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In-Depth Review

Endothelin and the podocyte

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Endothelins are ubiquitously expressed stress-responsive regulators acting in both a paracrine and autocrine fashion and are in involved in the pathogenesis of chronic kidney disease (CKD).

Glomerulosclerosis as the main underlying cause of CKD is associated with activation of endothelin, and its detrimental effects on renal structure and function are predominantly mediated by the endothelin ET_A receptor.

Podocytes are gatekeepers of the glomerular filtration barrier and express a fully functional endothelin system. CKD leads to podocyte effacement and disruption of the podocyte actin cytoskeleton. Blockade of endothelin ET_A receptors can prevent and reverse podocyte injury and actin cytoskeleton disruption.

Endothelin receptor antagonists interfere with glomerulosclerosis and proteinuria and may even cause reversal of renal disease under certain conditions by mechanisms that are only in part pressure-dependent.

Endothelin receptor antagonists are a new class of drugs undergoing clinical testing for the treatment of proteinuric renal disease. First clinical studies have shown reversal of proteinuria on top of standard RAAS inhibitor therapy in patients with diabetic nephropathy and those with non-diabetic CKD that were largely independent of systemic arterial blood pressure.

Abstract

In the past decade, research has advanced our understanding how endothelin contributes to proteinuria and glomerulosclerosis. Data from pre-clinical and clinical studies now provide evidence that proteinuric diseases such as focal segmental glomerulosclerosis and diabetic nephropathy as well as hypertension nephropathy are sensitive to treatment with endothelin receptor antagonists (ERAs). Like blockade of the renin–angiotensin system, ERA treatment—under certain conditions—may even cause disease regression, effects that could be achieved on top of renin–angiotensin–aldosterone system blockade, suggesting independent therapeutic mechanisms by which ERAs convey nephroprotection. Beneficial effects of ERAs on podocyte function, which is essential to maintain the glomerular filtration barrier, have been identified as one of the key mechanisms by which inhibition of the endothelin ET_A receptor ameliorates renal structure and function. In this article, we will review pre-clinical studies demonstrating a causal role for endothelin in proteinuric chronic kidney disease (with a particular focus on functional and structural integrity of podocytes *in vitro* and *in vivo*). We will also review the evidence suggesting a therapeutic benefit of ERA treatment on the functional integrity of podocytes in humans.

Keywords: diabetes; epithelial cell; focal segmental glomerulosclerosis; glomerular; hypertension

Introduction

The prevalence and incidence of chronic kidney disease (CKD), one of the most serious clinical consequences of patients with diabetes or hypertension, have been steadily rising over the past decades [1]. CKD is characterized by progressive loss of functional glomerular tissue, defects in the glomerular filter function, and subsequent proteinuria [2]. CKD also aggravates pre-existing cardiovascular risk factors such as hypertension and dyslipidaemia, and as a result, accelerates atherosclerosis progression; in fact most CKD patients die from cardiovascular complications [3]. In view of an ageing population and the predicted increase of the world's population from currently 7 to 9 billion people by the year 2050 [4], health care providers
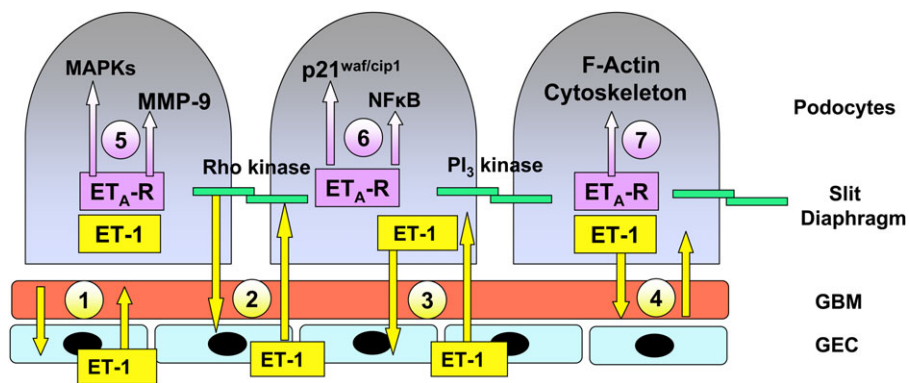
Fig. 1. Proposed interactions of endothelin (ET-1) with the glomerular basement membrane (GBM) of the glomerular capillary, its endothelial cells (GEC) and podocytes/slit diaphragm. ET-1 is produced by cells on both sides of the GBM, namely GEC and podocytes. ET-1 is released from GEC and may interact either directly with GBM (1) the slit diaphragm (2) or the podocyte, also in the reverse direction (3). Podocyte-derived ET-1 (4) may also affect the GBM and vice versa. ET-1 activates podocyte endothelin ET_A receptors (ET_A R) activating mitogen-activated protein kinases (MAPKs) p38 and p44/p42 (5), growth promoters (p21$^{waf/cip1}$), or inflammation (NF-kappaB) (6). ET-1 also causes disruption of the podocyte F-actin cytoskeleton (7) and slit diaphragm dysfunction via activation of rho kinase and PI$_3$-kinase. Figure reproduced from reference [11] with permission of the publisher.

and political decision makers need to emphasize the importance of primary and secondary prevention of CKD even more [4].

