therapy led to 3.59% (vs. 2.34%) discontinuation due to renal impairment, 3.37% (vs.1.72%) for hyperkalemia and 2.56% (vs. 1.58%) for hypotension.

These results clearly demonstrate that dual-blockade therapy increases the risk of adverse effects. However, considering that the meta-analysis for CV death or HF hospitalization suggests a beneficial effect within the association of ARB and ACEi or the addition of aliskiren to ARB/ACEI, it is recommended to evaluate the cost-benefit ratio on each patient, since these adverse effects can be monitored by routine exams.

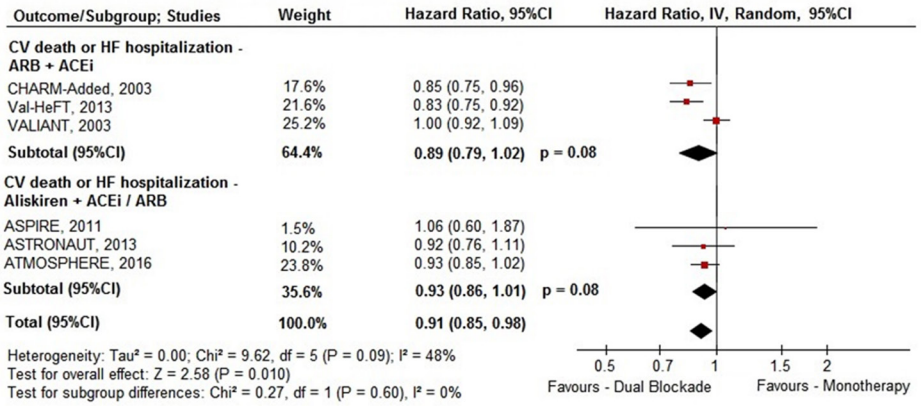

Figure 5. Multiple treatment meta-analysis to compare ARB combined with ACE inhibitors versus and aliskiren combined with ARB/ACEi on the effect of dual-blockade on cardiovascular death (CV) or heart failure (HF) hospitalization. The name and year of the studies are presented followed by the by weight of each study and the Hazard Ratio (HR) with 95% confidence interval (CI)

A new proposal for the dual RAS blockade in HF patients is currently being addressed with the novel angiotensin-neprilysin novel inhibitor LCZ696 (Sacubitril and Valsartan) [35]. The PARADIGM-HF study evidenced superiority of LCZ696 vs. enalapril in terms of death and hospitalization for heart failure, displaying a 20% risk reduction in death from cardiovascular causes or hospitalization for heart failure with similar results between groups with eGFR higher or lower than 60 mL/min/1.73m^2. The LCZ696 group had higher proportions of patients with hypotension and non-severe angioedema, but lower proportions of renal impairment, hyperkalemia, and cough when compared to the enalapril group [35].

Quality assessment

The risk assessment of bias by the Cochrane Collaboration tool for assessing risk of bias [36] (Figure 2) was mostly graded as low risk of bias. The evaluation of GRADE ranged from moderate to high according to the outcomes analysed (Table S4). Thus, these evaluations did not show important biases that could affect the interpretation of the meta-analysis results [37].

There was only one trial evaluated as high risk of bias (domain of selective reporting) for the Val-HeFT study (2013). However, this assessment was due to the lack of pre-specification in the protocol regarding CV death or HF hospitalization outcome [29]. Nevertheless, we cannot discard this study result for this reason, since its protocol was established within the analysis of death outcomes and morbid events. In addition, despite some differences among the studies, the main clinical characteristics of the

participants are similar for performing the meta-analysis.

Limitations

The limitations to be pointed out is the small number of studies available in the literature that have evaluated subgroups of patients with HF and RD. An additional limitation is that the results extracted for this review analyse subgroups of randomized clinical trials involving HF patients. Initially in the protocol of this review, it was planned to analyse patients with HF and concomitant chronic kidney disease, however, most of the studies did not evaluated the presence of proteinuria and normal eGFR.

There was also a variation of the average follow-up of the participants among the studies (Table 1). The lowest being the ARIANA-CHF-RD [19] with 6.1 months, ASTRONAUT [20] with 11.3 months, Val-HeFT with 23 months while the largest was the ATMOSPHERE with 36.6 months [19,20,27].

Conclusions

The present meta-analysis demonstrated that the use of dual RAS blockade in patients with heart failure and renal dysfunction reduces the risk of CV death and HF hospitalization by 11%. Furthermore, this benefit remains in patients with and without renal dysfunction. Even though the combination of RAS inhibition therapy increase the risk of adverse events such as hypotension, hyperkalemia, and renal impairment, only a small percentage of patients had to discontinue the treatment.

This meta-analysis contributes to the discussion whether dual RAS blockade in patients with heart failure and renal dysfunction should be recommended. Clinicians might be aware that dual RAS blockade in HF may not be categorically contraindicated and the relationship of the cost-benefit ratio for each patient should be evaluated.

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Supplemental Information

Supplementary Table S1. Search strategies with appropriated key words and MeSH terms.

