Key developments in renin–angiotensin–aldosterone system inhibition

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Abstract | The renin–angiotensin–aldosterone system (RAAS) was initially thought to be fairly simple. However, this idea has been challenged following the development of RAAS blockers, including renin inhibitors, angiotensin-converting-enzyme (ACE) inhibitors, type 1 angiotensin II (AT_1)-receptor blockers and mineralocorticoid-receptor antagonists. Consequently, new RAAS components and pathways that might contribute to the effectiveness of these drugs and/or their adverse effects have been identified. For example, an increase in renin levels during RAAS blockade might result in harmful effects via stimulation of the prorenin receptor (PRR), and prorenin—the inactive precursor of renin—might gain enzymatic activity on PRR binding. The increase in angiotensin II levels that occurs during AT_1-receptor blockade might result in beneficial effects via stimulation of type 2 angiotensin II receptors. Moreover, angiotensin 1–7 levels increase during ACE inhibition and AT_1-receptor blockade, resulting in Mas receptor activation and the induction of cardioprotective and renoprotective effects, including stimulation of tissue repair by stem cells. Finally, a role of angiotensin II in sodium and potassium handling in the distal nephron has been identified. This finding is likely to have important implications for understanding the effects of RAAS inhibition on whole body sodium and potassium balance.

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Introduction

In the past few decades, our understanding of the complexity of the renin–angiotensin–aldosterone system (RAAS) has increased considerably, largely as a result of knowledge obtained following the introduction of RAAS blockers, such as direct renin inhibitors, angiotensin-converting-enzyme (ACE) inhibitors, type 1 angiotensin II (AT_1)-receptor blockers (ARBs) and mineralocorticoid-receptor antagonists (MRAs). Although these agents do not always uniformly suppress angiotensin II and aldosterone levels or activity—breakthroughs, during which angiotensin II and aldosterone levels return to, or even increase above, their pretreatment levels often occur[1,2]—they have been used successfully to treat cardiovascular and renal diseases.[3] The efficacy of RAAS blockers despite the occurrence of angiotensin II and aldosterone breakthroughs suggests that their beneficial effects are not solely attributable to blockade of the angiotensin II–AT_1 receptor–aldosterone–mineralocorticoid receptor axis. For example, high angiotensin II levels, particularly during AT_1-receptor blockade, might stimulate activation of type 2 angiotensin II (AT_2) receptors, which are thought to oppose the effects of AT_1 receptor activation, including vasoconstriction, stimulation of growth and remodelling, sympathetic nervous system activation, and sodium and water retention.[4,5] Similarly, treatment with ACE inhibitors and ARBs results in the production of high levels of angiotensin II metabolites, most importantly angiotensin 1–7, which exerts cardioprotective and renoprotective effects via the Mas receptor (Figure 1).[6] Interactions between angiotensin 1–7 and the Mas receptor might also contribute to the efficacy of RAAS blockade.

RAAS blockade causes an increase in renin levels[7] as a result of interference with a negative feedback loop between angiotensin II and renin release. This effect explains, at least in part, why angiotensin II and aldosterone breakthroughs occur. In addition to renin, the levels of its precursor, prorenin, might also increase during breakthroughs. Since the discovery of the prorenin receptor (PRR),[8] which induces profibrotic effects *in vitro* in response to renin or prorenin binding, it has been proposed that increases in renin and prorenin levels will not only result in diminished RAAS suppression, but also in unwanted effects via PRR stimulation.[9]

Aldosterone interacts with angiotensin II in a synergistic manner and might exert nongenomic, acute effects via a mineralocorticoid-receptor-independent mechanism.[10–12] Unravelling this interaction at the level of the kidney is of importance to obtain a full understanding of the different effects of aldosterone during hypovolaemia (sodium retention and potassium conservation) and hyperkalaemia (potassium secretion), that is the aldosterone paradox. A better understanding of this paradox and of the specific roles of angiotensin II and aldosterone in sodium and potassium handling in the kidney is likely to be important to fully appreciate the effects of RAAS inhibition on sodium and potassium balance.

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In this Review, we describe the available evidence regarding the roles of the PRR, AT_2 receptor, angiotensin 1–7 and aldosterone–angiotensin II interactions in the RAAS cascade. Focusing mainly on the kidney, we discuss the potential of agents such as PRR blockers, AT_2-receptor agonists and Mas-receptor agonists to further enhance the degree of RAAS blockade with potentially beneficial effects in patients with cardiovascular and renal diseases.

Key points

- Interactions between renin, prorenin and the prorenin receptor (PRR) have not been confirmed *in vivo* and seem unlikely because of the low levels of renin and prorenin in blood
- Given the role of the PRR in V-type proton ATPase integrity and Wnt signalling, renin–angiotensin–aldosterone system (RAAS)-independent functions of the PRR seems more likely than RAAS-dependent functions
- Under certain pathological conditions, type 2 angiotensin II (AT_2) receptors mimic type 1 angiotensin II (AT_1) receptor function and exert detrimental effects including vasoconstriction and hypertrophy
- Stimulating angiotensin 1–7 generation or using stable angiotensin 1–7 analogues to activate the Mas receptor is a promising new strategy to improve tissue repair by stem cells
- Angiotensin II affects the activity of the main sodium and potassium transporters in the distal nephron: the sodium chloride cotransporter, epithelial sodium channel and renal outer medullary potassium channel
- Synergistic actions of angiotensin II and aldosterone on sodium and potassium transport in the distal nephron help to explain the effects of RAAS inhibition on renal sodium and potassium excretion