In the past decade, inhibitors of the renin–angiotensin–aldosterone system (RAAS) such as angiotensin-converting enzyme inhibitors (ACEIs) and angiotensin receptor blockers (ARBs) have been firmly established as anti-proteinuric therapeutics [5]. Because inhibitors of the renin–angiotensin system only partially interfere with progression of CKD of diabetic or hypertensive aetiology [5], further preventive measures and additional therapeutic approaches are urgently needed [6]—particularly in view of the ongoing obesity/diabetes pandemic. Inhibiting the cellular activity of endothelin-1 in renal disease through endothelin receptor antagonists (ERAs) may be a new and promising approach [7]. In several recent articles [8–14], on which the present article is based, we have addressed molecular and physiological mechanisms by which endothelin contributes to proteinuria and podocyte injury and reviewed the clinical trials on ERAs in patients with CKD ongoing at the time [8, 9, 12].

Podocyte dysfunction as a determinant of CKD

Podocyte injury and chronic proteinuric nephropathies

Podocyte loss and podocyturia have been proposed as potential diagnostic markers of glomerular disease severity [15, 16]. Indeed, as disease progresses, viable—in addition to senescent—podocytes are shed in the urine [17] and thus provide a sensitive indicator of the degree of glomerular injury [16]. CKD is characterized by mesangial cell injury as well as by defects of the glomerular filtration barrier [15]. This includes injury of the glomerular endothelial cells, the glomerular basement membrane (GBM), glomerular epithelial cells, and the slit diaphragm (Figure 1) [11, 15].

Glomerular epithelial cells can be divided into parietal and visceral (glomerular) epithelial cells [15]. Visceral epithelial cells have been termed 'podocytes' due the appearance of 'interdigiting' foot processes adhering to the glomerular capillary [13]. Podocyte injury is a hallmark of proteinuric renal disease such as diabetic nephropathy and focal segmental glomerulosclerosis (FSGS) [18, 19]. Although the term 'podocytopathy' has recently been used to describe proteinuria in association with podocyte injury [20], proteinuria in some cases may also occur in the ab-

sence of podocyte effacement [13, 21]. Since podocyte injury precedes the development of glomerulosclerosis, it not only represents a therapeutic target but can also be used for diagnostic purposes [22–26].

Previous dogma stated that because podocytes—once injured—cannot be replaced, glomerulosclerosis is progressive and irreversible [27, 28]. However, podocyte proliferation and migration have been observed under certain conditions, including inflammatory activation during renal injury [29, 30]; in the past decade numerous studies have clearly shown that glomerulosclerosis is a reversible disease condition [10, 31–40]. Podocytes have a defined lifetime since they are terminally differentiated, however, they may be continuously replaced by parietal epithelial cells (PECs) residing in the inner portion of Bowman's capsule, which can migrate onto the glomerulus [41, 42]. Thus far, whether migration of PECs is beneficial or detrimental for glomerular structure and function remains a matter of debate. These novel and unexpected findings have provided one explanation for many studies reporting reversal/regression of established glomerular disease using different pharmacological approaches [10, 31–40].

Podocyte function and the actin cytoskeleton

The podocyte actin cytoskeleton is being increasingly recognized as a key modulator of the functional integrity of the glomerulus [43]. Disruption of the actin cytoskeleton is a feature of podocyte injury in proteinuric nephropathies. The actin cytoskeleton of podocytes not only provides structural support for the cells but also contributes to podocyte signalling [43, 44]. Reorganization of the podocyte actin cytoskeleton appears to be a response to cell stress [45], and loss of podocytes results in uncoupling of podocyte-specific proteins in the remaining cells from the actin cytoskeleton [46, 47].

Podocytes are linked to the GBM through their foot processes and form the structural basis of the glomerular filtration barrier responsible for filtering proteins (Figure 1). Podocyte injury results in a reduction in podocyte number, which is characteristic of many forms of proteinuric renal disease [21, 48, 49] and subsequent increased accumulation of GBM matrix, which is derived from podocytes [30]. Accordingly, in cases of regression of proteinuria induced by pharmacological intervention, a reduction in the degree of

podocyte injury and of GBM thickening has been observed [39].

In addition to podocyte injury, proliferation of glomerular mesangial cells and hypertrophy of the GBMs contribute to the progression of proteinuric nephropathies [10]. Accordingly, mutations of the actinin-4 (*actn4*) gene, which encodes the podocyte-localized actin-binding protein α-actinin, have been linked to autosomal-dominant proteinuria associated with familial FSGS [50, 51]. Similarly, variants of the apolipoprotein L-1 (*apol1*) gene have been identified as a risk factor for FSGS and hypertension nephropathy in blacks; it is present in African chromosomes and absent in European chromosomes [52]. Black subjects are at higher risk to develop hypertension, diabetes and renal and vascular disease and hypertensive blacks show higher circulating endothelin-1 (ET-1) levels than Caucasians [53].

Integrin-mediated adhesion of podocytes to the basement membrane of the glomerular capillary has recently been reported to require the tretraspanin *cd151*, which protects from podocyte effacement and renal disease development in a podocyte-dependent fashion [27, 54]. It is well possible, if not likely, that anti-proteinuric effects of drugs such as ARBs, ACEIs, or ERAs involving drug-induced glomerular stabilization of preventing podocyte loss in end-stage kidney disease [55] involve mechanisms requiring integrins and/or *cd151* [56]. Research is ongoing to identify the mechanisms involved in podocyte injury, many of which converge at the level of the actin cytoskeleton [43]. One mechanism that was recently identified is podocyte-induced secondary injury of 'remnant' intact podocytes [57], which leads to podocyte depletion in CKD.