Database	Search
EMBASE Result: 7.886 Filters type of articles: Article Result: 3.208	(('heart failure'/exp OR 'heart failure' OR 'cardiac decompensation' OR 'cardiac failure' OR 'cardiac incompetence' OR 'cardiac insufficiency' OR 'cardiac stand still' OR 'cardial decompensation' OR 'cardial insufficiency' OR 'chronic heart failure' OR 'chronic heart insufficiency' OR 'decompensation, heart' OR 'heart backward failure' OR 'heart decompensation' OR 'heart incompetence' OR 'heart insufficiency' OR 'myocardial failure' OR 'myocardial insufficiency') AND ('Kidney Disease'/exp OR 'Kidney Disease' OR 'disease, kidney' OR 'kidney diseases' OR 'kidney disorder' OR 'nephropathy' OR 'renal disease' OR 'renal disorder')) AND ('renin angiotensin aldosterone system'/exp OR 'renin angiotensin aldosterone system' OR 'angiotensin renin system' OR 'renin aldosterone system' OR 'renin angiotensin system' OR 'renin-angiotensin system' OR 'system, renin angiotensin' OR 'system, renin angiotensin aldosterone' OR 'angiotensin 1 receptor antagonist'/exp OR 'angiotensin 1 receptor antagonist' OR 'angiotensin 1 receptor blocker' OR 'angiotensin 1 receptor blocking agent' OR 'angiotensin AT1 antagonist' OR 'angiotensin AT1 blocker' OR 'angiotensin AT1 blocking agent' OR 'angiotensin AT1 receptor antagonist' OR 'angiotensin AT1 receptor blocker' OR 'angiotensin AT1 receptor blocking agent' OR 'angiotensin II type 1 receptor antagonist' OR 'angiotensin II type 1 receptor blocker' OR 'angiotensin II type 1 receptor blockers' OR 'angiotensin II type 1 receptor blocking agent' OR 'AT1 antagonist' OR 'AT1 blocker' OR 'AT1 blocking agent' OR 'AT1 receptor antagonist' OR 'AT1 receptor blocker' OR 'AT1 receptor blocking agent' OR 'dipeptidyl carboxypeptidase inhibitor'/exp OR 'dipeptidyl carboxypeptidase inhibitor' OR 'ACE inhibitor' OR 'angiotensin converting enzyme inhibiting agent' OR 'angiotensin converting enzyme inhibitor' OR 'angiotensin converting enzyme inhibitors' OR 'angiotensin i converting enzyme inhibitor' OR 'angiotensin-converting enzyme inhibitors' OR 'converting enzyme inhibitor' OR 'dipeptidyl carboxypeptidase i inhibitor' OR 'kininase ii inhibitor' OR 'aliskiren'/exp OR 'aliskiren' OR 'aliskiren hemifumarate' OR 'aliskiren hydrochloride' OR 'enviage' OR 'rasilez' OR 'riprazo' OR 'sprimeo' OR 'tekturna' OR 'aliskiren plus valsartan'/exp OR 'aliskiren plus valsartan' OR 'aliskiren hemifumarate plus valsartan' OR 'aliskiren-valsartan' OR 'aliskiren/valsartan' OR 'valsartan plus aliskiren' OR 'valsartan plus aliskiren hemifumarate' OR 'valsartan-aliskiren' OR 'valsartan/aliskiren' OR 'valturna')
LILACS (May 20th, 2016)	(tw:((tw:((tw:((mh:("Heart Failure")) OR (mh:("Insuficiência Cardíaca")) OR (mh:("Insuficiencia Cardíaca")) OR (mh:("Insuficiência Cardíaca Congestiva")))) OR (tw:((tw:((tw:("Heart Failure")) OR (tw:("Insuficiência Cardíaca")) OR (tw:("Insuficiencia Cardíaca")) OR (tw:("Insuficiência Cardíaca Congestiva")))))))) AND (tw:((tw:((mh:("Kidney Diseases")) OR (mh:("Nefropatias")) OR (mh:("Doença Renal")) OR (mh:("Enfermedades Renales")))) OR (tw:((tw:("Kidney Diseases")) OR (tw:("Nefropatias")) OR (tw:("Doença Renal")) OR (tw:("Enfermedades Renales")))))))) AND (tw:((tw:((mh:("Angiotensin-Converting Enzyme Inhibitors")) OR (mh:("Inibidores da Enzima Conversora de Angiotensina")) OR (mh:("Inhibidores de la Enzima Convertidora de Angiotensina")) OR (mh:("Inibidores da ACE")) OR (mh:("Angiotensin II Type 1 Receptor Blockers")) OR (mh:("Bloqueadores do Receptor Tipo 1 de Angiotensina II")) OR (mh:("Bloqueadores del Receptor Tipo 1 de Angiotensina II")) OR (mh:("Bloqueador do Receptor Tipo 1 de Angiotensina II")) OR (mh:("Bloqueador do Receptor do Tipo 1 de Angiotensina II")) OR (mh:("Aliskiren")) OR (mh:("Alisquireno")))) OR (tw:((tw:("Angiotensin-Converting Enzyme Inhibitors")) OR (tw:("Inibidores da Enzima Conversora de Angiotensina")) OR (tw:("Inhibidores de la Enzima Convertidora de Angiotensina")) OR (tw:("Inibidores da ACE")) OR (tw:("Angiotensin II Type 1 Receptor Blockers")) OR (tw:("Bloqueadores do Receptor Tipo 1 de Angiotensina II")) OR (tw:("Bloqueadores del Receptor Tipo 1 de Angiotensina II")) OR (tw:("Bloqueador do Receptor Tipo 1 de Angiotensina II")) OR (tw:("Bloqueador do Receptor do Tipo 1 de Angiotensina II")) OR (tw:("Aliskiren")) OR (tw:("Alisquireno"))))))

