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Review Article

Renin inhibition in the treatment of diabetic kidney disease

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Abstract

Inhibition of the RAAS (renin–angiotensin–aldosterone system) plays a pivotal role in the prevention and treatment of diabetic nephropathy and a spectrum of other proteinuric kidney diseases. Despite documented beneficial effects of RAAS inhibitors in diabetic patients with nephropathy, reversal of the progressive course of this disorder or at least long-term stabilization of renal function are often difficult to achieve, and many patients still progress to end-stage renal disease. Incomplete inhibition of the RAAS has been postulated as one of reasons for unsatisfactory therapeutic responses to RAAS inhibition in some patients. Inhibition of renin, a rate-limiting step in the RAAS activation cascade, could overcome at least some of the abovementioned problems associated with the treatment with traditional RAAS inhibitors. The present review focuses on experimental and clinical studies evaluating the two principal approaches to renin inhibition, namely direct renin inhibition with aliskiren and inhibition of the (pro)renin receptor. Moreover, the possibilities of renin inhibition and nephroprotection by interventions primarily aiming at non-RAAS targets, such as vitamin D, urocortins or inhibition of the succinate receptor GPR91 and cyclo-oxygenase-2, are also discussed.

Key words: aliskiren, cyclo-oxygenase-2, diabetic nephropathy, prorenin, renin–angiotensin system (RAS), urocortin, vitamin D

INTRODUCTION

Inhibition of the RAAS (renin–angiotensin–aldosterone system) plays a pivotal role in the prevention and treatment of diabetic nephropathy and some other proteinuric kidney diseases. A spectrum of studies, including large multi-centre randomized trials in patients at various stages of kidney disease, have documented that treatment with RAAS inhibitors can slow the progressive decrease in GFR (glomerular filtration rate), reduce proteinuria and microalbuminuria, and reduce CV (cardiovascular) mortality and morbidity in diabetic patients. However, despite these documented beneficial effects of RAAS inhibitors in patients with CKD (chronic kidney disease), reversal of the progressive course of disorders, such as diabetic nephropathy, or at least long-term stabilization of renal function is often difficult to achieve, and many patients still progress to ESRD (end-stage renal disease). Consequently, we are witnessing an unrelenting quest for new approaches to treatment of diabetic nephropathy that would improve prognosis in these patients.

The present review focuses on the current status of renin inhibition in the treatment of kidney disease and selected areas of renin research that may in future help to recognize new approaches to RAAS inhibition. Considering the key position of RAAS inhibitors in the treatment of diabetic nephropathy, most of the evidence discussed in the present review will be relevant for this disorder, but references to other kidney diseases will be also made when appropriate.

Abbreviations: ACE, angiotensin-converting enzyme; ACEI, ACE inhibitor; ALTITUDE, Aliskiren Trial in Type 2 Diabetes Using Cardiorenal Disease Endpoints; Ang-(1–7), angiotensin-(1–7); AngI etc., angiotensin I etc.; AT_1, angiotensin type 1; ARB, AT_1 receptor blocker; AVOID, Aliskiren in the Evaluation of Proteinuria in Diabetes; BP, blood pressure; CKD, chronic kidney disease; COX, cyclo-oxygenase; coxib, COX inhibitor; CRE, cAMP-response element; CRF, corticotropin-releasing factor; CRFR, CRF receptor; CTGF, connective tissue growth factor; CV, cardiovascular; DRI, direct renin inhibitor; ERK, extracellular-signal-regulated kinase; ESRD, end-stage renal disease; GFR, glomerular filtration rate; eGFR, estimated GFR; GPCR, G-protein-coupled receptor; HRP, handle region peptide; $1,25(OH)_2D$, 1,25-dihydroxyvitamin D; 25(OH)D, 25-hydroxyvitamin D; JGC, juxtaglomerular cell; KO, knockout; ATKO, AT_{1a} receptor KO; MAPK, mitogen-activated protein kinase; PGE_2, prostaglandin E_2; PRA, plasma renin activity; (P)RR, (pro)renin receptor; RAAS, renin–angiotensin–aldosterone system; RAS, renin–angiotensin system; SBP, systolic BP; siRNA, short interfering RNA; STZ, streptozotocin; TGF-β, transforming growth factor-β; UACR, urine albumin/creatinine ratio; Ucn, urocortin; V-ATPase, vacuolar H^+-ATPase; VDR, vitamin D receptor; VDRA, VDR agonist; VEGF, vascular endothelial growth factor; VITAL, Vitamin D Receptor Activator (Paricalcitol) in Albuminuria Lowering.

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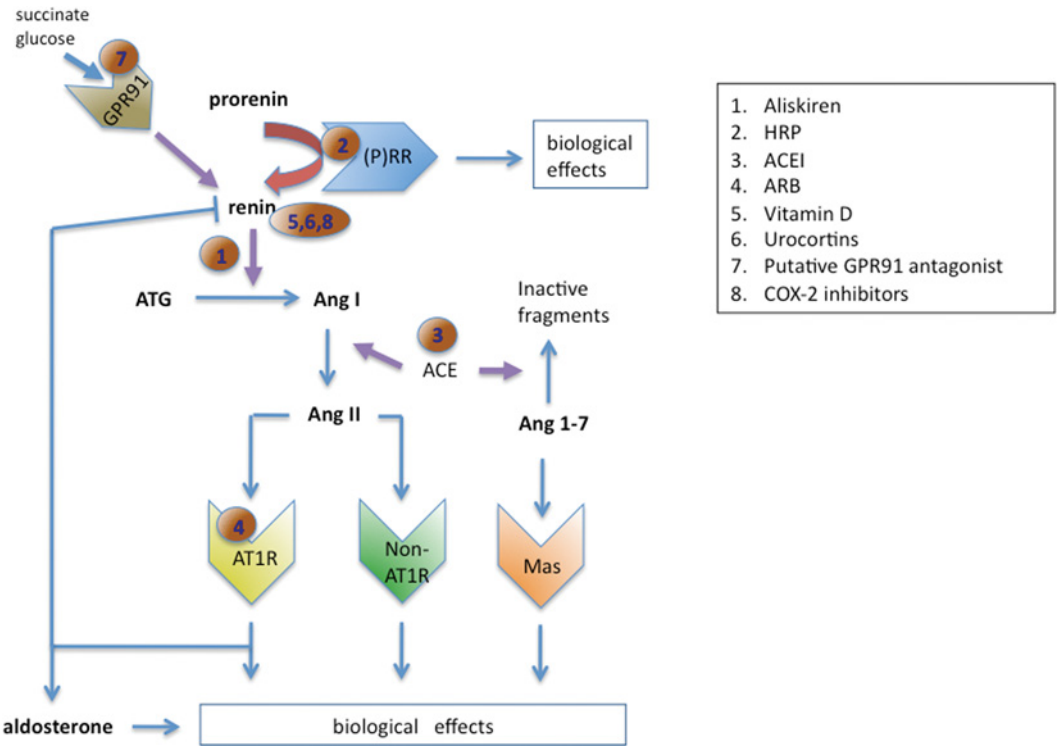

Figure 1 **Schematic presentation of the main steps in the RAAS cascade and sites of action of the RAAS inhibitors discussed in the present review**

Blue arrows indicate activation steps; and blue 'T' lines indicate an inhibitory step within the system. Purple arrows denote enzymatic actions. The sites of action of the RAAS inhibitors are in brown circles. ATG, angiotensinogen; Mas, Ang-(1–7) receptor.