Role for endothelin in the development of CKD

Endothelin-1: vasoconstrictor and promoter of inflammation and growth

Endothelins are ubiquitously expressed stress-responsive regulators acting in both a paracrine and autocrine fashion [58]. Within a year after Furchgott and Zawadzki's had discovered an endothelium-derived vasorelaxing factor (later identified as nitric oxide) [59–61], endothelium-derived vasoconstrictor activity was reported by de Mey and Vanhoutte [59, 60]. A potent peptidergic vasoconstrictor activity isolated from endothelial cell supernatants was reported in 1985 [62], and the gene and peptide sequence of this vasoconstrictor, named endothelin-1, were published by Yanagisawa *et al.* in 1988 [63]. Endothelin-1 is the biologically most relevant isoform of three endothelin isopeptides, which bind to endothelin receptors (designated ET_A and ET_B) [59], that were cloned in the early 1990s [58]. ET_A receptors have primarily vasoconstrictor and growth-promoting functions, whereas ET_B receptors mainly mediate vasodilation and inhibition of growth and inflammation, via release of nitric oxide and prostacyclin [58]. Identification of these receptors allowed the development of orally active ERAs, which are now firmly established in pulmonary medicine [9] and currently in clinical trials for CKD [9, 12].

Numerous endothelin-dependent mechanisms contribute to proteinuria and CKD [9, 64]. Endothelin promotes collagen production and stimulates glomerular fibronectin synthesis. Endothelin becomes activated under conditions associated with renal disease progression, such as diabetes, insulin resistance, obesity, dyslipidaemia, reactive oxygen species formation and inflammation [10]. In fact, inflammation may

be a unifying detrimental mechanism by which endothelin causes kidney injury. Indeed, inflammation is crucial for glomerulosclerosis progression and can be attenuated by ET_A receptor antagonist treatment, which reduces circulating cytokines in a model of acute allograft rejection after solid organ transplantation, even in the absence of immunosuppression [65]; ERA treatment also limits inflammation in experimental proliferative nephritis [66]. Consistent with these effects, chronic infusion of endothelin at non-pressor doses increases pro-inflammatory mediators such as intercellular adhesion molecule-1 (ICAM-1) and monocyte chemotactic protein-1 (MCP-1) and the number of macrophages in the renal cortex, effects that are largely abrogated by pre-treatment with an ET_A receptor antagonist [67], and similar effects were obtained in a model of diabetes-associated renal inflammation [68, 69]. Interestingly, only selective ET_A, but not non-selective ET, receptor antagonists inhibited the renal inflammatory response [70].

Endothelin also increases formation of other vasoactive and growth factors such as angiotensin II by increasing the activity of ACE [71]. On the other hand, angiotensin II activates renal endothelin formation [72], compatible with a vicious cycle between the renin–angiotensin–aldosterone and the endothelin systems [73]. Mesangial cell proliferation and GBM hypertrophy (Figure 1) are indirectly mediated via podocyte injury [44] and represent an important indicator of glomerular stability [18]. Wiggins *et al.* recently reported that combined ARB/ACEI treatment reduces podocyte loss and thereby contributes to glomerular stabilization in experimental end-stage renal disease [55].

Endothelins and endothelin receptors in the kidney

In the normal kidney, endothelin regulates blood pressure, vascular tone and natriuresis, the latter of which is mediated via the ET_B receptor [74], and is influenced by sex [75]. In the systemic and renal vasculature, endothelin exerts basal ('tonic') ET_A receptor-mediated vasoconstriction [74]. Under physiological conditions, endogenous renal endothelin controls water and sodium excretion and acid–base balance and maintains normal renal cell proliferation and tonic vasoconstriction [74]. Endothelin also stimulates proliferation of vascular smooth muscle cells, a cellular function facilitating the development of hypertension and renal disease [58]. Endothelial cell-derived endothelin controls blood pressure: mice with endothelial cell-specific deficiency of endothelin-1 are hypotensive [76], while those with endothelial cell-specific overexpression are hypertensive [77, 78].

With ageing, renal expression of endothelins increases at the messenger RNA (mRNA) and peptide level [79]. Radio-labelled ET-1 binds within 10 min after injection onto glomerular podocytes [80], cells that also produce and secrete endothelin [81, 82]. Endothelin binding to podocytes results in changes in intracellular calcium [83], and ET_B-specific binding on podocyte foot processes [84] and expression of mRNA for ET_A and ET_B receptors, preproendothelin-1, and endothelin-converting enzymes in podocytes have been reported [10]. The exact role of the podocyte endothelin system in podocytes warrants further investigation.