Cochrane CENTRAL (May 20th, 2016) PubMed (May 20th, 2016) Web of Science (May 20th, 2016)	((Heart Failure[MeSH Terms] OR "Heart Failure" OR "Cardiac Failure" OR "Heart Decompensation" OR "Right-Sided Heart Failure" OR "Right Sided Heart Failure" OR "Myocardial Failure" OR "Congestive Heart Failure" OR "Left-Sided Heart Failure" OR "Left Sided Heart Failure" OR "Chronic heart failure") AND (Kidney Disease[MeSH Terms] OR "Kidney Diseases" OR "Chronic kidney disease" OR "Chronic kidney diseases" OR "Kidney Disease" OR Renal Insufficiency[MeSH Terms] OR "Renal Insufficiency" OR "Renal Insufficiencies" OR "Kidney Insufficiency" OR "Kidney Insufficiencies" OR "Kidney Failure" OR "Kidney Failures" OR "Renal Failure" OR "Renal Failures" OR "Renal Dysfunction")) AND (Renin-Angiotensin System[MeSH Terms] OR "Renin Angiotensin System" OR Angiotensin II Type 1 Receptor Blockers[MeSH Terms] OR "Angiotensin II Type 1 Receptor Antagonists" OR "Type 1 Angiotensin Receptor Blockers" OR "Angiotensin receptor blocker" OR "Angiotensin 2 Type 1 Receptor Antagonists" OR "Type 1 Angiotensin Receptor Antagonists" OR "Selective Angiotensin II Receptor Antagonists" OR "Sartans" OR Angiotensin-Converting Enzyme Inhibitors[MeSH Terms] OR "Angiotensin-Converting Enzyme Inhibitors" OR "Angiotensin Converting Enzyme Inhibitors" OR "Kininase II Inhibitors" OR "Angiotensin I-Converting Enzyme Inhibitors" OR "Angiotensin I Converting Enzyme Inhibitors" OR "ACE-inhibitor" OR "ACE Inhibitors" OR "Angiotensin-Converting Enzyme Antagonists" OR "Angiotensin Converting Enzyme Antagonists" OR Aliskiren OR "rasilez" OR "Tekturna" OR "direct renin inhibitor" OR "Renin inhibitor")
International Clinical Trials Registry Platform (ICTRP) (May 20th, 2016)	heart failure AND kidney disease AND "Angiotensin II Type 1 Receptor Blockers" OR "Angiotensin I-Converting Enzyme Inhibitors" OR aliskiren
Clinicaltrials.gov (NCT) (May 20th, 2016) Open Grey (May 20th, 2016)	"Heart failure" AND "Angiotensin II Type 1 Receptor Blockers" OR "Angiotensin I-Converting Enzyme Inhibitors" OR aliskiren
Google Scholar (May 20th, 2016) Filters: 1)Relevance Results: First 100 citation AND 2)Year(2016) Results: First 100 citation	heart failure AND kidney disease "angiotensin ii type 1 receptor" OR "angiotensin Converting Enzyme Inhibitors" OR aliskiren

Supplementary Table S2. Excluded articles and reasons for exclusion (n=94)**.**

(1) Review (n = 6);
(2) Monotherapy (n = 20);
(3) Studies with only heart failure or renal disease (n = 23);
(4) Intervention with spironolactone (mineralocorticoid receptor antagonist) or other medicinal or medical procedures (n = 1);
(5) Triple therapy (n=0);
(6) Insufficient data (n = 42);
(7) Hemodialysis participants (n = 1).

Reference number	Author/Year	Reason for exclusion
1	Agarwal R., et al, 2001	3
2	Aleksova, A. et al, 2012	2
3	Anavekar, N. S., et al, 2004	6
4	Anavekar. N.S, et al, 2008	6
5	Azizi, M., et al, 2004	1
6	Azizi, M., et al, 2013	1
7	Becquemont, L., et al, 2015	2
8	Berl, T. et al, 2003	2
9	Berra, G., et al, 2015	2
10	Brunner-La Rocca, H. P., et al, 2015	6
11	Carr, A. A., et al, 2005	2
12	Damman, K., et al, 2016	6
13	de Zeeuw, D., et al, 2004	2
14	Desai, A. S., et al, 2007	6
15	Doulton, T. W. R.,et al, 2009	1
16	Dounaevskaia, V.,et al, 2011	6
17	Edner M, et al., 2015	6
18	Epstein M, et al., 2009	1
19	Evans KL, et al., 2014	2
20	Farcas, A., et al., 2016	6
21	Fernandez Juarez G, et al., 2013	3
22	Fragasso, G. et al, 2013	6
23	Frazee LA, et al., 2006	6
24	Fried LF, et al., 2013	6
25	Frimodt-Moller M, et al., 2010	3
26	Gilbert CJ, et al., 2013	6
27	Gjerde B, et al., 2012	6
28	Granger CB, et al., 2003	2

29	Hackam, D. G., et al, 2008	2
30	Heerspink, H. J. L. et al, 2014	6
31	Heerspink, H. J. L. et al, 2016	3
32	Heerspink, H. J. L. et al, 2016	3
33	Herget-Rosenthal S, et al., 2013	6
34	Hillege, Hans L, et al.,2006	6
35	Horita Y, et al., 2006	3
36	Imai, E. et al, 2011	3
37	Jackson CE, et al, 2011	6
38	Jhund, P. S. et al, 2015	3
39	Kao, D. P. et al, 2015	2
40	Karlsen FM, Kamper AI., 2003	1
41	Khan NA, et al., 2006	2
42	Kuijvenhoven MA, et al., 2013	2
43	Kurnik, D.,et al, 2011	6
44	Maggioni, A. P. et al, 2013	6
45	Maggioni, A. P. et al, 2008	6
46	Mancia G, et al. 2011.	3
47	Mann, Johannes FE, et al, 2008	6
48	Marre M, et al., 2004	2
49	Massie, B. M. et al, 2008	6
50	McAlister,F.A. ,et al,2011	6
51	McKelvie,R.S., et al, 1999	6
52	McMullan,C.J., et al, 2014	4
53	McMurray, J.J.V.,et al, 2003	6
54	McMurray, J.J.V.,et al, 2008	6
55	Mehdi UF, et al., 2009	3
56	Mitrovic, V. et al, 2009	3
57	Miura, M. et al, 2015	6
58	Miyagishima K, et al., 2009	6
59	Molnar,M.Z.,et al, 2014	6
60	Muzzarelli, S.,et al, 2012	6
61	Nagy,G., et al, 2011	3
62	Nakamura, T., et al, 2005	6
63	Noise P, et al., 2011	6
64	Obialo CI, et al., 2002	2
65	Ohsawa M, et al., 2013	3
66	Onuigbo MA, et al., 2008	6
67	Onuigbo MA, et al., 2008	3