The PRR

Activation of prorenin

The PRR is an almost ubiquitously expressed 350-amino-acid protein that can bind both renin and prorenin.[8,13,14] Binding to the PRR induces prorenin to undergo a conformational change that allows the prosegment to move out of the catalytic cleft, resulting in full, nonproteolytic activation of the protein.[8,13] This mechanism differs from the proteolytic activation of prorenin in the kidney, during which the prosegment is cleaved off by an as yet unidentified enzyme.[15] Given the much higher levels of prorenin (which is largely derived from the kidney, but also from extrarenal sources)[15] than renin in blood plasma, and the fact that prorenin–renin converting enzymes are only expressed in the kidney, the hypothesis that PRR binding enables the normally inactive prorenin to gain angiotensin-generating activity at tissue sites is attractive.[16] However, a study showed that following renin or prorenin exposure, the PRR directly activated the extracellular signal-regulated kinase 1/2 (ERK1/2) signalling pathway independently of the formation of angiotensin II.[8,17] This activation was not blocked by renin inhibitors, suggesting that binding of these inhibitors to prorenin does not prevent binding of prorenin to the PRR, although inhibitor binding did prevent the generation of angiotensin I.[13,17]

As the affinity of prorenin for the human PRR is threefold to fourfold higher than that of renin,[14] the assumption that the prosegment facilitates binding to the PRR seems reasonable. The prosegment contains a 'handle region' (peptides 10–19), which is thought to bind to the PRR and enable catalytic activation of prorenin.[18] Peptidic antagonists that mimic the handle region of prorenin, known as handle region peptides (HRPs), bind competitively to the PRR, thereby preventing PRR-mediated prorenin activation and reducing RAAS activity in tissues.[19,20] The structure of prorenin is highly species specific so different HRPs have been designed for use in humans, rats and mice.

RAAS-dependent functions

In rats, ubiquitous overexpression of the human PRR resulted in proteinuria, glomerulosclerosis[21] and renal cyclooxygenase-2 (COX-2, also known as prostaglandin G/H synthase 2) upregulation.[22] The human PRR binds, but does not activate rat prorenin,[21] therefore, it was not surprising that overexpression of the human PRR did not alter angiotensin levels in the plasma and tissues of transgenic rats.[22] However, these animals showed increased levels of aldosterone in their blood plasma.[23] This increase was unlikely to be the result of prorenin-induced PRR stimulation because neither renin nor prorenin affected aldosterone synthesis in the human adrenocortical cell lines H295R and HAC15.[23] An infusion of HRP prevented the development of glomerulosclerosis in transgenic rats that expressed the human PRR.[21]

In diabetic rats, HRP therapy normalized high renal angiotensin levels without affecting blood pressure, and prevented the development of nephropathy.[19] HRP therapy also prevented the development of nephropathy in diabetic type 1a angiotensin II (AT_{1A}) receptor-deficient mice,[20] suggesting that this effect was not dependent on the suppression of local angiotensin generation. Interestingly, ERK1 and ERK2 as well as phosphorylated p38 mitogen-activated protein kinase (MAPK) and phosphorylated c-Jun N-terminal kinase were upregulated in the diabetic kidneys of both wild-type and AT_{1A} receptor-deficient mice. HRP (but not ACE inhibitor) therapy fully normalized this increased phosphorylation.[20]

Evidence supporting a renin–PRR interaction came from a study that showed that renin activated ERK1 and ERK2 in mesangial cells, even in the presence of renin inhibitors, ACE inhibitors or ARBs.[24] This activation resulted in production of transforming growth factor β1 (TGF-β1) and the subsequent upregulation of genes encoding profibrotic molecules, such as plasminogen-activator inhibitor 1 (PAI-1), fibronectin and collagens.[8,24] ERK1/2 activation occurred in response to <1 nmol/l renin and could be fully prevented by PRR small interfering RNA (siRNA).[24] Similar profibrotic effects of both renin and prorenin were shown in human embryonic kidney cells, although 100 nmol/l of these enzymes were required to induce gene upregulation.[25] Remarkably, in podocytes, 2 nmol/l human prorenin increased ERK1/2 activation without affecting TGF-β1 or PAI-1 levels.[26] These data suggest that the PRR–ERK1/2-mediated activation of profibrotic pathways does not occur in all cell types. Interestingly, in cultured nephritic glomeruli from rats with glomerulonephritis,