RATIONALE FOR RENIN INHIBITION

Incomplete inhibition of the RAAS has been postulated as one of the reasons for unsatisfactory therapeutic responses to RAAS inhibition in some patients [1]. As reviewed previously [1–3], there are specific features of individual classes of RAAS inhibitors, reasons for incomplete blockade of the system by ACEIs [ACE (angiotensin-converting enzyme) inhibitors] or ARBs [AT_1 (angiotensin type 1) receptor blockers], and the rationale and potential benefits of renin inhibition in the treatment of hypertension and end-organ damage. The impact on the system downstream of inhibition differs between these individual classes. In brief, ACE inhibition leads to a reduced generation of AngII (angiotensin II) from AngI (angiotensin I), resulting in increases in the concentration of plasma AngI. ACEIs also lead to enhanced generation of Ang-(1–7) [angiotensin-(1–7)], which is considered to be one of the fragments with beneficial effects in CV system and kidney [4–7] (Figure 1). However, the ACEI-induced increase in plasma renin activity may ultimately lead to increased AngII generation by ACE-independent synthetic pathways, such as chymases [8], resulting in an escape from beneficial actions of ACEIs. ACE has many substrates other than AngI, including bradykinin [9,10], substance P, enkephalins and Ang-(1–7) [6,11]. Accumulation of those substances in response to treatment with the ACEI induces endothelium-dependent vasodilation and natriuresis mediated via NO (nitric oxide) and vasodilatory prostanoids further contributing to beneficial effects of ACEIs in the CV system and kidney. An additional line of evidence has shown that ACEIs increase the plasma levels of another ACE substrate *N*-acetyl-seryl-aspartyl-lysyl-proline [12], a peptide with antifibrotic actions [13].

In the case of ARBs, reactive increases in PRA (plasma renin activity) result in enhanced formation of AngII and all other angiotensin fragments, including AngIII (angiotensin III) and Ang-(1–7). This leads to stimulation of non-AT_1 receptors, resulting in effects that are not yet completely understood. For example, the increase in plasma AngII concentration after ARB intake stimulates AT_2 (AngII type II) receptors, which have been associated with beneficial, but also with detrimental, effects in target cells [14–17]. Moreover, increases in AngII may potentially override ARB blockade of AT_1 receptors.

The phenomenon of 'aldosterone breakthrough' has also been described in patients treated with ACEIs or ARBs [18]. The aldosterone breakthrough observed with ARB treatment may be related to increased AngII levels. ACEI therapy results in an initial decrease in plasma aldosterone level, but, in 30–40 % of patients, plasma aldosterone concentrations return to normal (or even above-normal) levels over a variable period of weeks or months [19]. In observational studies, patients with aldosterone breakthrough have worse clinical outcomes than do those without [20].

Inhibition of renin, a rate-limiting step in the RAAS activation cascade, is the logical approach to overcome at least some of the

abovementioned problems associated with the treatment with an ACEI or ARB and incomplete RAAS blockade. The introduction of aliskiren, a non-peptide orally active DRI (direct renin inhibitor), was a result of almost three decades of research in the field. Aliskiren occupies specific subsites within the enzymatic pocket of renin and blocks its enzymatic function, resulting in suppression of PRA. Unlike the ACEI or ARB, DRIs completely inhibit production of all angiotensin peptides [21]. Moreover, aliskiren possesses more sustained aldosterone-suppressive effects than other RAAS inhibitors [21,22]. On the other hand, aliskiren also lacks potentially beneficial endothelium-dependent actions of ACEIs. More detailed information about the history and pharmacology, as well as about CV and renal effects of renin inhibitors in non-diabetic context have been a subject of excellent reviews [1,3,23]. The antihypertensive effects of aliskiren have been well established, and summarized in two recent meta-analyses [24,25]. Detailed discussion of these effects is beyond the scope of the present review.

ALISKIREN IN THE TREATMENT OF KIDNEY DISEASE

Experimental evidence

The renin molecule displays high species specificity, and human renin inhibitors can be effectively tested only in the marmoset and the guinea pig. Experimental studies in models of kidney disease are not therefore as abundant compared with other RAAS inhibitors. To investigate the therapeutic effects of human renin inhibitors in more practical rodent models of hypertension and organ damage, researchers have utilized transgenic rats expressing the human renin and angiotensinogen genes [26]. As reported by Pilz et al. [27], aliskiren lowered BP (blood pressure) and significantly improved kidney function, albuminuria, cardiac morphology and mortality in this aggressive model of hypertensive end-organ damage. The effects were comparable with valsartan.

Several reports indicating the nephroprotective effects of aliskiren are available in models expressing mouse renin (mRen2). The mechanisms of the effects of aliskiren in this model of nephropathy have been reported by Feldman et al. [28]. In addition to an amelioration of proteinuria, and renal expression of TGF-β (transforming growth factor-β) and collagen I, the authors demonstrated a capability of aliskiren to bind both mouse renin and prorenin, and neutralize prorenin receptor-mediated activation of prorenin and the consequent gain in AngII-generating ability (see below). In more recent studies, Ren2 rats exhibited increases in SBP (systolic BP), albuminuria and renal 3-nitrotyrosine content, as well as ultrastructural podocyte foot-process effacement and diminution of the podocyte-specific protein nephrin. Structural and functional alterations were accompanied by increased renal cortical AngII and AT_1 receptors, as well as NADPH oxidase subunit (Nox2) expression compared with controls. Abnormalities were attenuated to a similar extent with both aliskiren and irbesartan treatment [29]. A more recent study by these authors [30] confirmed the beneficial effect of aliskiren on podocyte integrity, tubular injury, proteinuria and markers of renal oxidative stress in Ren2 rats, but did not document additive effects of a combination of aliskiren with the ARB compared with monotherapies. Aliskiren was also effective in treatment of models of normotensive tubulointerstitial fibrosis in collagen III-KO (knockout) mice [31] and, surprisingly, in rats with unilateral ureteral obstruction [32]. On the contrary, aliskiren failed to influence experimental chronic allograft nephropathy [33]. Unlike aliskiren, candesartan was effective in this model. The authors attributed the differences to lower generation of protective Ang-(1–7) in aliskiren-treated animals.