68	Oparil, S.,et al, 2007	3
69	Özdemir, M. et al, 2007	3
70	Parving, H. H. et al, 2009	3
71	Parving, H. H. et al, 2012	3
72	Parving, H. H. et al, 2008	3
73	Persson, H.H, et al, 2010	6
74	Pfeffer,M.A et al., 2003	6
75	Pfeffer,M.A et al., 2003	6
76	Remuzzi G, et al., 2004	2
77	Rossing K, et al., 2002	3
78	Russo D, et al., 2001	3
79	Sakata, Y., et al., 2015	6
80	Santana,C. et al,2012	2
81	Schindler C, et al., 2008	1
82	Scrutinio D, et al., 2011	2
83	Segura, J. et al, 2003	3
84	Solomon, S. D. et al, 2011	6
85	Szeto CC, et al., 2013	2
86	Tang,Y.X., et al, 2015	7
87	Tobe SW, et al., 2011	3
88	Ueno H, et al., 2010	6
89	Vaur L, et al., 2003	2
90	Vereijken TL., et al., 2007	6
91	Wetmore JB, et al., 2015	2
92	Wolf G, et al., 2008	6
93	Yusuf, S. et al, 2003	6
94	Yusuf, S. et al, 2008	3

Supplemental References

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Supplementary Table S3. Articles/Clinical trials with abstract or full-text not available

Nº reference	Author / Study protocol / Year	Reason for pending classification
1	1998	No access
2	Blandon, J. A., 2013	No access
3	JPRN-UMIN000002546, 2009	Not published
4	JPRN-UMIN000003678, 2009	Not published
5	JPRN-UMIN000005759, 2011	Not published
6	JPRN-UMIN000008615	No access
7	Kageyama, S. et al, 2008	No access
8	National Taiwan University H, Taipei Veterans General Hospital, 2010	Not published
9	Rutecki, G. W. 2011	No access
10	Yang YH, et al., 2009	No access

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Supplementary Table S4. Summary of the evaluation of the quality of evidence, according to GRADE (Grading of Recommendations Assessment, Development and Evaluation). a. The risk of bias in the report of high selective outcome for the Val-HeFT study. b. Meta-analysis with inconsistency. I^2 = 76%; c. Meta-analysis with moderate inconsistency. I^2 = 56%.

Nº of studies	Study design	Risk of bias	Inconsistency	Indirectness	Imprecision	Quality
Death according to the estimated glomerular filtration rate.						
2	randomised trials	not serious	not serious	not serious	not serious	HIGH
Death						
3	randomised trials	not serious	not serious	not serious	not serious	HIGH
Cardiovascular death or hospitalization for heart failure according to the estimated glomerular filtration rate.						
3	randomised trials	serious a	not serious	not serious	not serious	MODERATE
Renal impairment						
2	randomised trials	not serious	not serious	not serious	not serious	HIGH
Renal impairment - Discontinuation of treatment						
3	randomised trials	not serious	serious b	not serious	not serious	MODERATE
Hyperkalemia						
2	randomised trials	not serious	serious c	not serious	not serious	MODERATE
Hyperkalemia - Discontinuation of treatment						
2	randomised trials	not serious	not serious	not serious	not serious	HIGH
Hypotension						
2	randomised trials	not serious	not serious	not serious	not serious	HIGH
Hypotension - Discontinuation of treatment						
3	randomised trials	not serious	not serious	not serious	not serious	HIGH

Appendix 1. Risk of Bias Assessment using the Cochrane Collaboration's tool

N	Characteristic	Anand et al., 2009	Gheorghiade et al., 2013	Lesogor et al., 2013	McMurray et al, 2016	Schroten et al, 2015
1	Random Sequence Generation (selection bias)	Unclear	Unclear	Unclear	Low	Low
	Support for judgment	Not reported	Not reported	Not reported.	Computer system	Computer system
2	Allocation Concealment (selection bias)	Low	Low	Low	Low	Low
	Support for judgment	Probably done by the coordinating centers	Probably done by the committee	Probably done by the coordinating centers	Central	Central
3	Blinding of Participants and Personnel (performance bias) Primary and Secondary outcomes	Low	Low	Low	Low	Low
	Support for judgment	Double blind. it is unlikely that the blinding could have been broken	Double blind. it is unlikely that the blinding could have been broken	Double blind. it is unlikely that the blinding could have been broken	Double blind. it is unlikely that the blinding could have been broken	Double blind. it is unlikely that the blinding could have been broken
4	Blinding of Outcome Assessment (detection bias) Primary and Secondary outcomes	Low	Low	Low	Low	Low
	Support for judgment	Blinding of outcome assessment ensured, and unlikely that the blinding could have been broken	Blinding of outcome assessment ensured, and unlikely that the blinding could have been broken	Blinding of outcome assessment ensured, and unlikely that the blinding could have been broken	Blinding of outcome assessment ensured, and unlikely that the blinding could have been broken	Blinding of outcome assessment ensured, and unlikely that the blinding could have been broken

5	Incomplete Outcome Data (attrition bias) Primary and Secondary outcomes	Low	Low	Low	Low	Low
	Support for judgment	there was no loss of relevant data	there was no loss of relevant data	there was no loss of relevant data	Missing outcome data balanced in numbers across intervention groups, with similar reasons for missing data across groups;	there was no loss of relevant data
6	Selective Reporting (reporting bias)	Low	Low	High	Low	Low
	Support for judgment	The study protocol is available and it is clear that the published reports include all expected outcomes	The study protocol is available and it is clear that the published reports include all expected outcomes	The secondary outcome reported was not prespecified in protocol	The study protocol is available and it is clear that the published reports include all expected outcomes	The study protocol is available and it is clear that the published reports include all expected outcomes
7	Other Sources of Bias	Unclear	Unclear	Unclear	Unclear	Unclear
	Support for judgment	The study protocol excluded patients with creatinine greater than 2.5mg. The study was conducted 10 years ago.	Exclusion Criteria Patients with lower filtration rate 40 mL / min / 1.73 m2	The study protocol excluded patients with creatinine greater than 2.5mg. The study was conducted 10 years ago.	Exclusion Criteria Patients with lower filtration rate 40 mL / min / 1.73 m2	The study allow inclusion of patients with eGFR up to 75 mL / min / 1.73 m2
	Summary	High=0 Unclear=2 Low=5	High=0 Unclear=2 Low=5	High=1 Unclear=2 Low=4	High=0 Unclear=1 Low=6	High=0 Unclear=1 Low=6

Chapter 7

SUMMARY, CONCLUSIONS AND PERSPECTIVES

SUMMARY and CONCLUSIONS

More than a century has passed since the discovery of renin by Tigerstedt and Bergman in 1898. From that moment, research on the renin-angiotensin system (RAS) has never stopped growing, and notably, renin assumed a prominent position, as it is responsible for the first and rate-limiting step of the RAS cascade. It is now well-established that the RAS is a major player in blood pressure regulation and fluid electrolyte homeostasis, besides its participation in the pathophysiology of renal and cardiovascular diseases. Nevertheless, despite the extensive knowledge acquired so far, renin-producing cells (RPC) are still a conundrum, and only recently, these cells are starting to be deciphered. This thesis focused on RPC, their plasticity, novels aspects and function, beyond perspectives and insights related to these unique cells.