Endothelin contributes to glomerulosclerosis progression

For almost 20 years, different experimental models of renal disease (renal mass reduction, hypertension, salt-sensitive hypertension, RAAS-dependent hypertension, nitric oxide (NO)-deficient hypertension, ageing, FSGS)

have been studied to test whether endothelin inhibition can interfere with renal disease progression [85]. In 1993, Benigni *et al.* [86] published the first pre-clinical study to suggest a causal role for endothelin in chronic proteinuric renal disease. In a prevention-type study, remarkable reductions in proteinuria and glomerulosclerosis were observed after selective blockade of ET_A receptors in a rat renal mass reduction model of hypertensive glomerular disease [86]. Hocher *et al.* [87] subsequently demonstrated glomerulosclerosis in normotensive mice systemically overexpressing the human preproendothelin gene. Data from pre-clinical studies indicate that endothelin—via activation of the ET_A receptor—importantly contributes to renal disease progression under hypertensive [24, 34, 88, 89] as well as under normotensive conditions [39, 90].

## Involvement of endothelin in podocyte dysfunction and injury

Podocyte growth factor receptors and glomerular disease

Injury to podocytes results in glomerular filtration barrier dysfunction and subsequent proteinuria, a hallmark of all glomerular diseases [15]. Podocytes express receptors for vasconstrictors and growth factors such as angiotensin II, thromboxane A_2, endothelin-1 and prostaglandins [13]. Angiotensin II increases renal endothelin synthesis [10] and glomerular permeability for albumin, and causes podocyte actin cytoskeleton disruption and podocyte apoptosis [10]. A role for growth factor receptors is also suggested by the observation that either ET_A receptor [39] or angiotensin AT_1 receptor antagonists [49] prevent podocyte injury.

The activity of endothelin or angiotensin may also be inhibited indirectly. Nephroprotective peptides such as bone morphogenetic protein-7 (BMP-7) [91, 92] reduce expression of the growth-promoting ET_A receptor, both at the RNA and the protein level, alleviate hyperglycaemia-mediated podocyte injury and improve podocyte survival (reviewed in [10]). BMP-7 also stimulates glomerular capillary formation that has been implicated in ARB- or ACEI-induced regression of glomerulosclerosis [37, 93]. Similar to BMP-7, nephroprotective effects inhibition of epidermal growth factor (EGF) receptor transactivation in podocytes [94] (EGF stimulates endothelin-mediated cell growth and constriction [95, 96]) might also reduce renal injury—at least in part—via interfering with the endothelin-1-ET_A receptor axis.

### Podocyte injury involves endothelin signalling

We have previously proposed that the neighbouring cells in close proximity of podocytes—which also produce endothelin *in vitro* [81, 82]—affect podocyte function and structure through endothelin-mediated interactions [10, 13] (Figure 1). Disruption of the podocyte actin cytoskeleton has been observed following *in vitro* exposure to endothelin [39, 97, 98]. Interestingly, exposure of podocytes to protein *in vitro* induces synthesis of endothelin that results in increases in glomerular permselectivity, an effect antagonized by ET_A receptor blockade [97, 98]. These findings are reinforced by recent work from Pollock *et al.* demonstrating that exogenous endothelin—both acutely as well as chronically—increases glomerular permeability via ET_A receptor-mediated mechanisms [67]. This effect appears to be independent of whether the ET_B receptor is blocked
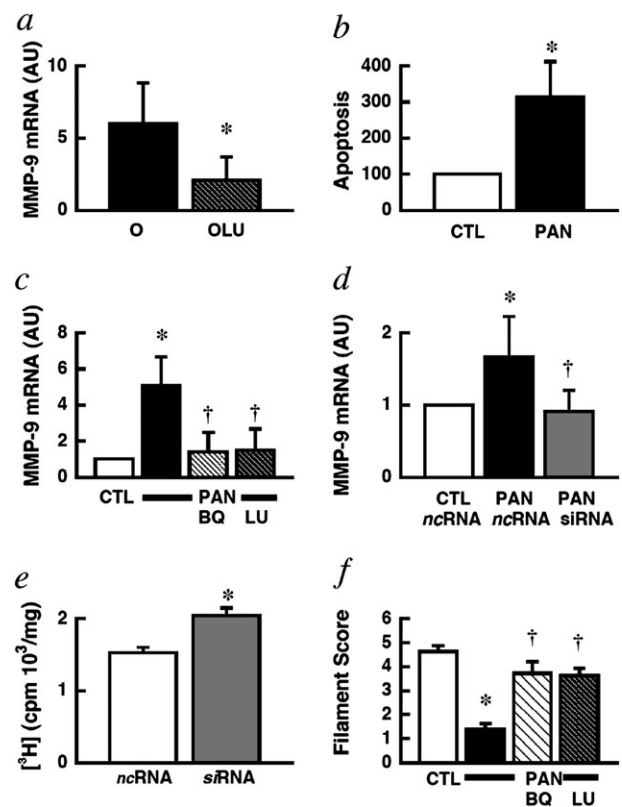
Fig. 2. Effect of ET_A receptor inhibition in laser-dissected glomeruli of rats with FSGS *in vivo* (**a**) and in podocytes *in vitro* (**b**-**f**). (**a**) Darusentan reduced MMP-9 gene expression (a marker of podocyte injury [103]) compared with placebo-treated rats. (**b**) *In vitro* treatment with puromycin aminonucleoside (PAN) caused podocyte apoptosis and up-regulation of MMP-9; (**c**) peptide (BQ) or non-peptide (LU, darusentan) ET_A receptor antagonists prevented this effect, as did ET_A receptor gene silencing (siRNA) (**d**). (**e**) Gene silencing of ET_A receptors increased podocyte growth as determined by *de novo* DNA synthesis. (**f**) Actin cytoskeleton disruption was largely prevented by ET_A antagonists; representative examples of these experiments are shown in Figure 3. ncRNA, non-coding RNA control; O, old; AU, arbitrary units; OLU, old, darusentan. *$P < 0.05$ versus control (CTL); †$P < 0.05$ versus PAN alone/old. Figure reproduced from reference [39] with permission of the publisher.

or not [70]. Thus, ET_A receptor-dependent effects mediate proteinuria.