In the diabetic context, aliskiren inhibited high glucose-induced generation of AngII by immortalized mouse podocytes *in vitro* [34]. *In vivo*, Kelly et al. [35] first observed the beneficial effects of aliskiren on albuminuria and renal structural parameters in diabetic (mRen-2)27 rats. The beneficial effects in the diabetic kidney were comparable with the ACEI perindopril, despite a weaker effect on BP. However, it should be noted that otherwise elegant studies with mRen rats have only limited relevance for the treatment of diabetic nephropathy, as most of the changes in the kidney are result of severe hypertension and intrarenal RAS (renin–angiotensin system) activation, with only a minor additive effect of diabetes [36].

Clinical evidence

Most of the evidence in the clinical arena is available in diabetic patients. Smaller short-term studies in Type 2 diabetic patients initially indicated an anti-albuminuric effect of aliskiren (44% decrease) after 4 weeks of treatment compared with baseline. These effects went beyond its BP-lowering actions and were comparable with previous studies with an ARB or ACEI in Type 2 diabetes [37]. After drug withdrawal, the antiproteinuric effect of aliskiren lasted longer than its effect on BP.

When directly compared with the ARB irbesartan (300 mg) in a double-blind randomized cross-over study in Type 2 diabetic patients with four 2-month treatment periods [38], aliskiren (300 mg) reduced albuminuria to a similar degree (48% compared with placebo) as irbesartan (58%). Combination treatment reduced albuminuria by 71%, significantly more than either monotherapy. The effect of aliskiren on 24-h BP was weaker compared with irbesartan and was not additive in the combination of both inhibitors. That study suggested that the combination of aliskiren and irbesartan could be more antiproteinuric in Type 2 diabetic patients with albuminuria compared with monotherapy.

Until recently, the AVOID (Aliskiren in the Evaluation of Proteinuria in Diabetes) trial [39] was the largest clinical trial evaluating the nephroprotective potential of aliskiren in diabetes. This randomized double-blind placebo-controlled multi-centre study, conducted in 15 countries, evaluated the effects of aliskiren in 599 patients with hypertension, Type 2 diabetes and manifest nephropathy already receiving established treatment with RAAS inhibitors. By the end of the 6 month study period, the patients treated with add-on aliskiren (150–300 mg) demonstrated a further reduction in the mean urinary UACR (urine albumin/creatinine ratio) by 20% compared with placebo. After adjustment for the change from baseline in SBP, the reduction was 17%. Moreover, UACR was reduced by at least 50% in 25% of patients treated with aliskiren, whereas this outcome occurred only in 13% of

those receiving placebo. The beneficial effects of aliskiren were associated with a slight, but statistically insignificant, reduction in SBP and DBP (diastolic BP) (2/1 mmHg). Aliskiren was well-tolerated, with a similar profile of adverse events as in the placebo group. In the original publication, severe hyperkalaemia (>6 mmol/l) occurred more frequently in the aliskiren group, but the difference was not statistically significant.

The authors concluded that aliskiren has nephroprotective effects independent of its BP-lowering effect in patients with Type 2 diabetes who are receiving the maximal recommended renoprotective treatment and optimal antihypertensive treatment.

Although the study demonstrated the promising additive effect of aliskiren in patients already treated with the maximal dose of an ARB, several criticisms were brought up after publication of the paper. Provided that the escape from the effects of the ACEI or ARB is a clinically significant phenomenon, one might have expected that adding a renin inhibitor to an ARB would be more effective than combinations of an ARB with other RAAS inhibitors, such as an ACEI or spironolactone. However, available evidence does not support this notion. For example, Rossing and Parving [40] demonstrated in a smaller study that adding spironolactone to maximal treatment with an ARB or ACEI achieved a further reduction in albuminuria by 33 %. It is likely that similar effects as reported in the AVOID trial would be achieved by combining aliskiren with traditional RAAS inhibitors, or even other hypertensive agents at much lower cost of treatment. Moreover, the study was too short and relied only on albuminuria as a primary end point, making the comparisons of nephroprotective potential of the aliskiren + losartan combination with losartan alone difficult. Although the trial did not document a significantly higher risk of hyperkalaemia in aliskiren-treated patients, the concerns about this adverse effect of aliskiren remained.

Several post-hoc analyses addressed some concerns raised by the original AVOID publication. The authors attempted to address the major issue of whether addition of aliskiren to an ARB could better preserve kidney function than the treatment with the ARB alone and provide harder evidence than the disputed antiproteinuric effect alone. When stratified for baseline kidney function, aliskiren-treated patients with CKD3 [eGFR (estimated GFR), 30–59 ml/min] demonstrated a lower frequency of renal dysfunction (prespecified as development of serum creatinine >176.8 μmol/l) [41]. Other analyses showed that renal function was better preserved with aliskiren in patients with insufficient BP control [42] and revealed a higher incidence of hyperkalaemia, particularly in the group of patients with CKD3 [41]. Finally, analysis of cost effectiveness was also published, indicating that the higher cost of the treatment with aliskiren could be in part compensated by saving money for renal replacement therapy [43]. These post-hoc analyses strengthened the original report; however, the data remained unconvincing as these studies provided data still within the 6-months follow-up period and the longer follow-up of kidney function is not available.

Some caveats of the AVOID trial, i.e. reliance on albuminuria as the main endpoint and short duration, were supposed to be addressed by the ALTITUDE (Aliskiren Trial in Type 2 Diabetes Using Cardiorenal Disease Endpoints) trial [44]. This large multicentre double-blind randomized placebo-controlled trial has included 8600 patients with Type 2 diabetes (age $\geqslant$35 years) at high risk of CV and renal events with at least one of the following inclusion criteria: persistent macroalbuminuria and eGFR $\geqslant$30 ml/min per 1.73 m^2, persistent microalbuminuria and a mean eGFR $\geqslant$30 and <60 ml/min per 1.73 m^2, a history of CV disease (e.g. myocardial infarction, stroke, heart failure or coronary artery disease) and a mean eGFR $\geqslant$30 and <60 ml/min per 1.73 m^2. Patients were treated with aliskiren titrated to 300 mg/day after 4 weeks or a placebo in addition to optimal CV treatment including an ACEI or ARB. The primary end point of that study was a doubling of serum creatinine, onset ESRD, CV-related death, myocardial infarction and stroke.