Chapter 1 provides a broad review about the current knowledge on RPC. It summarizes not only classical concepts like the so-called "recruitment" phenomenon, growth and developmental processes, but also novel contexts such as inflammation, cell repair mechanisms and tissue regeneration. **Chapter 2** describes a new approach to understand RPC biology. It is the first report of a gene expression catalogue of human renin-producing tumors, or reninomas, that culminated in the discovery of 36 genes that are new to the RPC gene signature. Noteworthy, the cell models available to study RPC are mouse-derived, and therefore, proper knowledge of human RPCs is limited, relying on speculation on the basis of murine models. This fact makes our approach unique, and furthermore, among the genes identified, the platelet-derived growth factor (PDGF) β - PDGF β receptor signaling pathway was revealed as a promising RPC regulator.

In **chapter 3**, we investigated the existence of the brain RAS, whose activity relies on the assumption that local (pro)renin synthesis by RPC would be a *sine qua non* condition, since the presence of the blood-brain barrier would preclude (pro)renin uptake from blood by central nervous system cells. We were unable to find evidence to support the aforementioned concept. Instead, our data suggest that renin in the brain represents trapped plasma renin and/or locally activated trapped plasma prorenin. These findings uphold the conception that RPC in the kidney are the only renin source in the body.

Chapter 4 discusses the plausibility of 'open' prorenin (prorenin in an open conformation, capable of displaying angiotensin-generating activity) release by renin and/or prorenin-synthesizing cells. After many decades, prorenin's physiological role remains a riddle: is it just renin's inactive precursor or more than that? Juxtaglomerular cells (JGC), i.e. RPC located at the juxtaglomerular apparatus, are the main source of prorenin and the only source of renin in circulation under unstressed conditions. In addition, other tissues are also able to synthesize and release prorenin. Under physiological conditions (pH 7.4, 37°C), prorenin is present in a 'closed' conformation, which cannot result in angiotensin generation. We speculated that prorenin might exert local angiotensin-generating effects, at the site of its release, provided that is released in an open conformation from prorenin-synthesizing cells, before returning to the closed

conformation in the extracellular milieu. Our in-vitro study did not reveal any evidence to support this conjecture, and, thus, at least under cell culture conditions, RPC and other prorenin-synthesizing cells do not release open (active) prorenin.

Chapter 5 investigates renin and prorenin reabsorption by human conditionally immortalized proximal epithelial tubule cells (ciPTEC). The reabsorption of RAS components like angiotensinogen, renin and prorenin occurs in the kidney proximal tubule, in a megalin-dependent manner, and may underlie renal angiotensin production. Patients with Dent's disease or Lowe syndrome (resulting in impaired megalin function) exhibit an increase in urinary renin and angiotensinogen levels, suggestive for diminished reabsorption. Strikingly, the defective reabsorption also allowed the detection of urinary prorenin, which is normally undetectable. We demonstrated that ciPTEC bind and internalize renin and prorenin in a temperature-dependent manner, resulting in prorenin activation at 37°C. This process involved megalin and/or mannose-6-phosphate (M6P) receptors, and surprisingly, the M6P receptor antagonist M6P interfered with megalin endocytosis.

RAS blockade is a cornerstone in the treatment of cardiovascular and renal diseases. In **chapter 6**, we performed a systematic review and meta-analysis of randomized clinical trials in patients with concomitant heart failure and renal dysfunction under dual RAS blockade to analyze death, cardiovascular death, heart failure hospitalization and adverse events such as worsening in renal function, hypotension and hyperkalemia. Even though the number of available trials was limited, our analysis supports the potential benefits of dual RAS blockade, despite the increased frequency of adverse events. Dual RAS inhibition might therefore be used cautiously in certain patient groups, of course keeping in mind that chronic RAS blockade triggers RPC "recruitment" along the afferent arteriole.

FUTURE PERSPECTIVES

Beyond the main function of the RAS as the major regulator of blood pressure and fluid electrolyte homeostasis, RPCs play a much more in-depth role than simply producing and releasing renin. Their huge phenotypical plasticity, exemplified in the "recruitment phenomenon", allows them to work as pluripotent progenitors for different kidney cell types.

Just recently, RPCs have been connected to tissue regeneration, inflammation and possibly fibrosis. Interestingly, our findings have revealed the PDGF β - PDGF β receptor signaling pathway as a potential regulator of the RPCs expression, able to shift their classical renin phenotype into an inflammatory phenotype. Yet, PDGF β is a pericyte marker, and the latter cells are known to wrap vascular walls and to possess a high phenotypical plasticity. Not surprisingly, RPCs are able to differentiate into pericytes and vice versa.

It's noteworthy that our findings do not support the concept of extrarenal sites of renin production, in particular in the brain. Multiple papers have speculated about

(pro)renin production in the brain, possibly intracellularly, and about locally synthesized prorenin displaying activity following its binding to the brain (pro)renin receptor. However, no paper actually quantified brain (pro)renin in an appropriate manner. Our data now support that there is no (pro)renin in the brain except for the small amounts of renin and prorenin in trapped blood in brain tissue (which disappeared after buffer perfusion), thus arguing strongly against angiotensin production at brain tissue sites. Even if prorenin is released locally (e.g., in the ovary), we were unable to show that such prorenin would display activity at its site of release. Together with the recent insight that the (pro)renin receptor has functions beyond the RAS, and is unrelated to prorenin activation, this implies that we need to find novel mechanisms supporting a role for prorenin, if any, in local angiotensin generation. Thus, JGC firmly remain the only source of renin in the body, on which angiotensin generation depends, and whether prorenin generates angiotensins locally under physiological or even pathological conditions remains to be proven.