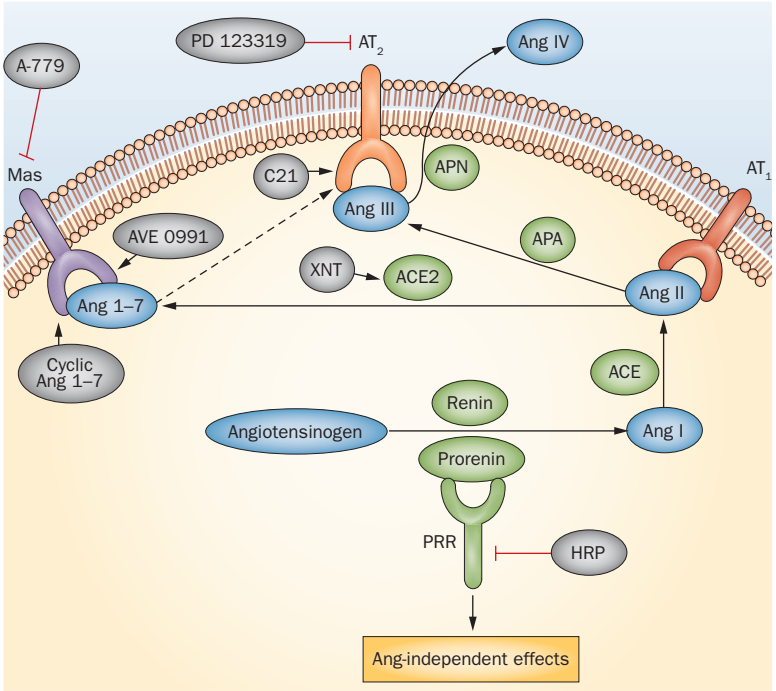

Figure 1 | New developments in the renin–angiotensin-system cascade. Binding of the renin precursor, prorenin, to the PRR activates the protein, enabling prorenin to cleave angiotensinogen and generate angiotensin 1. Renin and prorenin also act as agonists of the PRR and induce angiotensin-independent effects, such as ERK1/2 activation. APA generates angiotensin III from angiotensin II, and APN subsequently degrades angiotensin III to angiotensin IV. Binding of angiotensin II to the AT_1 receptor induces vasoconstriction, stimulation of growth and remodelling, sympathetic nervous system activation, and sodium and water retention. Angiotensin III is thought to be the preferred endogenous agonist of the AT_2 receptor, which mediates effects that either resemble or oppose those of AT_1-receptor activation. ACE2 generates angiotensin 1–7 from angiotensin II, and this metabolite activates the Mas receptor (and possibly AT_2 receptors), thereby inducing beneficial effects (such as improvement in endothelial function, reduced fibrosis and enhanced tissue repair by stem cells) that oppose those of AT_1-receptor activation. New drugs in development include antagonists of the PRR (HRP), AT_2 receptor (PD123319) and Mas receptor (A779), and activators of the AT_2 receptor (C21), Mas receptor (AVE 0991, cyclic angiotensin 1–7) and ACE2 (XNT). Abbreviations: ACE, angiotensin-converting enzyme; ACE2, angiotensin-converting-enzyme 2; Ang, angiotensin; APA, aminopeptidase A; APN, aminopeptidase N; AT_1, type 1 angiotensin II; AT_2, type 2 angiotensin II; ERK1/2, extracellular signal-regulated kinase 1/2; HRP, handle region peptide; PRR, prorenin receptor.

concomitant PRR inhibition (using siRNA) and angiotensin II blockade (using an ACE inhibitor) had a greater suppressive effect on TGF-β1 and PAI-1 expression than either inhibitor alone.[27] The authors attributed this effect to the fact that ACE inhibition increases renin mRNA levels, thereby counteracting the consequences of angiotensin II suppression, since renin upregulates TGF-β1 and PAI-1 by stimulating the PRR.[27]

In a gene expression profiling study, treatment of human mesangial cells with renin (20 nmol/l) and prorenin (50 nmol/l) activated pathways implicated in organ damage.[28] For example, upregulation of COX-2 and activation of the TGF-β1–PAI-1 pathway were observed. Although the effects of renin were greater than the effects of prorenin, there was extensive overlap between the gene signatures, indicating that renin and prorenin act through similar pathways. In podocytes, COX-2 overexpression exacerbated diabetic nephropathy by increasing PRR expression.[29] Thus, not only does PRR stimulation result in COX-2 upregulation,[22] but the converse is also true, possibly because products generated by COX-2, such as prostaglandins, promote PRR activation.[29] Indeed, the presence of this positive feedback loop was confirmed in a study that showed that high glucose levels upregulated expression of the PRR and prorenin in rat mesangial cells, thereby facilitating generation of angiotensin II, which subsequently induced expression of COX-2 and interleukin 1β.[30] In addition, COX-2 inhibition reduced glucose-induced PRR upregulation, suggesting that products generated by COX-2 upregulate the PRR.[29,30] Expression of the PRR in glomeruli and renal tubules was increased in diabetic rats, possibly as a result of enhanced AT_1 receptor and NADPH oxidase activity.[31] In diabetic rats, HRP treatment did not seem to affect PRR expression *in vivo*, whereas ARB therapy reduced PRR expression.[32]

Effects of RAAS blockade

Consistent with the stimulatory effects of AT_1 receptors on PRR expression,[31] RAAS blockade reduced PRR expression in the kidneys of diabetic rats.[33] However, in a Goldblatt rat (high angiotensin II) model of hypertension, parallel increases in PRR expression and renin levels were observed.[34] PRR upregulation also occurred in the remnant kidneys (particularly in the tubular cells) of five-sixths nephrectomized rats,[35] and in the kidneys (mainly in the tubular cells and collecting ducts) of patients with end-stage renal disease caused by diabetic nephropathy.[36] These contrasting findings suggest that PRR expression might also be regulated in a RAAS-independent manner, for example via the NADPH oxidase-dependent generation of reactive oxygen species.[25,31]