In December 2011, the Data Monitoring Committee recommended early termination of the trial after 18–24 months. According to its interim analysis, it became apparent that aliskiren was unlikely to have additional beneficial effects when combined with standard treatment, including an ACEI or an ARB. There was increased incidence of renal complications, hyperkalaemia, hypotension and, surprisingly, non-fatal strokes with aliskiren compared with placebo. In April 2012, Novartis announced that will remove its combination product Valturna® (which contains aliskiren and valsartan) from U.S.A. markets. Aliskiren (Tekturna®) remained on the market.

Subsequently, the ASPIRE (Aliskiren Study in Post-MI Patients to Reduce Remodeling) study conducted in high-risk post-myocardial infarction patients also reported a significantly increased incidence of renal failure, hypotension and hyperkalaemia with the combination of aliskiren and an ACEI or ARB [45]. Interestingly, preliminary termination of the ALTITUDE study coincided with the publication of a meta-analysis of studies focusing on the risk of hyperkalaemia with the combination of aliskiren with an ACEI or an ARB [46]. This meta-analysis of ten randomized controlled trials conducted in a heterogeneous population of almost 5000 patients with hypertension, diabetes, heart failure or myocardial infarction confirmed the increased risk of hyperkalaemia (serum potassium, >5.5 mmol/l) associated with a 8–36-week exposure to dual RAS blockade with aliskiren (usually at maximal doses) and ACEIs or ARBs (at various doses) compared with monotherapies. This work did not detect an increased risk of acute renal failure (serum creatinine, >176.8 μmol/l) with dual RAAS blockade with aliskiren, but the data were obtained mostly from patients with normal renal function at baseline.

Most recently, investigators of the ongoing large multi-centric APOLLO (Aliskiren Prevention of Later Life Outcomes) trial exploring the safety and CV and renal effects of aliskiren in combination with amlodipine or with HCTZ (hydrochlorothiazide) in elderly patients were informed about the decision by Novartis to terminate the study. This decision was apparently motivated by safety concerns raised by the ALTITUDE study, in particular in a subset of patients also receiving another RAAS inhibitor.

At this stage, introduction of aliskiren does not seem to represent a significant progress in the treatment of diabetic kidney disease and proteinuric CKD. However, it represents an important addition to the pool of therapeutic agents, which might be useful especially for patients who have intolerance/allergy to ACEIs or ARBs.

Although more complete blockade of the RAAS may suppress some pathophysiological processes operating in the kidney, these beneficial effects are, in clinical practice, offset by a high risk of hyperkalaemia and kidney failure. In addition to traditional causes for these complications, such as suppression of aldosterone production and altered renal haemodynamics, the lack of additive protective effects of aliskiren when combined with other RAAS inhibitors may be attributable to reduced generation of Ang-(1–7) and, in particular, to reactive hyper-renaemia, the consequences of which are discussed in the following section.

(P)RR [(PRO)RENIN RECEPTOR] AND ITS INHIBITION

The (P)RR field has been extensively reviewed and analysed [47], including a recent excellent overview in *Clinical Science* [48]. In the present review, I will focus only on the evidence related to nephroprotection in diabetes and other chronic kidney diseases.

Both renin and prorenin bind the (P)RR, which was first described by Nguyen et al. [49]. This receptor acts as a (pro)renin co-factor on the cell surface by enhancing renin catalytic activity. Aliskiren does not inhibit the (pro)renin–(P)RR interaction [28,50]. (Pro)renin has an N-terminal 'handle region' that covers the enzymatic site, rendering this molecule inactive in terms of angiotensinogen cleavage. When prorenin occupies (P)RR, the molecule is non-proteolytically activated via opening of the 'handle region' of the prosegment and becomes capable of cleaving angiotensinogen. In addition to its roles in enhancing (pro)renin enzymatic activity towards AngI generation, both *in vitro* studies in renal cells and *in vivo* studies in models of kidney disease have shown that (P)RR binding triggers intracellular signalling cascades involved in the pathophysiology of kidney diseases, independently of AngII actions.

Each class of RAAS inhibitors, including aliskiren, causes reactive increases in PRA, as well as secretion of renin and prorenin due to interruption of the AngII-induced feedback control of renin synthesis and release. As renin/prorenin accumulate upstream of blocked RAAS, the (P)RR may attain paramount importance, and its stimulation triggered by accumulating (pro)renin could explain the lack of additive renal and CV protection in patients treated with combinations of RAAS inhibitors or even lower protective potential of this approach compared with monotherapies with these agents. Thus, despite remaining entirely in the area of basic research, this signifies the high clinical relevance of (P)RR research.

Indeed, the potentially deleterious effects of RAAS-inhibitor-induced renin activity have been demonstrated by the results of the ALOFT (Aliskiren Observation of heart Failure Treatment) trial [51], which have shown that active plasma renin concentration is a strong indicator of mortality in patients with chronic heart failure treated with an ACEI or ARB, in particular in those with lower kidney function.

Specific (P)RR inhibition is particularly attractive in diabetes. As demonstrated in seminal studies by Luetscher et al. [52], diabetic patients display a 2–3-fold elevation in circulating prorenin, and prorenin levels are considered to be a risk factor for the development of microvascular complications in Type 1 diabetes [53].

Considering the a possible consequences of (P)RR interaction with renin or prorenin described above, it is not surprising that studies exploring the nephroprotective potential of PRR inhibition followed shortly after the discovery of the receptor. Thus far, the effort to design competitive antagonists of the (P)RR has been unsuccessful. However, investigators have found a way to bypass this problem. Suzuki et al. [54] observed that an antibody directed against the 'handle' region of the prosegment of human prorenin was able to open and non-proteolytically activate this part of the prorenin molecule in a similar manner to its binding to the (P)RR. On the basis of this finding, Ichihara et al. [55] designed a decapeptide, which encompassed the handle region (HRP), as an inhibitor of prorenin–(P)RR binding. *In vitro,* these original studies showed that HRP competitively inhibited prorenin binding to the (P)RR [55]. *In vivo*, the effects of HRP were first investigated in models of diabetic nephropathy, a logical approach considering the stimulatory effects of diabetes on circulating prorenin [55]. In this first study, HRP was remarkably effective in preventing proteinuria, glomerulosclerosis and glomerular type IV collagen expression in STZ (streptozotocin)-diabetic rats over the period of 6 months, without affecting BP and metabolic control. The treatment with HRP did not influence PRA or plasma concentrations of prorenin and angiotensin peptides. However, the treatment normalized renal tissue levels of angiotensin peptides.