Within the kidney, apart from JGC, also tubular cells have been proposed to synthesize prorenin. Surprisingly, angiotensin stimulates rather than inhibits this production (positive feedback, as opposed to its negative feedback in JGC), and there is no evidence for tubular renin production. To what degree this prorenin truly contributes to intrarenal angiotensin generation is still controversial; it was even suggested to act on angiotensinogen of tubular origin, for instance when bound to the (pro)renin receptor. Yet, making use of transgenic rodents not expressing renal angiotensinogen it became clear that renal angiotensin production depends entirely on plasma-derived angiotensinogen, i.e., angiotensinogen of hepatic origin. Studies in patients with defective proximal tubular reabsorption support megalin-dependent reabsorption of filtered angiotensinogen, as well as renin and prorenin, and our current ciPTEC data allow a detailed insight into the mechanism of this phenomenon. Unexpectedly, there may be an additional role for M6P receptors in the reabsorption process, and/or M6P itself blocks megalin-mediated processes. Considering the massive (up to 40-fold) rise in RAS components levels in urine in patients with Dent's disease or Lowe syndrome, the idea of tubular synthesis and release of renin, prorenin and/or angiotensinogen needs to be reconsidered. Most likely, changes in urinary (pro)renin and angiotensinogen levels reflect changes in megalin-dependent reabsorption (regulated, among others, by angiotensin!) instead of independent tubular release of RAS components.

The benefit of RAS blockade in cardiovascular and renal disease is indisputed, but what is still controversial is the optimal degree of RAS blockade. A high degree of RAS blockade may have additional beneficial effects, but also results in a higher incidence of side effects. The degree of blockade can be estimated from the increase in circulating renin, depicting the "recruitment" of new RPC along the afferent arterioles. The long-term consequences of this process are still unknown, nor do we known if it contributes to renal impairment in the future. Besides renin production, RPC would participate directly in the concentric vascular hypertrophy, despite the control of the blood pressure levels. Furthermore, many questions remain unanswered. Would these cells contribute to a fibrosis process, leading to architecture disruption and extracel-

lular matrix production? If these cells possess tissue repair mechanisms, why are they switched off or dysfunctional? To what degree is tissue regeneration truly effective to restore the normal function? Would such regeneration be persistent or transient? Answering these questions, combined with a full understanding of local (renal) angiotensin production will ultimately allow more effective anti-hypertensive therapies, and better outcomes in patients with cardiovascular and renal pathologies.

SAMENVATTING en CONCLUSIES

In 1898, meer dan een eeuw geleden, werd renine ontdekt door Tigerstedt en Bergman. Vanaf dat moment is het onderzoek naar het renine-angiotensine systeem (RAS) continu gegroeid, en met name renine heeft hierin een prominente positie ingenomen, daar dit enzym verantwoordelijk is voor de eerste en snelheidsbepalende stap in de RAS keten. Inmiddels staat vast dat het RAS een belangrijke rol speelt in de bloeddrukregulatie en vloeistof- en elektrolythuishouding, naast het leveren van een bijdrage aan de pathofysiologie van nierziekten en hart- en vaataandoeningen. Echter, ondanks de gedetailleerde kennis die tot nu toe is opgedaan, zijn renine-producerende cellen (RPC) nog steeds een raadsel, en pas recentelijk is men begonnen met het ontrafelen van deze cellen. Dit proefschrift richt zich op RPC, hun plasticiteit, nieuwe aspecten en functies, naast diverse andere inzichten in relatie tot deze bijzondere cellen.

Hoofdstuk 1 biedt een breed overzicht van de huidige kennis omtrent RPC. Het beschrijft niet alleen het zgn. "recruitment" fenomeen (het aanzetten van RPC op grote schaal), maar toont ook aan dat deze cellen een rol spelen bij ontsteking, cel-reparatiemechanismen en weefselvernieuwing. **Hoofdstuk 2** beschrijft een nieuwe aanpak om de RPC biologie beter te begrijpen. Het is de eerste rapportage van een genexpressie catalogus van humane renine-producerende tumoren, of reninoma's, welke geresulteerd heeft in de ontdekking van 36 genen die representatief zijn voor RPC. Hierbij moet opgemerkt worden dat de tot dusver beschikbare celmodellen voor de bestudering van RPC zijn afgeleid van de muis, waarbij de relevantie voor de mens twijfelachtig is. Dit maakt onze aanpak uniek en bovendien bleek onder de geïdentificeerde genen platelet-derived growth factor (PDGF) β een veelbelovende RPC regelaar te zijn.

In **hoofdstuk 3** onderzochten we het bestaan van het RAS in de hersenen, waarvan de activiteit is gebaseerd op de aanname dat lokale (pro)renine synthese door RPC een *sine qua non* conditie is, aangezien de aanwezigheid van de bloed-hersen barrière de opname van (pro)renine uit het bloed door cellen van het centraal zenuwstelsel in de weg zou staan. We konden echter geen bewijs voor dit concept vinden. In plaats daarvan suggereren onze data dat renine in de hersenen gewoon het renine is dat in bloed voorkomt, aangezien hersenweefsel ook bloed bevat. Dit betekent dat RPC in de nieren de enige bron voor renine in het lichaam zijn.

