the Renin-Angiotensin System in Hypertension and Diabetes

FROM MAN TO RODENT AND BACK

Lodi Roksnoer

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HET RENINE-ANGIOTENSINE SYSTEEM IN HYPERTENSIE EN DIABETES

van mens naar knaagdier en terug

Proefschrift

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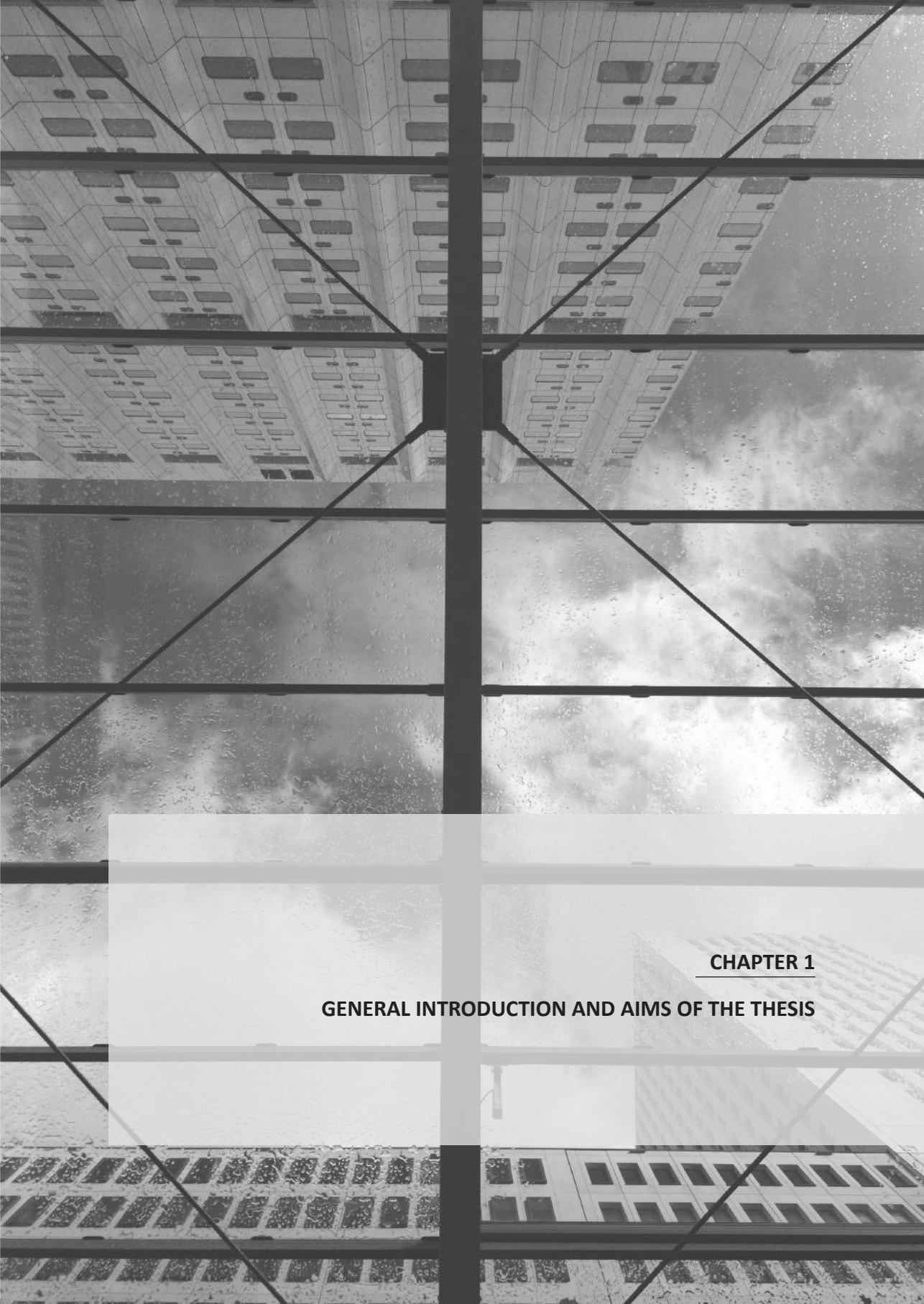