Despite the above data, we still do not know for certain whether prorenin–PRR interactions occur *in vivo*, and whether the therapeutic effects of HRPs are attributable to interference with such interactions. Importantly, the affinity of the PRR for renin and prorenin is in the high nanomolar range (possibly even 20 nmol/l for renin),[14] which is difficult to reconcile with the picomolar levels of renin (~0.5 pmol/l) and prorenin (~5 pmol/l) in extracellular fluid.[37] However, this affinity is consistent with the findings that PRR overexpression did not alter the levels of RAAS components in rats,[22,38] and that in most *in vitro* studies in which prorenin–PRR interactions were investigated, high nanomolar prorenin concentrations (≤100 nmol/l) were required for activation of ERK1/2 and upregulation of profibrotic genes.[6,25,28,39] Moreover, in rodents, prorenin overexpression, which resulted in an up to 400-fold increase in plasma prorenin levels, increased blood pressure in an angiotensin-dependent manner[40–42] but did not cause the fibrosis or glomerulosclerosis that was expected based upon the findings of *in vitro* studies with renin and prorenin.[17,25,28,39] Even larger increases in plasma prorenin levels might be required to produce such effects. However, under pathological conditions and/or during RAAS blockade,

prorenin and renin levels do not normally increase more than three orders of magnitude above baseline. Whether such high levels occur in prorenin-synthesizing tissues, such as in renal interstitial fluid, is not known. The maximum increases in plasma prorenin levels that have been shown in humans—for example during severe heart failure or RAAS blockade—are ~50–100-fold for renin (although the increase is normally well below 10-fold), and twofold to threefold for prorenin.[1,37,43]

Not surprisingly, as a result of the negative feedback loop between angiotensin II and renin, the largest increases in renin levels occur during the highest levels of RAAS blockade. Consequently, dual or even triple RAAS blockade (using a combination of RAAS blockers) resulted in a greater increase in renin levels than did RAAS blockade using a single RAAS blockade approach.[44] Studies of spontaneously hypertensive rats kept on a low-sodium diet, showed that dual RAAS blockade resulted in massive increases (up to several-hundred-fold) in both plasma and renal renin levels, and depletion of angiotensinogen.[44] As a consequence, a large decrease in blood pressure and severe renal failure occurred. These findings are reminiscent of those of the ALTITUDE study, in which the efficacy of the renin inhibitor aliskiren was evaluated versus placebo in high-risk patients with type 2 diabetes mellitus and renal dysfunction, whose blood pressure was normalized using one or more antihypertensive drugs, including ACE inhibitors and ARBs.[45] The trial was stopped prematurely because of a lack of beneficial effects and an increased incidence of serious adverse events, including hypotension, hyperkalaemia and renal complications, in the patients who received aliskiren. The latter adverse-effect profile is entirely consistent with the consequences of angiotensin II and/or aldosterone depletion, and thus deleterious PRR stimulation in response to increased renin and/or prorenin levels during combined aliskiren and ACE inhibitor or aliskiren and ARB treatment does not need to be invoked to explain these adverse effects. Moreover, although the levels of prorenin and renin in the patients who received aliskiren in the ALTITUDE trial have not yet been reported, it is highly unlikely that they were ≥10,000 times greater than the levels in untreated patients and, therefore, within the range required to stimulate the human PRR *in vivo*.[39] In addition, as discussed above, RAAS blockade in rodents induced a decrease, rather than an increase, in PRR expression.[31–33]

RAAS-independent functions

If prorenin levels *in vivo* are too low to induce PRR stimulation, even during RAAS blockade or under pathological conditions, the phenotype that develops in response to PRR overexpression *per se* must be the result of RAAS-independent effects of PRR stimulation. Colocalization of the PRR with V-type proton ATPase (V-ATPase) in the kidney has been reported.[46] This colocalization might occur because the 8.9 kDa accessory protein ATP6AP2 of V-ATPase is a post-translationally truncated version of the PRR, which resembles the C-terminal domain of the receptor.[14] V-ATPases have an important role in the acidification of subcellular compartments and the PRR is indispensable for V-ATPase integrity; in mice with podocyte-specific PRR knockout, the abundance of several V-ATPase subunits in podocytes was reduced, resulting in defective autophagy, severe endoplasmatic stress, and podocyte necrosis.[47] As a consequence, the mice died at 2–4 weeks of age due to renal failure. The PRR also functions as an adaptor between V-ATPase and receptors for members of the Wnt family of signalling molecules.[48] These findings clearly indicate the importance of the PRR beyond renin/prorenin binding, and raise doubt that the PRR binds to and activates prorenin *in vivo*. However, they do not rule out the possibility that the PRR has an important role in tissue damage, or that the beneficial effects of RAAS blockade occur as a result of a reduction in PRR expression.

The available data on the effects of HRPs are inconclusive, mainly because no study has convincingly shown that these drugs block PRR–prorenin interactions *in vivo*. A possibility exists that HRPs are partial PRR agonists that exert their effects independently of renin and prorenin,[49] thus explaining some of the conflicting results. However, given the interference with V-ATPase, PRR blockade *per se* would not necessarily have beneficial effects.