Hoofdstuk 4 bespreekt de mogelijkheid van 'open' prorenine (prorenine in een open conformatie, in staat tot angiotensine vorming) afgifte door renine en/of prorenine-synthetiserende cellen. Na vele decennia blijft de fysiologische rol van prorenine een mysterie: is het alleen renine's inactieve voorvorm of is het meer dan dat? Juxtaglomerulaire cellen (JGC), dat wil zeggen RPC die zich in het juxtaglomerulaire apparaat bevinden, zijn de belangrijkste bron van prorenine en de enige bron van renine in de bloedsomloop onder normale omstandigheden. Diverse andere organen zijn ook in staat om prorenine te synthetiseren en af te geven aan het bloed. Onder fysiologische omstandigheden (pH 7.4, 37°C) is prorenine aanwezig in een 'gesloten' conformatie, hetgeen niet kan leiden tot de vorming van angiotensine. We speculeerden

dat prorenine mogelijk lokaal angiotensine kan vormen, op de plaats waar het wordt uitgescheiden, mits deze afgifte geschiedt in een open conformatie voordat het terugkeert naar de gesloten conformatie in de extracellulaire ruimte. Onze in-vitro studie leverde echter geen bewijs op dat deze aanname ondersteunt, en daarom, in ieder geval onder celkweek-omstandigheden, lijkt er geen afgifte van open prorenine door RPC en andere prorenine-synthetiserende cellen te geschieden.

Hoofdstuk 5 onderzoekt de reabsorptie van renine en prorenine door proximale tubulus epitheelcellen uit de nier van de mens. De reabsorptie van RAS componenten, zoals angiotensinogeen, renine en prorenine, vindt plaats in de proximale tubulus van de nier, via het eiwit megaline, en dit proces ligt mogelijk ten grondslag aan de produktie van angiotensine in de nier. Patiënten met een verstoorde megalinefunctie hebben sterk verhoogde renine en angiotensinogeen spiegels in hun urine, wat lijkt te wijzen op een verminderde reabsorptie. Het is opvallend dat een dergelijke verstoorde reabsorptie ook leidt tot de detectie van prorenine in de urine, hetgeen normaliter niet meetbaar is. We vonden dat de epitheelcellen renine en prorenine op een temperatuur- en tijdsafhankelijke manier internaliseerden, waarna bij 37°C prorenine in de cel werd omgezet in renine. Zowel megaline als mannose-6-fosfaat (M6P) receptoren zijn hierbij betrokken, en onverwacht bleek de M6P receptor antagonist M6P ook megaline-afhankelijke endocytose te blokkeren.

Blokkade van het RAS staat centraal bij de behandeling van cardiovasculaire aandoeningen en nierziekten. In **hoofdstuk 6** hebben we alle studies in patiënten met gelijktijdig hartfalen en nierdysfunctie onder duale RAS blokkade (i.e., die 2 RAS blokkers tegelijk kregen) geanalyseerd om tot een uitspraak te komen of de gunstige cardiovasculaire effecten van een dergelijke krachtige RAS blokkade opwegen tegen de bijwerkingen (dood, ziekenhuisopname, verslechtering van de nierfunctie, hypotensie en hyperkaliëmie). Hoewel het aantal beschikbare studies beperkt was, ondersteunt onze analyse de potentiële voordelen van duale RAS blokkade, ondanks de toegenomen frequentie van bijwerkingen. Duale RAS remming kan daarom voorzichtig gebruikt worden bij bepaalde groepen patiënten, uiteraard rekening houdend met het feit dat chronische RAS blokkade RPC "recruitment" induceert.

TOEKOMSTPERSPECTIEVEN

Naast de hoofdfunctie van het RAS als de belangrijkste regelaar van de bloeddruk en water- en electrolythuishouding, spelen RPC een veel diepgaandere rol dan simpelweg het produceren en afgeven van renine. Hun enorme fenotypische plasticiteit, geïllustreerd door het "recruitment" fenomeen, stelt hen in staat om te werken als pluripotente stamcellen voor verschillende celtypen in de nier.

Onlangs is er een relatie gevonden tussen RPC en weefselregeneratie, ontsteking en mogelijk fibrose. Het is hierbij interessant dat onze bevindingen de profibrotische factor PDGF β als een mogelijke regelaar van de expressie van RPC laten zien. Tevens is PDGF β een marker voor pericyten, en van de laatstgenoemde cellen is bekend dat zij aanwezig zijn in de vaatwand en een grote fenotypische plasticiteit

bezitten. Het lijkt daarom geen verrassing dat RPC kunnen differentiëren tot pericyten en vice versa.

Onze bevindingen bieden geen ondersteuning aan het concept dat er renine geproduceerd wordt buiten de nieren, in het bijzonder in de hersenen. In vele artikelen is gespeculeerd over de produktie van (pro)renine in de hersenen, mogelijk intracellulair, en over lokaal gesynthesiseerd prorenine dat activiteit vertoont nadat het zich gebonden heeft aan de (pro)renine receptor in de hersenen. Geen enkel artikel heeft echter daadwerkelijk (pro)renine in de hersenen gekwantificeerd. Onze data tonen nu aan dat er geen (pro)renine in de hersenen is, op de kleine hoeveelheden renine en prorenine na die zich nu eenmaal bevinden in het beetje bloed dat zich in hersenweefsel bevindt (wat verdwijnt na buffer-perfusie), en dit is een sterk argument tegen de produktie van angiotensine in hersenweefsels. Zelfs als prorenine lokaal wordt afgegeven (bijvoorbeeld in de eierstokken), waren wij niet in staat om aan te tonen dat dit prorenine activiteit vertoonde op de plek van afgifte. Samen met het recente inzicht dat de (pro)renine receptor functies heeft die niets met het RAS te maken hebben, impliceert dit dat we nieuwe mechanismen moeten vinden om te begrijpen hoe prorenine bijdraagt aan de lokale produktie van angiotensine. Derhalve blijven JGC vooralsnog de enige bron van renine in het lichaam, waarvan de produktie van angiotensine volledig afhangt. Of prorenine lokaal angiotensine aanmaakt onder fysiologische, of zelfs pathologische omstandigheden, moet nog bewezen worden.