Similarly, ET_A receptor antagonists prevent disruption of the podocyte actin cytoskeleton following exposure to puromycin aminonucleoside [39] (Figures 2 and 3), an effect that can also be obtained with angiotensin AT_1 receptor antagonists [46, 99, 100]. Puromycin aminonucleoside (which causes FSGS *in vivo*) [101, 102] results in podocyte apoptosis and up-regulation of matrix metalloproteinase-9 (MMP-9) as a marker of podocyte injury [103]; up-regulation of MMP-9 and disruption of the actin cytoskeleton can be completely prevented by ET_A receptor (but not ET_B) antagonists or ET_A receptor gene silencing (Figure 2 and 3) [39]. These observations were confirmed by Benigni's group [39, 97] showing similar cytoskeleton-protective effects of ET_A receptor blockade [98]. Little is known about the mechanisms that would explain how endothelin might regulate podocyte proliferation or motility [44]. We found that ET_A receptor gene silencing stimulates podocyte growth (Figure 2E), a finding compatible with the notion that endogenous endothelin produced by the podocyte itself [81, 82] acts as a negative regulator of podocyte cell proliferation.
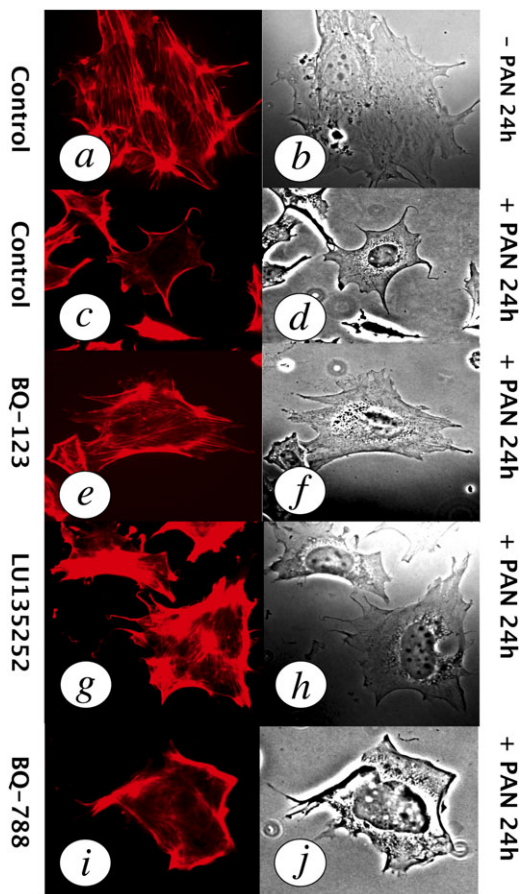
Fig. 3. Endothelin mediates podocyte injury *in vitro*. (**a, b**) Actin-phalloidin immunofluorescence (left) and phase-contrast images of actin cytoskeleton fibres and cell morphology (right panels) in a normal podocyte. (**c, d**) Puromycin aminonucleoside (PAN)-induced foot process effacement, cytoskeleton disruption (disappearance of actin fibres) and cell body shrinkage. (**e, f**) Prevention of PAN-induced injury by the ET$_A$ antagonist BQ-123. (**g, h**) Prevention of PAN-induced injury by the ET$_A$ antagonist darusentan/ LU135252. (**i, j**) The ET$_B$ receptor antagonist BQ-788 had no effect on PAN-induced injury. Figure reproduced from reference [39] and reproduced with permission of the publisher.

CKDs involving podocyte injury and endothelin

Assessing podocyte injury in patients is difficult and so far can be done only indirectly through measurements of proteinuria or albuminuria. Since most of the recent renal trials with ERAs have been extensively discussed in our previous articles, we will only briefly address clinical outcomes.