In the following study, Ichihara et al. [56] investigated the contribution of AngII-independent (P)RR-dependent pathways in the development of diabetic nephropathy. The therapeutic effects of the HRP were compared with the effects of the ATKO (AT_{1a} receptor KO) and ACE inhibition in STZ-diabetic mice. Compared with wild-type mice, ATKO slightly attenuated the development of proteinuria, but had minimal effect on glomerulosclerosis at 24 weeks after induction of diabetes. Treatment with HRP prevented the development of proteinuria and glomerulosclerosis in ATKO mice. An ACEI failed to influence these parameters. Interestingly, HRP was more effective in the prevention of nephropathy than an ACEI even in wild-type STZ-diabetic mice. These studies suggested the importance of AngII-independent components of (P)RR signalling for the pathogenesis of diabetic nephropathy, at least in mice. The same group also documented the beneficial effects of HRP in uninephrectomized STZ-diabetic rat [57] and in *db*/*db* mice, a model for Type 2 diabetes [58].

It cannot be overlooked that the original evidence about the nephroprotective effects of HRP and nephropathic (P)RR signalling came from a limited number of investigators. However, more recently, other groups have also communicated evidence consistent with the beneficial effects of HRP in the diabetic kidney, specifically its effects on TGF-β and CTGF (connective tissue growth factor) expression, ERK activation, and (P)RR expression and phosphorylation *in vitro* in mesangial cells exposed to high glucose and *in vivo* in STZ-diabetic rat kidneys [59,60]. These effects were comparable with valsartan [60].

The relevance of HRP treatment in the diabetic context has been also supported by a study showing its beneficial effects in

the diabetic rodent retina [61]. The authors showed that HRP reduced leukostasis in retinal vasculature of diabetic rats and mice. This effect was stronger than in animals treated with the ARB losartan in wild-type mice and was also observed in AT_1-receptor-KO mice. (P)RR inhibition also reduced the diabetes-induced elevation in retinal expression of VEGF (vascular endothelial growth factor) and ICAM-1 (intracellular adhesion molecule-1) in wild-type diabetic animals. Similar to renal cells, HRP reduced the diabetes-induced elevation in retinal expression of phosphorylated MAPK (mitogen-activated protein kinase).

In a non-diabetic context, the observations supporting the pathophysiological roles of (P)RR in the kidney and CV system and HRP-induced nephroprotection include the beneficial actions of the peptide in (i) end-organ damage in transgenic rats expressing prorenin in the liver [62], (ii) higher BP in transgenic rats with human (P)RR targeted to the vascular smooth muscle [63], (iii) HRP-inhibitable proteinuria and glomerulosclerosis in rats with ubiquitous expression of the human (P)RR [64], (v) attenuation of cardiac fibrosis in HRP-treated stroke-prone SHRs (spontaneously hypertensive rats) [65], and (vi) attenuation of slowly progressive nephropathy by HRP in non-clipped kidneys [66] in 2K1C Goldblatt hypertensive rats.

Although impressive, the results of studies with HRP have been accepted from the beginning with some caution. The renal structural data in models of diabetes in the studies mentioned above were not sufficiently convincing. This would be particularly welcome in studies in mice models that display some degree of resistance to the development of glomerulosclerosis. Considering the antiproteinuric effects of HRP in diabetes, analysis of podocyte morphology and the expression of podocytic proteins was not provided in the earlier studies nor were the long-term effects of HRP on the decline in GFR compared with other RAAS inhibitors reported.

Further scepticism has been fuelled by disparate findings in *in vitro* studies and in some models of non-diabetic kidney disease. Other groups have not confirmed the inhibition of prorenin binding to the (P)RR by HRP [67–69] *in vitro*, and HRP binding was also detected in cells devoid of the (P)RR [68]. HRP is also unlikely to block renin binding to the (P)RR.

In vivo, short-term treatment with HRP, i.e. during the renin-dependent stage of hypertension and organ damage, was not effective in the prevention of glomerulosclerosis in the clipped kidney in Goldblatt hypertensive rats [66,69,70], which is in contrast with their finding of long-term beneficial effects in non-clipped kidneys [66]. In this model, HRP did not affect BP and proteinuria. Another line of evidence casting doubt on the physiological relevance of the renin/prorenin–(P)RR interaction was obtained in transgenic rats with inducible prorenin expression, which displayed no renal involvement despite 200-fold higher prorenin levels [71,72].

Moreover, recent studies in cardiomyocytes and in the kidney have suggested additional physiological functions of the (P)RR unrelated to the RAAS. The (P)RR co-localizes with V-ATPase (vacuolar H^+-ATPase) in the kidney [73]. V-ATPases are complex proton pumps found in most cell types that play an important role in the acidification of subcellular compartments. Further studies have demonstrated that the receptor is indispensable for the stability and integrity of V-ATPase, a phenomenon related to the fact that accessory protein ATP6AP2 (V-ATPase lysosomal accessory protein 2) of the V-ATPase is a post-translationally truncated version of the (P)RR, resembling its C-terminal domain. In cardiomyocyte-specific (P)RR-KO mice, the abundance of several V-ATPase subunits is decreased in cardiomyocytes, resulting in the development of heart failure due to defective autophagy and ultimately cell death [74]. The (P)RR also functions as an adaptor between V-ATPase and receptors for members of the Wnt family of proteins [75], which are important for kidney development. Two recent studies have in parallel demonstrated that (P)RR is indispensable for kidney or podocyte development and function, an effect related to V-ATPase function. Working with mice with a conditional podocyte-specific (P)RR KO, Oshima et al. [76] and Reidiger et al. [77] described early mortality, massive proteinuria and devastating structural changes involving practically all renal compartments by the end of the 4-week period after birth.

The idea of (P)RR inhibition as one of the means of nephroprotection has been somewhat revived by studies utilizing (P)RR siRNA (short interfering RNA). In unstimulated rat mesangial cells, (P)RR siRNA suppressed ERK1/2 activation, cell proliferation and TGF-β1 expression [59]. In high-glucose-stimulated mesangial cells, (P)RR knockdown decreased the expression of TGF-β1 and CTGF [60]. In these studies, the effects of (P)RR knockdown were comparable with the effects of HRP. A suppression of TGF-β1 and PAI (plasminogen-activator inhibitor)-1 has also been reported in (P)RR siRNA-treated cultured glomeruli from rats with glomerulonephritis [78]. In complex studies by Cheng et al. [79], mice with STZ-induced diabetes and selective COX (cyclo-oxygenase)-2 overexpression in podocytes displayed an accelerated course of nephropathy compared with diabetic wild-type animals. These transgenic mice had a more rapid progression of albuminuria, more advanced mesangial expansion, GBM (glomerular basement membrane) thickening and podocyte effacement. This was associated with increased podocytic (P)RR expression, indicating that COX-2 metabolites stimulate receptor expression in these cells. Treatment of COX-2-overexpressing podocytes with (P)RR siRNA decreased high-glucose-stimulated renin activity, activation of ERK and p38 MAPK, restored α-actinin 4 expression and reduced apoptosis.