In de nieren is geopperd dat naast JGC ook tubulus cellen prorenine synthetiseren. Opmerkelijk genoeg heeft angiotensine op deze produktie juist een stimulerend effect (positieve feedback, in tegenstelling tot de negatieve feedback in JGC), en is er geen bewijs voor de produktie van renine in de tubulus. In hoeverre dit prorenine daadwerkelijk bijdraagt aan angiotensine produktie in de nieren is nog niet duidelijk; mogelijk vertoont het een interactie met angiotensinogeen afkomstig uit de tubulus, bijvoorbeeld na binding aan de (pro)renine receptor. Het gebruik van transgene dieren die geen angiotensinogeen konden maken in hun nieren maakte echter duidelijk dat de angiotensine produktie in de nier volledig afhankelijk is van gefilterd angiotensinogeen uit plasma, dat wil zeggen: angiotensinogeen van hepatische oorsprong. Studies in patiënten met een verstoorde reabsorptie in de proximale tubulus ondersteunen het concept van megaline-afhankelijke reabsorptie van gefilterd angiotensinogeen, renine en prorenine, en onze huidige data in epitheelcellen laten gedetailleerd zien welk mechanisme hieraan ten grondslag ligt. Verrassend genoeg blijkt dat er mogelijk een bijkomende rol is weggelegd voor M6P receptoren in het reabsorptieproces, en/of dat M6P zelf de megaline-gemedieerde processen remt. Gezien de enorme stijging van de hoeveelheid RAS componenten in urine (tot 40-voudig) in patiënten met een verstoorde megaline functie, moet het idee van synthese en afgifte van renine, prorenine en/of angiotensinogeen in de tubulus heroverwogen worden. Hoogstwaarschijnlijk wijzen veranderingen (pro)renine- en angiotensinogeen spiegels in urine op veranderingen in megaline-afhankelijke reabsorptie (gereguleerd, onder andere, door angiotensine!) in plaats van onafhankelijke afgifte van RAS componenten vanuit de tubulus.

De voordelen van RAS blokkade bij cardiovasculaire aandoeningen en nierziekten zijn onbetwist, maar hoeveel blokkade optimaal is, is nog altijd onbekend. Een hoge mate van RAS remming kan leiden tot gunstiger effecten, maar resulteert ook in een hogere prevalentie van bijwerkingen. De mate van remming kan worden ingeschat op basis van de toename van renine in het bloed, een parameter voor de "werving" van nieuwe RPC. De lange termijngevolgen van dit proces zijn nog onbekend, noch weten we of het bijdraagt aan toekomstige nierfunctiestoornissen. Naast de produktie van renine, zouden RPC ook direct kunnen bijdragen aan vasculaire hypertrofie, zelfs als de bloeddruk normaal is. Nog altijd zijn vele vragen onbeantwoord. Dragen deze cellen bij aan het fibroseproces? Indien deze cellen weefsel-herstelcapaciteit bezitten, waarom zijn ze dan soms uitgeschakeld of dysfunctioneel? In hoeverre is weefselherstel daadwerkelijk effectief bij het herstellen van de normale functie? Zou dit herstel voortdurend plaatsvinden of is het van tijdelijke aard? Het beantwoorden van deze vragen, evenals een volledig begrip van de lokale angiotensine produktie (in de nieren), zal uiteindelijk leiden tot meer effectieve antihypertensieve therapieën, en betere resultaten bij patiënten met cardiovasculaire aandoeningen en nierziekten.

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CURRICULUM VITAE

Alexandre Goes Martini was born on the July 7th of 1977 in Curitiba, Brazil. He graduated in Medicine in 2003, and finished his medical training in Internal Medicine and Nephrology in 2009. He obtained his Master degree (MSc) in 2012 at the University of Brasília (UnB), where afterward he started his PhD at the laboratory of Molecular Pharmacology. In 2015, he came to the Netherlands to receive further training in kidney physiology in the Department of Internal Medicine, Division of Vascular Medicine and Pharmacology, at Erasmus Medical Center, Rotterdam. His research focused on the renin-angiotensin system, notably renin-synthesizing cells. The results are displayed in the present thesis.

1- "Do prorenin-synthesizing cells release active, 'open' prorenin?"
Journal of Hypertension. 2017;35:330-337

2- "Aliskiren, Enalapril, or both in Heart Failure"
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High Blood Pressure and Cardiovascular Prevention. 2017;24:231-242

6- "Dibutyltin compounds effects on PPAR/RXRa activity, adipogenesis, and inflammation in mammalians cells
Frontiers in Pharmacology. 2017; in press

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Ensembl Workshop	2017	0,6
Annual Course in Molecular Medicine	2017	0,6
Intensive Care Research Part I COEUR Course	2017	0,5
Intensive Care Research Part II COEUR Course	2017	0,5
Cardiovascular Imaging and Diagnostics Part I COEUR Course	2017	0,5
Cardiovascular Imaging and Diagnostics Part II COEUR Course	2017	0,5
Congenital Heart Disease Part I COEUR Course	2017	0,5
Translation Imaging Workshop by AMIE	2017	1,4
Biostatistical Methods I (CC02A)	2017	2,0
Workshop on Microsoft Access 2010: Basic	2017	0,3
Workshop on Microsoft Access 2010: Advanced	2017	0,3
Workshop on Photoshop and Illustrator CS6	2017	0,3
Workshop on Indesign CS6	2017	0,3
Basic Course on R	2017	1,8
Survival Analysis Course	2017	0,6
Research Integrity Course	2017	0,3
Teaching Autonomic Nervous System	2015-2016	0,6
Conferences, seminars and symposia		
International Symposium on Renin Angiotensin System, SP	2014	0,4
Brazilian Congress of Hypertension, Rio de Janeiro	2015	0,6
OMICS in Cardiovascular Medicine	2017	0,2
Endovascular Thrombectomy in Acute Ischemic Stroke Seminar	2017	0,2
MRI in Carotid Arteries Symposium	2017	0,2
Science Day, ErasmusMC Internal Medicine, Antwerp	2016-2017	1,0

Internal Medicine Research Symposium	2016-2017	0,9
COEUR PhD Day	2017	0,3

Presentations		
American Society of Nephrology, ASN, Atlanta	2014	0,5
Council on Hypertension, AHA, Orlando	2016	0,5
Council on Hypertension, AHA, San Francisco	2017	0,5
European Society of Hypertension, ESH, Milan	2017	0,5
Internal Medicine Research Symposium	2017	0,4
PLAN (platform AIOS Nephrology) Day	2017	0,4
Presentations at the Division of Pharmacology	2015-2017	1,8