Hypertension nephropathy

Hypertensive renal disease was one of the first targets in pre-clinical studies to explore the therapeutic potential of ERAs. Renal preproET-1 mRNA expression and urinary ET-1 excretion increase in hypertension due to renal mass reduction, a condition associated with proteinuria and impairment of renal function (reviewed in [13]). Using the same experimental model, Benigni *et al.* [86] demonstrated that selective ET$_A$ receptors antagonists reduce the degree of glomerulosclerosis and proteinuria, albeit with treatment also having anti-hypertensive effects. Clozel *et al.* [104], using combined blockade of ET$_A$ and ET$_B$ receptors, found only protective effects on renal function,
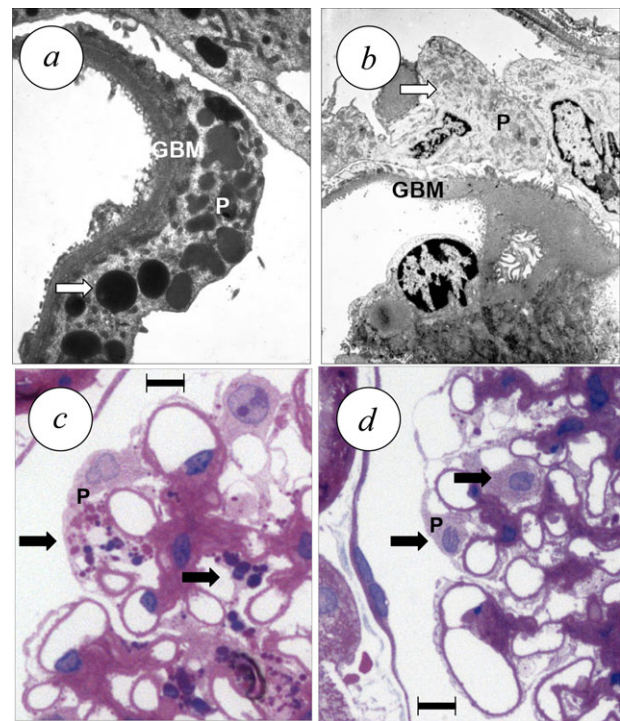
Fig. 4. Effect of 4 weeks of treatment with the ERA darusentan on renal structure in established FSGS [39]. (**a**) Untreated animal, transmission electron microscopy demonstrates GBM hypertrophy and podocyte injury with diffuse foot process effacement and vacuolar degeneration involving autophagy [113]. (**b**) ERA-treated animal, showing regression of GBM hypertrophy and disappearance of podocyte vacuoles. (**c**) Untreated animal, light microscopy image (haematoxilin/eosin) demonstrating hypertrophy of podocytes with enlarged nuclei, prominent nucleoles and vacuolar degeneration due to autophagy. (**d**) Treated animal, showing normal sized podocyte nuclei and virtually complete disappearance of vacuolar degeneration (arrows). Scale bar, 10 μm (**c, d**). Panels adapted from reference [39] and reproduced with permission of the publisher.

but no effect on proteinuria, suggesting possible therapeutic advantages of selective ET$_A$ selective antagonists. Other prevention-type studies found nephroprotective effects in a variety of forms of hypertension (angiotensin-II-dependent, renin-dependent, salt-loaded renin-dependent, aldosterone-induced, genetically salt-sensitive and deoxycorticosterone–salt-induced) as well as chronic NO deficiency, with effects usually being associated with a partial reduction in blood pressure [105].

The first study to demonstrate reversal of indices of hypertensive nephropathy using ERAs was performed by Boffa *et al.* [34] in a rat model of NO-deficient hypertension. The same group also demonstrated that established glomerulosclerosis caused by chronic hypertension due to NO deficiency was also reversed by non-selective ERA in a manner that was independent of blood pressure [40]. In this model, increased ET-1 synthesis in the kidney was found primarily in pre-glomerular arterioles and a downstream sclerotic action on glomerular cells was unraveled with non-selective ERAs [106]. Vaneckova *et al.* have provided evidence that selective ET$_A$ receptor blockade maintains its profound anti-proteinuric effects even when initiated in the presence of established malignant hypertension and that an attenuation of podocyte injury (which precedes proteinuria) can be observed even in the absence of glomerulosclerosis, i.e. at early stages of renal disease [24, 89]. In contrast, non-selective ERA treatment did not prevent podocyte injury nor did it reduce proteinuria.

Importantly, the protective effects were observed in the presence of sustained hypertension [24, 89].

Podocyte injury before the development of glomerulosclerosis has also been observed in aldosterone-infused rats [26] and in hypertensive Dahl salt-sensitive hypertensive rats [23, 107]. Podocyte protection in Dahl rats was achieved by either inhibition of the mineralocorticoid receptor [23] or the endothelin ET_A receptor [107]. In contrast, simple anti-hypertensive treatment of these animals with hydralazine had no effect on podocyte injury [23], indicating that the nephroprotective effects of aldosterone blockade and endothelin blockade are specific for these vasoactive and growth-promoting factors and in part blood pressure independent. The concept of a blood pressure-independent mode of action of ERAs is further supported by the glomerulosclerosis despite normotension in endothelin-overexpressing mice [87] and blood pressure-independent nephroprotective effects of ERAs in diabetes [58]. A reduction in proteinuria has also been observed in patients with resistant hypertension on triple anti-hypertensive therapy additionally treated with the ET_A antagonist darusentan [108].

Focal segmental glomerulosclerosis

FSGS is a widely varying, clinicopathological entity characterized by injury of the glomerular filtration barrier [19]. Urinary excretion of ET-1 increases in primary FSGS patients and glomerular ET-1 expression is enhanced in experimental FSGS (reviewed in [13]). Podocyte-specific mechanisms have been proposed as mechanisms underlying FSGS development [22, 109].

Ageing in rodents and humans is associated with spontaneous development of FSGS [22], which is characterized by podocyte injury and hypertrophy, glomerular enlargement and glomerulosclerosis [110, 111]. Pharmacologically induced FSGS results in podocyte injury leading to cytoplasmic accumulation of functional synaptic-like vesicles, which occurs even before detachment of the cells from the GBM [112]. These vesicles or vacuoles are also present in spontaneous age-dependent FSGS [39] (Figure 4, untreated animals, left panels). Interestingly, susceptibility to develop age-dependent FSGS has recently been linked to autophagy-related mechanisms controlling podocyte vacuolation [113].