AT_2 receptors

AT_2 receptors are generally thought to oppose classical AT_1-receptor-mediated effects, such as vasoconstriction, sodium retention and inflammation. In the kidney, AT_2 receptors are expressed in the proximal tubules, collecting duct and renal resistance arteries,[4] suggesting a role in renal haemodynamics and tubular function. Pathological conditions, such as diabetes and kidney injury, usually increase renal AT_2-receptor expression, and concomitant ARB treatment further enhances this upregulation.[50–52] AT_2-receptor-knockout mice had either increased blood pressure at baseline or an increased hypertensive response to angiotensin II compared with wild-type mice, supporting a vasodilatory role of AT_2 receptors.[53,54] The knockout mice also showed a rightward shift of the pressure-natriuresis curve and a more pronounced antinatriuretic response to angiotensin II than wild-type mice, again supporting a counter-regulatory role of AT_2 receptors.[55] Moreover, unilateral urethral obstruction resulted in increased renal interstitial fibrosis and apoptosis in AT_2-receptor-knockout mice compared with wild-type mice.[56,57] However, an important consideration is that AT_2-receptor-null mice showed increased expression of the AT_1 receptor, which might explain these findings.[58]

Effects of stimulation

ARB pretreatment is usually required to allow angiotensin II-induced, AT_2-receptor-mediated hypotensive and natriuretic effects to be observed in wild-type animals,[4,59] suggesting that the opposing actions of the AT_1 receptor usually predominate. Interestingly, in rats angiotensin III (angiotensin 2–8) rather than

angiotensin II seems to be the preferred agonist of the AT_2 receptor (Figure 1);[60] blockers of aminopeptidase type A, which converts angiotensin II into angiotensin III, inhibited AT_2-receptor-mediated natriuresis, whereas blockers of the degradation of angiotensin III (by aminopeptidase N) enhanced this effect.[61,62]

Development of the selective AT_2-receptor agonist C21 has made investigation of the effects of AT_2-receptor stimulation possible without concomitant ARB treatment. In obese Zucker rats, C21 induced natriuresis in a nitric oxide (NO) and cyclic guanosine monophosphate (cGMP)-dependent manner.[63,64] This effect was prevented by pre-infusion of the AT_2-receptor antagonist PD123319.[63,64] C21 infusion also increased the fractional excretion of lithium, suggesting involvement of the proximal tubules in AT_2-receptor-mediated natriuresis.[63] Although AT_2-receptor stimulation has been reported to increase renal blood flow in Sprague–Dawley rats, AT_2-receptor stimulation did not affect glomerular filtration rate, and thus altered renal haemodynamics are unlikely to underlie the increased natriuresis.[65] AT_2-receptor-mediated anti-inflammatory effects have been observed in the rat 2-kidney, 1-clip model of hypertension; C21 decreased the renal interstitial levels of tumour necrosis factor, interleukin 6, and TGF-β1, and activated the NO/cGMP cascade in a blood-pressure-independent manner.[64]

Based upon findings in AT_2 knockout animals, AT_2-receptor-mediated natriuresis, and C21-induced activation of the NO/cGMP cascade, one would expect that C21 infusion lowers blood pressure. Remarkably, however, the majority of studies have shown no effect of C21 on blood pressure, whereas other studies showed hypertension or hypotension in response to C21 treatment.[66] Increases in blood pressure in response to C21 treatment might be a result of nonselective AT_1-receptor stimulation occurring at high C21 doses.[4] C21-induced decreases in blood pressure could not be blocked by PD123319, and might be the result of a C21-dependent blockade of calcium transport into the cell.[66]

Effects of blockade

Studies of the effects of AT_2-receptor blockade using PD123319 have also yielded conflicting results. In subtotally nephrectomized rats, PD123319, alone or in combination with ARB treatment, decreased proteinuria as well as monocyte and macrophage infiltration into the remnant kidney.[67] Similarly, dual AT_1-receptor blockade and AT_2-receptor blockade, using PD123319 and the AT_1-receptor antagonist losartan, prevented activation of the NFκB pathway in the mouse unilateral ureteral obstruction model of renal injury,[68] and in rats, the glomerular infiltration of monocytes and macrophages following angiotensin II infusion depended on AT_2-receptor-mediated chemokine induction.[69] In a rat renal-wrap-hypertension model, angiotensin II–AT_2 receptor interactions increased renal interstitial bradykinin levels, thus activating the protective NO/cGMP pathway.[70] In rats, however, PD123319 treatment following renal ablation increased renal damage and blood pressure, most likely because AT_2-receptor-mediated vasodilation was prevented, and, therefore, ischaemic damage was increased in the remnant tissue.[50] Finally, AT_2-receptor stimulation in stroke-prone, spontaneously hypertensive rats reduced monocyte and macrophage infiltration in the aorta and the kidney.[71]

Detrimental effects

Although data from a wide range of studies in healthy and diseased animal models support a beneficial role of AT_2-receptor stimulation, resulting in anti-inflammatory, hypotensive and natriuretic effects,[50,53–65,71] the opposite has also been reported.[66–69] One explanation for these contrasting data is that the phenotype of AT_2 receptors is altered under pathological conditions and/or with age. Indeed, AT_2-receptor-mediated vasoconstriction has been observed in spontaneously hypertensive rats,[72] and only lowering blood pressure enabled the return of bradykinin/NO-mediated relaxant responses.[73] The change of phenotype might relate to the location of the receptor (endothelium or smooth muscle cell), as well as its capacity to heterodimerize with AT_1 receptors.[4] At this stage, the clinical application of an AT_2-receptor agonist seems premature, particularly because a comparison of the effects of ACE inhibitors and ARBs in humans has not revealed major outcome differences.[74] Nevertheless, the availability of a selective AT_2-receptor agonist will help obtain a better understanding of the location, age and disease-dependent roles of the AT_2 receptor and determine in which diseases such drugs might be applied.