These experiments with (P)RR siRNA are consistent with some previous positive observations with HRP *in vitro* and provide further support that (P)RR could play a role in stimulation of at least some of the molecular markers and mediators of diabetic nephropathy and progressive kidney disease. However, this approach could not help to resolve the issue as to whether long-term PRR inhibition results in kidney protection.

Thus it remains unclear whether PRR inhibition will expand our battery of approaches to RAAS inhibition in clinical medicine in the future. The current evidence reviewed above suggests that (P)RR inhibition might be nephroprotective in adult organisms in some kidney diseases, but the receptor is necessary for appropriate kidney development and its inhibition is detrimental in the developing organism, a situation resembling the developmental and pathophysiological roles of the AT_1 receptor. Assuming that (pro)renin binding to (P)RR has detrimental consequences in the

CV system and in the kidney, (P)RR inhibition might be beneficial in combination with RAAS inhibitors. However, these hypotheses are still waiting for confirmation in appropriately designed studies. Importantly, there is still no evidence indicating the importance of (P)RR stimulation and downstream signalling in humans. The potential clinical relevance of (P)RR inhibition warrants further investigation upon the availability of specific agents, such as non-peptide antagonists, to interrupt this system.

RENIN INHIBITION BY TREATMENTS PRIMARILY AIMING AT NON-RAAS TARGETS

Previous sections have discussed approaches that have been designed to directly target renin (and prorenin) actions. The following sections will review approaches primarily aiming at non-RAAS targets, which also interfere with the actions or synthesis of renin (see Figure 1). These interventions may act as adjuvant renin inhibitors in combination with ACEIs or ARBs.

Vitamin D and VDRAs [VDR (vitamin D receptor) agonists]

In addition to its well-known effects on calcium and bone metabolism, vitamin D and other VDRAs have been shown to possess potent nephroprotective effects. Studies in mouse models for both Type 1 [80] and Type 2 [81] diabetes have demonstrated that the combined treatment with losartan and paricalcitol completely prevented albuminuria, suppressed the induction of fibronectin, TGF-β and MCP (monocyte chemoattractant protein)-1, and reversed the decline of slit diaphragm proteins, leading to the restored glomerular filtration barrier structure and markedly reduced glomerulosclerosis. The combined treatment was significantly more effective compared with treatment with losartan or paricalcitol alone, suggesting synergistic effects with RAAS inhibitors. Similar beneficial actions have been reported in models of non-diabetic glomerulosclerosis [82–84], experimental glomerulonephritis [85] and tubulointerstitial fibrosis [86].

In accordance with abundant experimental evidence, several clinical studies have also suggested that administration of vitamin D and other VDRAs might possess nephroprotective actions. Smaller retrospective and prospective studies in patients with CKD, including those treated with RAAS inhibitors, originally detected short-term antiproteinuric effects of VDRAs [87–89]. More recently, significant reduction of albuminuria in conjunction with decreased urinary excretion of TGF-β has been reported in Type 2 diabetic patients with nephropathy after 4 months of treatment with cholecalciferol [90]. Calcitriol has a modest antiproteinuric effect in patients with IgA nephropathy and persistent proteinuria despite ACEI or ARB therapy [89].

The VITAL [Vitamin D Receptor Activator (Paricalcitol) in Albuminuria Lowering] study [91] has been thus far the largest trial in this field conducted in patients with diabetic nephropathy already treated with RAAS inhibitors. Although the study has demonstrated a borderline anti-albuminuric effect in the overall population and a significant anti-albuminuric effect in patients with serum creatinine >178 μmol/l, it was too short to evaluate whether the treatment was associated with better preservation of GFR. In fact, the eGFR was lower in paricalcitol-treated patients at the conclusion of the 6-month follow-up period.

Inhibition of renin has been the most frequently quoted mechanism of VDRA-induced nephroprotective actions. The relationship between vitamin D levels and PRA has been recognized for more than two decades. Studies conducted in normotensive, as well as hypertensive, patients on different sodium diets have shown an inverse relationship between PRA and vitamin D levels [92,93]. In a study by Forman et al. [94], lower plasma 25(OH)D [25-hydroxyvitamin D] levels in 184 normotensive individuals in high sodium balance were associated with significantly higher circulating AngII concentrations, as well as with a blunted RPF (renal plasma flow) response to infused AngII. Most recently, the data from these smaller studies have been supported by observations in a large cohort of patients ($n>3000$) in the LURIC (LUdwigshafen RIsk and Cardiovascular Health) study that have shown that both serum 25(OH)D as well as $1,25(OH)_2D$ [1,25-dihydroxyvitamin D] levels are independently and inversely associated with plasma renin and AngII concentrations [95]. These findings support the concept that vitamin D deficiency may be associated with an up-regulation of the RAS.

Li et al. [96,97] provided experimental evidence for the link between VDR and renin synthesis/secretion in mice lacking the *VDR* gene. Absence of vitamin D signalling in these animals led to an increase in renin gene expression and circulating AngII levels. When placed on a high-salt diet, these KO mice had slight reductions in renin and AngII, but maintained levels substantially higher than wild-type mice on a similar diet [96]. Furthermore, renin levels remained elevated despite normalization of plasma calcium concentrations, and injection of $1,25(OH)_2D$ reduced renin expression in wild-type mice. Overexpression of the VDR in JGCs (juxtaglomerular cells) resulted in renin suppression in transgenic mice [98]. Vitamin D repression of renin expression was independent of calcium metabolism, volume- and salt-sensing mechanisms and the AngII-feedback regulation. Supporting these findings, mice lacking the gene encoding 1α-hydroxylase, an enzyme responsible for vitamin D hydroxylation, have a similar phenotype [99].

Further studies by the same group have demonstrated that $1,25(OH)_2D_3$ (1,25-dihydroxyvitamin D_3) down-regulates renin gene transcription by suppressing, at least in part, CRE (cAMP-response element)-mediated transcriptional activity in the renin gene promoter [97]. As CRE is activated by cAMP/PKA (protein kinase A) signalling, that study identified a major regulatory pathway as the target in vitamin D inhibition of renin synthesis. Notably, there are additional mechanisms whereby vitamin D can interfere with RAAS activity, for example via inhibition of the expression of angiotensinogen by inhibition of NF-κB (nuclear factor κB) [100].

Other groups have demonstrated VDRA-induced suppression of renin in association with protective effects in the CV system and in the kidney. Bodyak et al. [101] demonstrated a reduction in renin levels in paricalcitol-treated Dahl SS (salt-sensitive) rats in conjunction with beneficial effects on cardiac hypertrophy.