Pre-clinical studies in aged rats suggest that treatment with ET_A receptor antagonists can in part restore podocyte morphology (Figure 4, treated animals, right panels), reverse FSGS and proteinuria independently of renal or systemic haemodynamics or renal function [39]. Disease regression was associated with reduced glomerular expression of MMP-9, a podocyte injury marker [39] and of cortical p21$^{waf/cip1}$ [39], a cdk-inhibitor and promoter of glomerulosclerosis [114]. Most notably, the regression of FSGS achieved by ERA treatment was associated with normalization of the thickness of the hypertrophied GBM and almost complete disappearance of podocyte vacuolization [39] (Figure 4).

We have recently proposed mechanisms that could contribute to the partial restoration of podocyte and glomerular structure and protein loss after ERA treatment [8, 10]. Though terminally differentiated, podocytes may be replaced from PECs [41, 115, 116]. This concept, initially put forward by Fogo *et al.* using podocyte-specific Lac-Z expression and demonstrating that PECs replace glomerular epithelial cells [42], was independently confirmed by Appel *et al.* [41]. However, they are still lacking evidence that former PECs migrating to the glomerular capillaries can become functional podocytes. In fact, studies from

Bart Smeets and Marcus Moeller suggest that PECs' invasion into the glomerular capillary tuft promotes scarring and FSGS. After these recent studies, we can wonder whether manipulation of the endothelin system would affect migration of PECs and their subsequent fate towards a desirable podocyte phenotype or a scarring phenotype. In addition, improved glomerular capillary architecture may result from ERA therapy and thereby accelerate disease regression [69].

Clinical data regarding effects of ERAs in FSGS patients are scarce. In patients with non-diabetic renal disease on standard anti-proteinuric RAAS inhibitor therapy, ET_A antagonism reduces proteinuria [117] independent of changes in glomerular filtration rate (GFR). The same authors reported that the effect depends on the degree of RAAS inhibition in this particular patient population with renal disease of different aetiologies [118], which complicates reaching a conclusion regarding the mechanisms involved.

Diabetic Nephropathy

Diabetes remains the main cause of CKD and is likely to increase even further in prevalence due to the ongoing obesity pandemic [119]. Hyperglycaemia—like endothelin [58]—results in disassembly of the podocyte actin cytoskeleton [120] as well as podocyte depletion and apoptosis involving reactive oxygen species (ROS)-dependent mechanisms [121]. In animals with diabetic nephropathy, even viable podocytes are shed in the urine [17, 122]. Nephrin, a podocyte-specific protein contributing to functional stability of the slit diaphragm (Figure 1), is involved in podocyte shedding as recombinant endothelin triggers nephrin loss from human podocytes, which is prevented by ET_A receptor antagonists [123]. A large number of experimental prevention studies demonstrated beneficial effects of ERA therapy on diabetic nephropathy and proteinuria [90, 124]. Whether these effects are pressure dependent is not clear: in some studies, partial or even complete normalization of blood pressure was observed [125, 126], while in other studies, little or no effect on blood pressure or GFR was observed, suggesting possible non-haemodynamic but rather structural effects. Indeed, Gross *et al.* [126] reported that darusentan was as effective as ACE inhibition in reducing glomerulosclerosis in insulin-treated streptozotocin-diabetic rats, but that ACE inhibition produced greater reductions in blood pressure, proteinuria, and glomerular volume. However, only ET_A receptor blockade completely restored the number of glomeruli per kidney to the normal expected number and increased the number of podocytes per glomerulus, suggesting that endothelin blockade has specific glomeruloprotective effects at the level of the pocoyte [126]. Indeed, Gagliardini *et al.* [69] found that ERA treatment in part prevents glomerular podocyte loss in experimental diabetes and reduced podocyte injury despite having only negligible effects on arterial blood pressure [127]. Thus, anti-proteinuric effects are likely to be due to structural or anti-inflammatory [124] properties of ERAs. A pressure-independent mode of action would also be compatible with findings from recent clinical trials in patients with diabetic nephropathy in which impressive reversal of proteinuria was observed [74, 119, 128].

Obesity nephropathy

Obesity has become a major health issue in all parts of the world and is increasingly affecting children and juveniles [129, 130]. Other diseases such as arterial hypertension