CHAPTER 1

GENERAL INTRODUCTION AND AIMS OF THE THESIS

The renin-angiotensin system in health and disease

Hypertension is a major risk factor for heart disease, stroke, and chronic kidney disease. It is the leading risk factor for cardiovascular disease, which is the main contributor to global disease burden (expressed in Disability-Adjusted-Life-Years), and it accounts for more than 10 million deaths worldwide every year.[1] The renin-angiotensin system (RAS) is the body's most important system to regulate blood pressure. When a low pressure is detected in the arterioles that deliver blood to the kidney's glomeruli, or NaCl delivery to the macula densa (in the thick ascending limb of the loop of Henle) decreases, cells in the juxtaglomerular (JG) apparatus release renin into the blood. Renin cleaves liver-derived angiotensinogen to generate angiotensin I. Angiotensin I is then converted into angiotensin II (Ang II) by angiotensin-converting enzyme (ACE), that is expressed i.a. on vascular endothelial cells. Ang II raises blood pressure through binding to the angiotensin type 1 (AT_1) receptor. Activation of this receptor leads to vasoconstriction, sodium reabsorption, sympathetic nervous stimulation and the release of both aldosterone from the adrenal cortex, as well as antidiuretic hormone (ADH) from the pituitary (*Figure 1*).

Adverse Ang II actions include induction of vascular and cardiac hypertrophy, fibrosis, and inflammation.[2-4] Not surprisingly, inhibition of the RAS has become the leading therapeutic strategy in slowing the progression of heart failure and proteinuric chronic kidney disease. The antiproteinuric effect can be attributed to both direct hemodynamic effects as well as other effects on the glomerular filtration barrier, that result from inhibition of AT_1 receptor stimulation.[5] Current RAS inhibiting drugs include renin inhibitors, ACE inhibitors, and AT_1 receptor blockers (ARB).

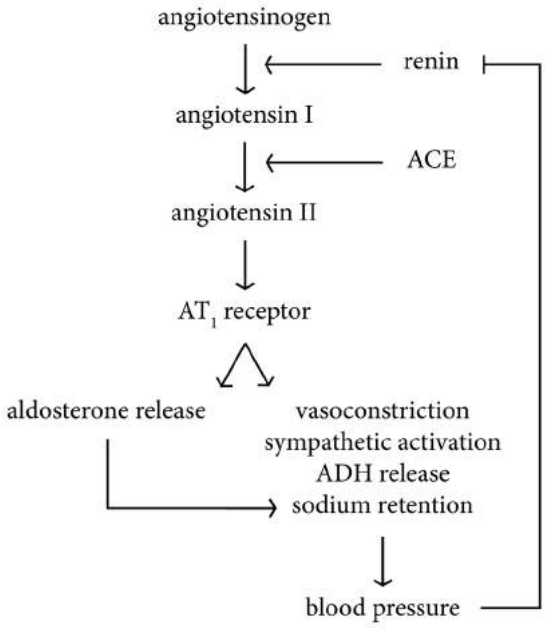

Figure 1. Schematic overview of the renin-angiotensin system (RAS). Angiotensin-converting enzyme (ACE), angiotensin type 1 (AT_1) receptor, antidiuretic hormone (ADH).

Renin

Renin is synthesized as a preprohormone, but the 'pre' region is enzymatically cleaved as soon as preprorenin enters the endoplasmic reticulum.[6] Prorenin can be reversibly activated by low pH, which causes a conformational change, exposing the active site (this is the so-called 'open conformation' of prorenin).[7] The irreversible conversion of prorenin into renin occurs by removal of the 'pro' segment by an endogenous protease.[8] Mature renin is stored in secretory granules in the JG cell and released by stimuli like low blood pressure. Parallel to this regulated pathway, the kidney also secretes prorenin via a constitutive pathway. In fact, plasma prorenin levels are usually 10-fold higher than renin levels. Approximately 2% of this prorenin is in the open conformation. JG cells are the only cells that secrete renin, but prorenin is secreted by a number of cell types in extrarenal tissues.[9] Recently, (pro) renin expression was demonstrated in the distal nephron, i.e. the collecting duct.[10] Ang II stimulates (pro)renin expression at this site, in contrast to its negative feedback effect on JG renin.[11] In diabetic patients with microvascular complications, the plasma levels of prorenin are greatly increased.[12] It has been suggested that this prorenin is derived from the collecting duct.[13] In urine, prorenin is usually not detectable, unless reabsorption of proteins is blocked with lysine.[14] Renin is detectable in urine, at levels that are ~6-7% of its plasma levels.[15, 16] This urinary renin might represent 1) cleaved (i.e., activated) prorenin that is produced and secreted in the collecting duct, 2) cleaved, filtered (i.e., plasma-derived) prorenin, and/or 3) filtered renin. In case of the latter, excreted renin would simply be plasma-derived renin that has escaped reabsorption in the proximal tubule. Given the natural changes that occur in glomerular filtration and tubular reabsorption (e.g., under pathological conditions), even this source might result in varying urinary renin levels. Ang II decreases proteinuria by decreasing glomerular filtration in the acute phase, however, sustained elevated renal Ang II levels induce proteinuria accompanied by glomerular damage.[17] Ang II also inhibits tubular reabsorption of proteins.[18, 19] As a consequence, RAS blockers may reduce proteinuria independently of blood pressure. Along the same line of reasoning, intrarenal RAS activation would increase proteinuria, and consequently, reninuria. Whether or not urinary renin, or other urinary RAS components, might serve as a biomarker for the activity of the intrarenal RAS, or the efficacy of RAS blockade in the kidney, is still under investigation.