In another study, paricalcitol treatment was shown to significantly reduce the mRNA levels and protein expression of angiotensinogen, renin and the renin receptor in subtotally nephrectomized rats [102]. Glomerular and tubulointerstitial damage, hypertension, proteinuria and the deterioration of renal function resulting from renal ablation were all similarly and significantly improved.

Direct effects of VDRA administration on the components of the renin–angiotensin axis in humans have been less studied. In a small non-controlled study conducted in patients on haemodialysis, intravenous calcitriol led to a significant reduction in plasma renin and AngII levels, and a significant regression of LVH (left ventricular hypertrophy) [103]. Sugden et al. [104] randomized 34 patients with Type 2 diabetes mellitus and 25(OH)D levels <20 ng/ml to receive either 100 000 units of vitamin D_2 or placebo. At 8 weeks of follow-up with supplementation, SBP declined by 14 mmHg compared with placebo. In addition, vitamin D-treated patients had a decrease in AngII levels compared with the placebo group. On the other hand, there were no differences in PRA or aldosterone in the VITAL trial, but it could be argued that the study was not designed to address this question.

These studies suggest that the beneficial effects of VDRAs in chronic renal failure are due, at least in part, to down-regulation of the renal RAAS. Inhibition of renin synthesis by VDRAs would be a welcome mechanism of action as opposed to all classes of currently used inhibitors of RAAS, which invariably cause hyperreninaemia with potential consequences (as discussed above in more detail).

Although the relationship between vitamin D and renin synthesis has been well established, some caveats remain. The long-term nephroprotection by VDRAs, as well as persuasive evidence indicating the potential of these compounds to suppress renin synthesis or PRA in clinical settings, in particular in patients treated with RAAS inhibitors, have not been documented yet. Interestingly, treatment with VDRAs in combination with RAAS inhibitors has not been shown to be associated with hyperkalaemia and reductions in GFR, as would be expected in the light of recent clinical trials. At this stage, we can only speculate whether this is an important advantage of VDRAs compared with the combination of traditional RAAS inhibitors or a result of insufficient renin-inhibiting actions with little clinical relevance.

Furthermore, in the light of recent evidence by Wang et al. [105] the mechanism of action whereby VDRAs induce the suppression of renin synthesis remains obscure. Interestingly, these authors did not detect VDR expression on JGCs, an observation in contrast with the abundant presence of this molecule in the distal tubule and to a lesser degree in the proximal tubule. It is possible that VDR expression in JGCs is different in some kidney diseases or that VDRA-induced suppression of renin synthesis occurs via an indirect mechanism, independent of VDR signalling in JGCs. Low expression of VDR on JGCs may require high doses of VDRAs to achieve the relevant therapeutic effect.

This leads to another shortcoming of VDRAs, i.e. their relatively narrow therapeutic window due to the risk of hypercalcaemia. Progress in the field may be achieved by introduction of new VDRAs with weaker hypercalcaemic effects. More studies are needed to evaluate further this attractive approach to RAAS modulation in diabetes and CKD.

Ucn (urocortin) peptides

Ucn1 and Ucn2 belong to a family of peptides with CRF (corticotropin-releasing factor). The Ucn peptides signal through the two GPCRs (G-protein-coupled receptors) CRFR1 and CRFR2 (CRF receptor 1 and 2 respectively). CRFR2 is widespread throughout the periphery, expressed in the heart and systemic vasculature, and mediates the CV effect of these peptides, which include vasodilation and positive inotropic and chronotropic effects in normal animals [106,107]. Endogenous production of Ucn peptides acting via CRFR2 plays a role in the pathogenesis of heart failure [108]. Acute administration of Ucn peptides has beneficial effects in experimental and clinical heart failure both on cardiac parameters as well as on vasoconstrictor and sodium/volume-retaining hormonal systems, including the RAAS and renal function [109,110]. In turn, inhibition of the actions of Ucn peptides with a CRFR2 antagonist peptide in experimental heart failure [108] is associated with marked elevations of the already activated PRA, aldosterone and ET (endothelin)-1 systems compared with controls. A similar, but greatly attenuated, response was observed in the normal state. Whether the increase in PRA [occurring despite increases in arterial pressure and plasma concentrations of ANP (atrial natriuretic peptide)] reflects inhibition by the endogenous Ucn peptides or is related to some other PRA-stimulatory mechanism(s) remains to be seen.

Importantly, Ucn peptides also block ACEI-induced PRA activity [111], and studies in rats have shown that, unlike renin inhibitors, Ucn peptides increase tissue concentrations of Ang-(1–7) [112]. In a recent study, Rademaker et al. [113] investigated more prolonged effects of Ucn2 administration (4 days) in sheep with heart failure. In accordance with their previous evidence, the beneficial haemodynamic, renal and hormonal effects of Ucn2 were associated with suppressed PRA and aldosterone.

Taken together, considering their actions, Ucn peptides represent almost ideal substances for combination with RAAS inhibitors, including the risky conditions with lower GFR. Further clinical research in this direction is highly warranted. The mechanisms of actions underlying renin suppression remain unclear – direct effects are possible considering the inhibitory effects of Ucn peptides on ACEI-induced PRA, but improved haemodynamics, GFR and changes in NaCl macula densa delivery have been suggested as contributing factors.

Relevant for nephroprotection in diabetes, two studies by Li et al. [114,115] conducted in *db*/*db* mice and STZ-diabetic rats have demonstrated that treatment with Ucn1 could indeed ameliorate glomerular extracellular matrix expansion, albuminuria, expression of TGF-β, CTGF, VEGF and markers of oxidative stress in the kidney. Unfortunately, PRA or other parameters of the RAAS were not measured in these studies and it remains unknown whether the beneficial effects of Ucn peptides in experimental diabetic nephropathy are attributable to suppression of renin. The clinical efficacy of Ucn peptides may be diminished by their negative effects on insulin signalling [116]; however, the data in the study mentioned above in experimental Type 2 diabetic mice suggest that this notion may be an unsubstantiated concern.