ACE2–angiotensin 1–7–Mas receptor axis

The ACE homologue ACE2 cleaves angiotensin II to generate angiotensin 1–7, which opposes AT_1-receptor-mediated effects via stimulation of the Mas receptor (Figure 1).[6] Angiotensin 1–7 can also be generated from angiotensin I, via angiotensin 1–9, again with the help of ACE2.[75] According to some studies, angiotensin 1–7 binds to AT_2 receptors, and high levels of angiotensin 1–7 might stimulate AT_1 receptors.[76] ACE2 and Mas receptors are expressed in kidney, heart and vascular tissue, suggesting that angiotensin 1–7 might have a physiological, protective role in these organs.[77] Indeed, in rodent models of cardiac ischaemia and heart failure, angiotensin 1–7 infusion or ACE2 overexpression preserved cardiac and endothelial function, and in models of hypertension and diabetes, such treatment prevented renal and cardiovascular anomalies.[77,78] In spontaneously hypertensive rats, the Mas-receptor antagonist A779 abrogated the antihypertensive and antiproteinuric effects of ACE inhibitors, suggesting that these effects depend, at least in part, on activation of the Mas receptor.[79,80] Mas-receptor activation increased endothelial NO release and reduced oxidative stress, thereby eliminating the pro-oxidant properties of angiotensin II, and thus resulting in anti-hypertrophic, antifibrotic and renoprotective effects.[81–83] Somewhat confusingly, transfection of the Mas receptor into murine renal proximal tubular cells attenuated angiotensin II-stimulated TGF-β1 expression, whereas in human mesangial cells, angiotensin 1–7 stimulated the TGF-β1-dependent profibrotic pathway—an effect that

was inhibited by A779.[84,85] These conflicting data, which are reminiscent of the contrasting data regarding AT_2-receptor-mediated effects, might relate to the existence of Mas–AT_1 receptor heterodimers.[86]

Role in stem cell regulation

In the bone marrow, haematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs) differentiate into cardiovascular and renal progenitor cells, which have a major role in tissue repair. Importantly, clinical studies have shown that cardiac grafting of autologous bone marrow cells improved cardiac performance in patients with ischaemic heart disease, an effect that appeared to be mainly due to improved angiogenesis.[87] Similarly, MSC infusion improved kidney function in patients with chronic kidney disease.[88,89] Given that all of the major components of the RAAS are present in bone marrow,[90] this system is now believed to have an important role in HSC and MSC regulation. In mice, treatment with angiotensin 1–7 after irradiation or chemotherapy stimulated regeneration of bone marrow.[91,92] Another rodent study showed that activation of the angiotensin 1–7–Mas-receptor pathway stimulated bone marrow cells to form early endothelial progenitor cells,[93] although, paradoxically, antiangiogenic properties of angiotensin 1–7 have also been reported.[94] Nevertheless, stimulation of Mas receptors in bone marrow might be a strategy to improve tissue repair by stem cells.

Novel therapies

Given the rapid breakdown of the peptide angiotensin 1–7 *in vivo*, novel therapies are now being developed to stimulate the ACE2–angiotensin 1–7–Mas-receptor axis. Angiotensin 1–7 encapsulation in the oligosaccharide hydroxypropyl β-cyclodextrin offers protection during gastrointestinal passage, and oral application of encapsulated angiotensin 1–7 was cardioprotective in infarcted rats.[95] Cyclic angiotensin 1–7, an angiotensin 1–7 analogue with a thioether bridge between amino acid residues 4 and 7, is resistant to metabolism, and can be delivered via oral and pulmonary routes.[96] Interestingly, cyclic angiotensin 1–7 selectively activated Mas receptors without binding to AT_1 or AT_2 receptors,[97] and improved cardiac remodelling and endothelial function after myocardial infarction.[98] Furthermore, the nonpeptide angiotensin 1–7 analogue AVE 0991 prevented end-organ damage in spontaneously hypertensive rats treated with an inhibitor of NO synthesis[80] and also enhanced acetylcholine-induced vasodilation in Wistar rats.[99] Finally, the ACE2 activator XNT improved cardiac function, and reduced cardiac, renal and pulmonary fibrosis in diabetic rats and in spontaneously hypertensive rats.[100,101]

In summary, Mas-receptor stimulation is a promising new approach to improve tissue repair by stem cells. Current strategies are directed either at generating high endogenous levels of angiotensin 1–7 using ACE2 activators, administering stable angiotensin 1–7 analogues or encapsulating angiotensin 1–7 in oligosaccharides to slow down its breakdown following oral administration.

Angiotensin–aldosterone interactions

The classical paradigm

Physiologically, the RAAS operates in the kidney to increase renal sodium retention during hypovolaemia and potassium secretion during hyperkalaemia. The classical paradigm states that angiotensin II primarily increases sodium reabsorption by the proximal tubule, whereas aldosterone stimulates sodium uptake and potassium secretion in the more distal parts of the nephron—the distal convoluted tubule, connecting tubule, and collecting duct.[102]

Sodium and potassium transporters

In the past two decades, the major sodium and potassium transport proteins in the nephron have been cloned and characterized. In the proximal tubule, angiotensin II mediates sodium bicarbonate reabsorption through sodium/hydrogen exchanger 3,[103] and in the distal nephron, the sodium chloride cotransporter (NCC),[104] epithelial sodium channel (ENaC),[105] and the renal outer medullary potassium channel (ROMK)[106,107] have all been shown to be sensitive to aldosterone. The NCC is located in the 'early' distal convoluted tubule, whereas the ENaC is located in the 'late' distal convoluted tubule, connecting tubule and collecting duct, and the ROMK is expressed along the entire distal nephron.[108] These aldosterone-sensitive transporters are important clinically because they are direct or indirect targets of several antihypertensive drugs, including ACE inhibitors, ARBs, thiazide diuretics, amiloride and MRAs.