ACE

ACE is abundantly present on the vascular endothelium of the lung, but also on the luminal side of the brush border of the kidney's proximal tubule.[20] It has been suggested that renal ACE might be key in the effects of ACE inhibition, mainly because ACE inhibitors continue to lower blood pressure even when plasma Ang II levels have returned to normal (phenomenon known as "angiotensin II escape").[21] This has been a topic of investigation for several decades. Unfortunately, total body ACE knockout mice display abnormal kidney development.[22] The latest studies make use of transgenic mice with markedly reduced renal ACE levels.[23-25] However, these studies raise a lot of issues, since it is unclear if and how other RAS components, and other pathways that influence the RAS, are affected by these low renal ACE levels.

Dual AT_1 receptor-neprilysin inhibitors

RAS blockers are highly effective for the treatment of hypertension, heart failure, and proteinuric renal disease. Still, current therapeutic strategies fail to stop the progression these diseases. Recently, a new drug has been developed that combines an ARB with neprilysin (NEP) inhibition.[26] NEP is involved in the breakdown of a variety of substrates,

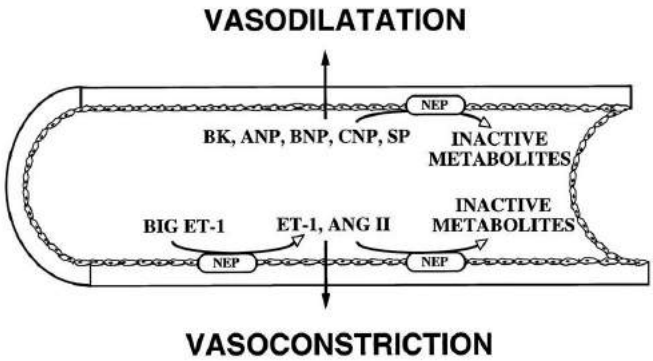

Figure 2. Neprilysin (NEP) substrates. Bradykinin (BK), atrial, brain, and C-type natriuretic peptides (ANP, BNP, and CNP resp.), substance P (SP), endothelin-1 (ET-1), and angiotensin II (Ang II). (Ferro et al., Circulation 1998)

such as natriuretic peptides, angiotensin, endothelin-1, bradykinin, and adrenomedullin. Among these substrates are both vasodilators and vasoconstrictors. Therefore, the net effect of NEP inhibition depends on the relative dominance of these substrates (*Figure 2*).[27] When NEP inhibition is combined with a blocker of the RAS, the balance should shift to the positive, blood pressure-lowering side. Combining an ARB with a NEP inhibitor (the combination is referred to as ARNI) could provide additional beneficial effects on blood pressure and heart function in patients with hypertension and/or heart failure, compared to ARB alone.[28, 29] It has been suggested that NEP is upregulated in diabetes, and that NEP inhibition improves vascular function in diabetes.[30] Recently, it has been shown that ARNI decreases NT-proBNP, a marker for the severity of heart failure, more so in diabetic patients than in non-diabetic patients.[31] The effects of ARNI on the microvascular complications of diabetes, such as diabetic nephropathy and retinopathy are not clear yet. In patients with heart failure and chronic kidney disease, ARNI treatment resulted in a better preservation of estimated glomerular filtration rate (eGFR).[32] However, ARNI also increased proteinuria, compared with patients treated with ARB treatment alone. The latter contradicts the results of our preclinical work in diabetic rats.[33]

Aims of the thesis

In this thesis we aimed to:

1. Summarize the current evidence for the existence of an intrarenal RAS, and give an overview of what is known about RAS components in urine that might serve as a markers for the activity of the intrarenal RAS (*Chapter 2*)
2. Compare the classical ways of measuring renin with a new renin ELISA (*Chapter 3*)
3. Delineate the origin of urinary renin (*Chapter 4*)
4. Discuss findings from recent studies on the role of renal ACE in hypertension (*Chapters 5 and 6*)
5. Compare the effects of a low and a high dose of NEP inhibition in combination with ARB on blood pressure, natriuresis, diuresis, cardiac hypertrophy and vascular reactivity, and obtain a better understanding of the biochemical pathways that are involved (*Chapter 7*)
6. Assess the effects of ARNI and ARB on blood pressure, cardiac hypertrophy , and nephropathy in a diabetes model, and gain a better understanding of the underlying mechanism (*Chapter 8*)
7. Assess the effects of ARNI and ARB on diabetic retinopathy (*Chapter 9*)