The succinate receptor GPR91 (GPCR 91)

The discovery of the GPCR GPR91 [117], which is activated by the citric acid (tricarboxylic acid) cycle intermediate succinate established a novel mechanism of RAAS activation. Intravenous injection of succinate into rats and mice elevated BP within 1–2 min via renin release and RAAS activation, and this response was abolished in GPR91-deficient mice [117]. More recently, this topic has been developed further by Peti-Peterdi and co-workers. These authors have identified a new direct link between diabetic hyperglycaemia and renin release [118,119]. The elevations in renal and plasma renin and prorenin detected in diabetic mice were abolished in diabetic GPR91-KO mice [118]. *In vitro*, in microperfused JGA (juxtaglomerular apparatus) preparations, which are free of systemic influences, high glucose or succinate treatment induced GPR91-mediated renin release within 1–2 min after administration [118]. The paracrine signalling mechanism involved increases in macula densa and endothelium-derived PGE_2 (prostaglandin E_2) and NO synthesis and release mediated by intracellular Ca^{2+} (in endothelium) and p38 MAPKs and phospho-ERK1/2 (in macula densa) [118,119]. PGE_2 and NO also caused afferent arteriole vasodilation, which may contribute to glomerular hyperfiltration in early diabetic nephropathy, an observation in agreement with our previous haemodynamic studies [120,121]. These effects of succinate are interesting as they can explain the rather counterintuitive parallel presence of hyperfiltration and activation of the RAAS in the early stage of diabetic nephropathy.

Succinate accumulation and GPR91 signalling are early and potentially important events in the diabetic kidney, which may be related to an imbalance between energy demands in cells and the food and oxygen supply [117,118]. In line with this hypothesis, Toma et al. [118] detected substantially higher levels of succinate in the urine and kidney homogenates of diabetic animals 1 week after STZ injection compared with controls. GPR91-dependent pathologies have also been found in the retina [122], suggesting that GPR91 may be a molecular link between the two most common diabetic complications, diabetic nephropathy and retinopathy.

As a molecule triggering important signalling pathways and linking several pathophysiological processes in the diabetic kidney, GPR91 is a good future target for nephroprotective interventions in diabetes, as well as in a spectrum of other kidney diseases. Recently, the discovery of selective non-peptide GPR91 antagonists has been published [123]. Some of these compounds blocked the acute effects of succinate on BP in Wistar rats. However, the effects of these molecules on renin synthesis and secretion and their nephroprotective potential remain to be established.

COX-2 inhibition

Although traditionally viewed as an inducible enzyme, COX-2 is constitutively expressed in the kidney in the macula densa and occasional cells of the TALH (thick ascending limb of Henle), and plays a role in regulation of renal function [124,125]. Apart from other actions in the kidney, COX-2-derived metabolites have been implicated in the stimulation of renin release. Several groups have demonstrated that stimulation of renin by a low-sodium diet can be blocked by selective COX-2 inhibitors [126,127], but not by selective COX-1 inhibition. The renin secretory response to a low-sodium diet is attenuated in COX-2-KO mice [128]. COX-2 inhibition or genetic deletion of COX-2 attenuate renin release in other high renin states, such as treatment with RAAS inhibitors [129,130]. Co-ordinated expression of COX-2 and renin has been found in models of renovascular hypertension [131,132], and selective COX-2 inhibition reduced renin mRNA expression and markedly decreased BP in renovascular hypertensive rats [132]. Increased macula densa COX2 expression has also been demonstrated in humans with high renin levels [133].

In parallel, our studies, as well as work by others, have described increased renal cortical COX-2 expression and function in the diabetic kidney cortex and a spectrum of nephroprotective actions of the selective COX2 inhibitors. In experimental diabetic nephropathy [120,134,135] these have included reductions in proteinuria, glomerulosclerosis, molecular markers and mediators of the disease. As already discussed, COX-2-derived metabolites may also increase (P)RR expression in the kidney [79].

Combining this evidence, COX-2-derived metabolites may act as important modulators of renin release and expression in diabetes and in CDK in general, and constitute a new therapeutic target to achieve better inhibition of the RAAS and enhance the therapeutic efficiency of RAAS inhibitors. However, the enthusiasm about this prospect has been diminished by several caveats. To explore the role of COX-2-derived metabolites in ACEI-induced renin stimulation in diabetes, we determined the effects of the selective COX-2 inhibitor MF-tricyclic on plasma renin concentration and renal renin expression in STZ-diabetic rats treated with enalapril [136]. COX-2 inhibition for 10 days did not influence the ACEI-induced increase in renal renin mRNA expression or the plasma and renal renin concentrations in diabetic rats, despite an observed reduction in renal prostanoid synthesis. In addition, we observed no effect of COX-2 inhibition on basal unstimulated renal renin mRNA expression and protein concentrations [136]. Possible reasons for this study outcome are beyond the scope of the present review, but the renin-lowering effect of COX2 inhibitors have not been a uniform finding. COX-2 inhibitors were without effect on PRA and renin expression in healthy humans and dogs fed on a low-salt diet [137,138] or in normal rats fed on a low-salt diet or ACEI [139], despite a marked reduction in renal prostanoid synthesis. Similar negative observations were reported in the ischaemic kidney shortly after renal artery clipping, despite co-ordinated renin and COX-2 expression [140] or in hypercalcaemia-induced stimulation of renal COX-2 [141].

More importantly, testing the selective COX-2 inhibition with coxibs in patients with diabetic nephropathy and hyperreninaemic states has been thwarted by detection of adverse CV effects associated with long-term treatment with these compounds. Rofecoxib was withdrawn voluntarily by Merck from the market in September 2004 following the increased CV risks observed in the APPROVe (Adenomatous Polyp Prevention on Vioxx) study [142]. Subsequently the sale of Bextra (valdecoxib) was also suspended by Pfizer in 2005. Recent meta-analysis of the adverse CV effects of NSAIDs (non-steroidal anti-inflammatory drugs), including the coxibs, showed a fairly wide range of CV risks of these compounds [143]. Consequently, in addition to

class effects, the design of COX-2 inhibitors may be important, supported by the more favourable CV risk profile of celecoxib compared with other agents in this class [144].

Despite these caveats, COX-2 remains a target for nephroprotective treatments, with beneficial actions, at least in part, attributable to renin reduction. Future research may introduce new COX-2 inhibitors devoid of thrombotic CV side effects allowing clinical testing of this approach.

CONCLUSIONS

Studies with aliskiren in a variety of clinical and experimental settings could offer an answer to the crucial question of whether more complete blockade of RAAS enhances the protective effects of the RAAS. However, current experimental and, in particular, clinical evidence does not support this notion. The inhibition of the system at its rate-limiting step has not fulfilled original expectations with respect to improved CV and renal protection. Renin inhibition, in particular when combined with other RAAS inhibitors and additional agents acting on the CV system, such as β-blockers, could in susceptible individuals limit the ability to properly react to some stresses, such as decreases in BP or extracellular volume contraction. A lack of the observed beneficial effects of aliskiren and its combination with other RAAS inhibitors may be also attributable to hyper-reninaemia and consequent stimulation of (P)RR. However, this area is controversial and still remains to be studied in the clinical arena. The treatments that influence not only PRA, but also inhibit renin synthesis and decrease renin mRNA expression may be preferable options for combinations with currently used RAAS inhibitors. However, these approaches also await confirmation by larger clinical trials.

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