Angiotensin II actions in the distal nephron

The paradigm that angiotensin II acts proximally in the kidney has now been challenged. Several studies, including ours, have shown that angiotensin II also regulates sodium and potassium handling in the distal nephron.[109–115] Although abundantly located in the proximal tubule, ^{125}I–angiotensin II binding studies have shown that AT receptors are also present in the thick ascending limb, distal convoluted tubule and collecting duct.[116] Wang and Giebisch were among the first to investigate the effects of angiotensin II on sodium and potassium transport in the distal tubule.[117] Using isolated perfused tubules, they showed that administration of angiotensin II increased sodium transport and decreased potassium transport in the early and late distal convoluted tubule.[117] Treatment with either an ARB or the ENaC blocker, amiloride, abolished these effects.[117] These data suggest that angiotensin II activates the ENaC and NCC, although the effect of NCC blockade using thiazides was not investigated. More compelling evidence that angiotensin II activates the NCC came from a rat study, which showed that ACE inhibition stimulated acute trafficking of the NCC from the apical plasma membrane to subapical cytoplasmic vesicles.[109] This effect was reversed by angiotensin II infusion. However, because angiotensin II also induces aldosterone secretion and aldosterone also activates NCC,[104] the effect of angiotensin II on NCC was still not proven unequivocally.

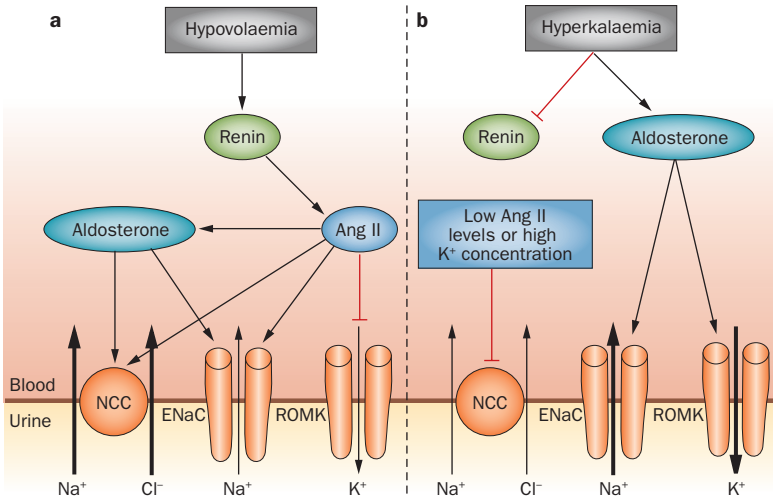

Figure 2 | The aldosterone paradox. Aldosterone exerts differential effects during hypovolaemia and hyperkalaemia. **a** | During hypovolaemia, angiotensin II and aldosterone act synergistically to increase the activity of the NCC and the ENaC. Angiotensin II also inhibits the activity of the ROMK. These actions results in maximal sodium reabsorption to correct hypovolaemia and conserve potassium. **b** | Hyperkalaemia directly stimulates aldosterone secretion independently of renin and angiotensin II. In the absence of high angiotensin II levels the ROMK is not inhibited and hyperkalaemia reduces the activity of the NCC. However, the ENaC and the ROMK are maximally activated by aldosterone. These combined effects result in maximal sodium delivery to the ENaC, which facilitates the coupled secretion of potassium through the ROMK. Abbreviations: Ang, angiotensin; ENaC, epithelial sodium channel; NCC, sodium chloride cotransporter; ROMK, renal outer medullary potassium channel.

To more clearly determine the effect of angiotensin II and aldosterone on the NCC, we treated adrenalectomized rats with a chronic infusion of either aldosterone or angiotensin II.[110] The latter therapy increased the levels of both total NCC and phosphorylated NCC in plasma membrane fractions independently of aldosterone. We also showed that angiotensin II caused sodium retention and that this was reversed by thiazides.[110] In a subsequent study, we investigated whether aldosterone required angiotensin II to activate the NCC.[111] We showed that aldosterone treatment increased the expression of NCC and ENaC in adrenalectomized rats, even in the presence of losartan; therefore, aldosterone alone is sufficient for these effects. However, the addition of losartan to the infusion of aldosterone reduced the levels of phosphorylated, and, therefore, active, NCC. These data suggest that angiotensin II might have an additive effect on aldosterone-induced NCC activation.

In our studies, the effects of angiotensin II on ENaC expression were less pronounced in terms of plasma membrane abundance than the effects on NCC expression; angiotensin II increased the expression of the β-subunit of the ENaC only.[110] However, more functional studies have shown that angiotensin II increases the open probability of the ENaC.[112,113] In freshly isolated, split-opened murine distal nephrons, angiotensin II acutely increased the open probability of the ENaC, whereas more prolonged exposure to angiotensin II induced a translocation of α-ENaC to the apical plasma membrane.[112] Interestingly, aldosterone did not acutely increase the open probability of the ENaC and the effect of angiotensin II on the ENaC persisted when mineralocorticoid receptors were saturated during the infusion of high doses of aldosterone. Thus, angiotensin II seems to have an additive effect to that of aldosterone in the activation of both the NCC and the ENaC.[111,112] The effects of angiotensin II on the ROMK have also been investigated using patch clamp recordings in split-opened collecting ducts.[114] Angiotensin II dose-dependently inhibited ROMK channel activity in rats on a low potassium diet, but not in rats on a normal potassium diet.[114] Conversely, a high potassium diet inhibited NCC and increased ROMK activity.[114,118] In summary, the observations of Wang and Giebisch[117] can now be explained by a stimulatory effect of angiotensin II on the NCC and the ENaC, and an inhibitory effect on the ROMK.