and diabetes, which are independent risk factors for CKD [131, 132] are aggravated by obesity. Secondary FSGS has been linked to obesity nephropathy [133, 134]. Accordingly, podocyte loss has been described in patients with obesity nephropathy [135] as well as in obese rodents [121]. In patients with obesity nephropathy, albuminuria is inversely correlated with plasma levels of the adipocytokine adiponectin [136]. On the other hand, albuminuria in adiponectin-deficient mice can be normalized by administering recombinant adiponectin [137]. Endothelin, which promotes proteinuria [67] and increases locally in the kidney [73, 138], inhibits adiponectin production [139], suggesting a possible mechanistic link. Obesity-associated diabetes often is associated with high circulating levels of insulin, a potent stimulus of endothelin production [58]. Furthermore, endothelin-1 has insulin-antagonizing properties [140]; thus, high local tissue or circulating levels of endothelin-1 could promote the development of insulin resistance thus promoting hyperglycaemia and subsequent podocyte injury. Another common concern of obesity-associated FSGS as well as with diabetic nephropathy is the initial chronic hyperfiltration leading to increased glomerular capillary pressure and likely podocyte stretch. ERA therapy may alleviate such local change in the glomerular haemodynamics. Finally, renal ACE becomes activated in obesity, an effect that can be completely prevented by ET_A receptor antagonists [73]. This suggests that ERAs function as ACEIs under certain pathological conditions and reinforcing the use of ACEI in patients with obesity nephropathy. Importantly, in most of the clinical ERA studies in patients with diabetic nephropathy showing beneficial effects on proteinuria, patients were either overweight or obese [74, 119, 128]. Thus, future studies in renal patients should also assess and compare effects of ERA therapy in individuals with normal and pathological body-mass indices. It should also be noted that in experimental studies, calorie restriction can limit or even largely prevent FSGS-associated podocyte injury [110, 111] suggesting that calorie restriction and/or weight loss are likely to be of great importance for the primary and possibly secondary prevention of CKD in obese patients.

Immune-mediated and minimal-change nephropathies

The mitogenic and co-mitogenic effects of ET-1 on mesangial cells were reported early [10]. In the model of glomerulonephritis with mesangial proliferation induced by the injection of anti-thymocyte antibodies, an increase is observed in the glomerular expression of ET-1 and ET_B receptors [141]. ET_A antagonists also prevent proteinuria and histological lesions due to mesangial proliferation following the repeated systemic injection of ovalbumin in rats [66]. Work by Benigni's group suggests that albumin causes podocyte cytoskeleton disruption and up-regulation of podocyte ET-1 expression [97].

Glomerulonephritis due to streptococcal infection is characterized by intense endocapillary mesangial proliferation [13]. In children with streptococcal infection, investigators found positive correlations between plasma ET-1 concentration and arterial blood pressure, consistent with a role for endothelial activation and endothelin participating in the disease process of acute post-streptococcal glomerulonephritis [142]. It is likely that plasma concentrations only in part reflect local tissue concentrations also in this disease, given the paracrine actions of this peptide [76]. Increased mesangial expression of ET-1 in humans with lupus-associated nephropathy with moderate me-sangial proliferation and after kidney transplantation with cyclosporine treatment has been reported (reviewed in [13]). Remarkably, even in the absence of immunosuppressant, ET_A receptor blockade can alleviate much of the transplant-associated mesangial proliferation and glomerulosclerosis [143].

Minimal-change disease represents a podocyte disease without an inflammatory infiltrate [13, 19]. Semi-quantitative morphological and immunohistochemical studies assessed renal ET-1 expression patients with minimal-change disease in the absence or presence of acute renal failure [144]. Renal failure patients showed higher ET-1 levels in glomerular and tubular endothelial cells. Thus, local increase in renal ET-1 production, which has been found in experimental studies of acute renal failure [145], appears to be involved in acute renal failure also in this setting.

Sickle cell nephropathy

FSGS is becoming epidemic in subjects affected by sickle cell disease (SCD) as life expectancy increases. In a case-control study comparing patients with chronic renal insufficiency and healthy subjects, urinary ET-1 excretion levels were significantly higher in the patients than the controls [146]. High urinary endothelin concentrations have also been reported in subjects with SCD and microalbuminuria with glomerular hyperfiltration. In these subjects, the elevated urinary ET-1 excretion, which was four times higher than that in carefully matched healthy controls, was correlated with microalbuminuria, suggesting a pathological link between the loss of glomerular permeability/selectivity and the renal synthesis of ET-1 in this context preceding progression to FSGS [147]. No studies have yet addressed possible interactions between the ET system and polymorphisms of the *apoL1* gene, which is associated with an increased risk of FSGS in carriers of African chromosomes [52]. Notably, SCD condition with initial hyperfiltration and glomerulomegaly may be related in part to the pathophysiology of incipient diabetic nephropathy and obesity-related FSGS as discussed above. ET_A or combined ET_A/ET_B blockade displayed contrasted effectiveness in blunting albuminuria and injury in experimental diabetic models with initial hyperfiltration [124, 148]. The increase in renal (mostly endothelial) ET-1 synthesis has been demonstrated in a mouse model of SCD [149] characterized by glomerulomegaly and glomerular hyperfiltration complicated by FSGS may be due to mechanically transduced stimulation. To date, clinical studies testing the potential anti-proteinuric and nephroprotective efficacy of ERAs in SCD patients have not been performed.

Conclusion and perspective

Endothelin is essential in the physiological regulation of renal blood flow and cell growth. There is solid pre-clinical and beginning clinical evidence that ERAs bear the potential as anti-proteinuric drugs and which also have beneficial effects at the level of the podocyte and the glomerular capillary in diseases such as diabetic nephropathy, hypertension nephropathy, FSGS and sickle cell nephropathy [150]. One of the current unsolved limitations of ERA therapy in patients is oedema formation. This appears to involve ET_A and ET_B receptor-dependent mechanisms [150, 151], which, however, can be circumvented by carefully adjusting diuretic therapy [12, 119]. Provided that clinical

drug development continues at a careful level, the addition of ERAs to the therapeutic armamentarium of nephrologists might ultimately allow the reversal—at least in part—proteinuria and possible glomerulosclerosis.

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