Regulation of sodium and potassium transporters

In addition to receptor–transporter interactions, the intracellular signalling pathways that regulate sodium and potassium transporters have also been a matter of intense research. This interest was stimulated by the discovery that mutations in with-no-lysine (WNK) kinases directly affected transporter activity and caused hypertension.[119] The current model of kidney transporter regulation by kinases has evolved to a complex kinase network that also includes the serine/threonine-protein kinase Sgk1 (SGK1) and STE20/SPS1-related proline-alanine-rich protein kinase (SPAK). This kinase network regulates the NCC, ENaC, and ROMK.[120] For example, in oocytes[115] and WNK4-knockout mice,[121] angiotensin II has been shown to activate the NCC through a SPAK–WNK4-dependent pathway. In rodents, a low sodium diet or an infusion of aldosterone or angiotensin II increased the SPAK-mediated phosphorylation of NCC.[110,122] The response of NCC to dietary sodium and potassium is mediated by SGK1,[123] whereas the inhibition of ROMK activity by angiotensin II is mediated by both SGK1 and WNK4.[124] These kinases are, therefore, obvious drug target candidates.[125]

The aldosterone paradox

The findings discussed above increase our understanding of the aldosterone paradox; the question as to how the same hormone can cause sodium retention during hypovolaemia and potassium secretion during hyperkalemia.[126,127] During hypovolaemia, levels of both angiotensin II and aldosterone are increased, causing a synergistic activation of both NCCs and ENaCs, which promotes maximal sodium reabsorption (Figure 2). At the same time, potassium is conserved because sodium reabsorption through the NCCs is electroneutral and does not require potassium secretion through the ROMK, which is inhibited by angiotensin II.[114,124] Conversely, during hyperkalaemia, aldosterone levels are maximally increased, whereas angiotensin II levels are suppressed, resulting in NCC inhibition and increased sodium delivery to ENaCs. Maximum ENaC activity facilitates electrochemical sodium reabsorption that promotes kaliuresis through the ROMK. Hyperkalaemia or

a high potassium diet might also directly inhibit NCC activity by increasing expression of WNK4.[128]

A better understanding of the aldosterone paradox is not only important physiologically, but also to understand the effects of RAAS inhibition on sodium and potassium homeostasis. For example, the synergistic activation of NCCs and ENaCs by angiotensin II and aldosterone could provide a rationale for dual blockade, that is, a combination of an ARB with an MRA to inhibit the combined effects of angiotensin II and aldosterone on NCCs and ENaCs. Notably, high doses (>25–50 mg per day) of the MRAs spironolactone and eplerenone might be required to establish a diuretic effect.[129] However, any combination of RAAS inhibitors confers a risk of hyperkalaemia and acute or chronic kidney injury, as discussed above.[45] Whether more selective inhibition of NCCs or ENaCs using drugs that interfere with WNK and SPAK kinases is a promising strategy for the treatment of hypertension remains to be determined. Finally, it should be noted that in addition to their natriuretic and antihypertensive effects, RAAS inhibitors also exert renoprotective effects by suppressing renal fibrosis and improving glomerular and podocyte function.[130]

Conclusions

The PRR was initially thought to be the key to prorenin activation at tissue sites. However, recent findings suggest that the PRR has other, perhaps more important, RAAS-independent functions. Contradictory data regarding the effects of HRPs—the only PRR blockers that currently exist—are therefore, perhaps unsurprising. Given the lethal consequences of PRR knockout, whether PRR blockade should be a therapeutic target is questionable. Similarly, the contrasting data regarding AT_2-receptor function suggest that a complete understanding of why the actions of this receptor differ depending on age, disease and location is required before AT_2-receptor agonists can be utilized in the clinic. Compelling evidence suggests a beneficial effect of Mas-receptor stimulation on stem-cell-mediated tissue repair. Thus, stable agonists are required to either stimulate the receptor or activate ACE2, the enzyme that generates the endogenous Mas-receptor agonist, angiotensin 1–7. If acting locally, such ACE2 activators might overcome the rapid metabolism of angiotensin 1–7 *in vivo*. Finally, the emerging role of angiotensin II in sodium and potassium handling in the distal nephron is likely to improve our understanding of the effects of RAAS inhibition on total body sodium and potassium balance. For example, the synergistic actions of angiotensin II and aldosterone on sodium and potassium transport in the distal nephron could be targeted to better treat diseases characterized by avid sodium reabsorption, including salt-sensitive hypertension and the cardiorenal syndrome, while at the same time preventing hyperkalaemia.

Review criteria

A search for original full-text English language papers was performed in PubMed. The search terms used were "angiotensin II", "angiotensin III", "renin", "prorenin", "prorenin receptor", "type 1 angiotensin II receptor", "type 2 angiotensin II receptor", "Mas receptor", "angiotensin 1–7", "ACE2", "aldosterone", "mineralocorticoid receptor", "WNK", "NCC", "ROMK" and "ENaC", alone and in combination. We focused primarily on work published in the past 5 years. Papers were also selected for inclusion based on our own knowledge of the literature and work in the field, and as a consequence of attending meetings where relevant data were presented. We also searched the reference lists of identified articles for further relevant papers.

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