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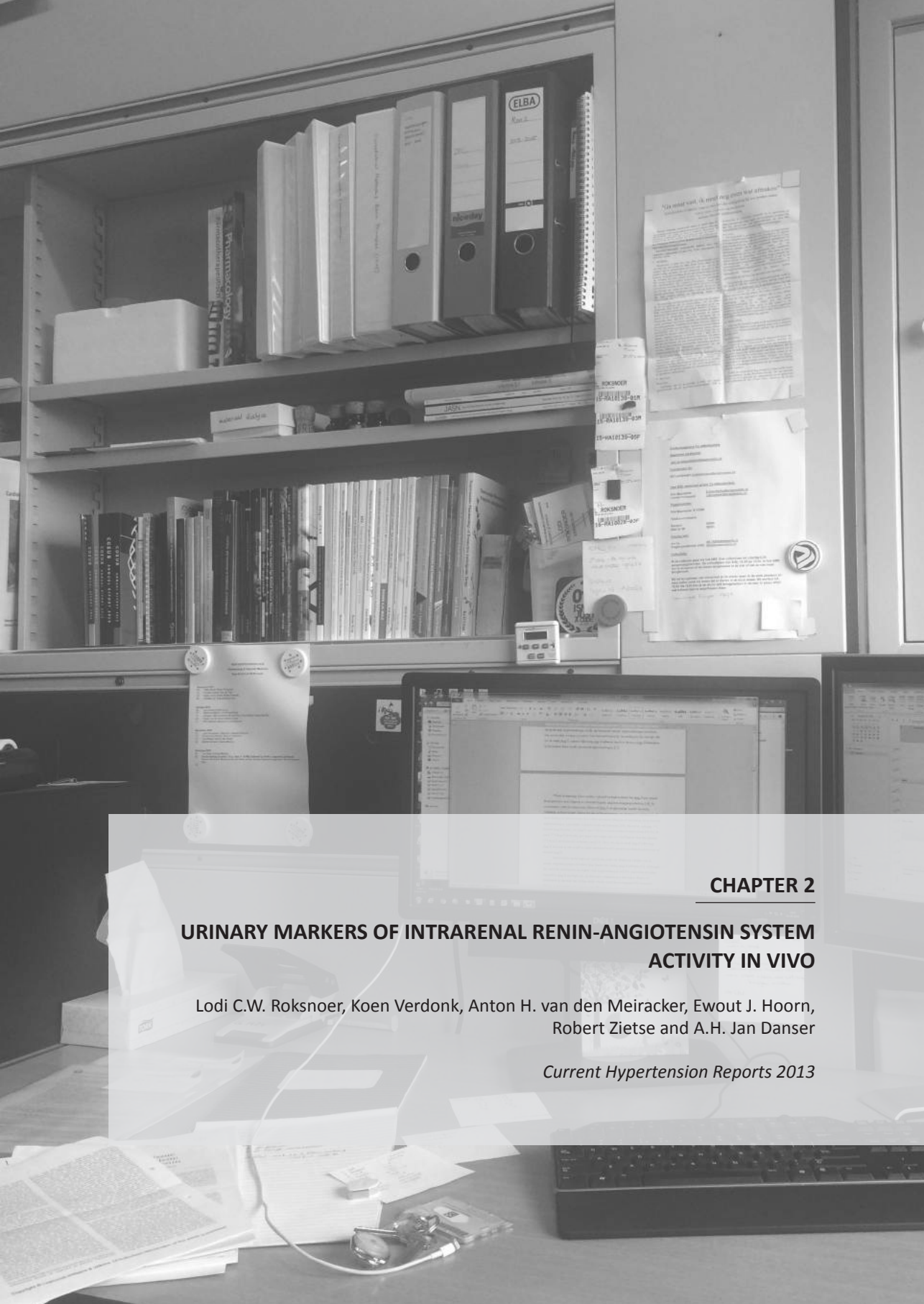
PART I



CHAPTER 2

URINARY MARKERS OF INTRARENAL RENIN-ANGIOTENSIN SYSTEM ACTIVITY IN VIVO

Lodi C.W. Roksnoer, Koen Verdonk, Anton H. van den Meiracker, Ewout J. Hoorn, Robert Zietse and A.H. Jan Danser

Current Hypertension Reports 2013

Abstract
Recent interest focuses on urinary renin and angiotensinogen as markers of renal renin-angiotensin system activity. Before concluding that these components are independent markers, we need to exclude that their presence in urine, like that of albumin (a protein of comparable size), is due to (disturbed) glomerular filtration. This review critically discusses their filtration, reabsorption and local release. Given the close correlation between urinary angiotensinogen and albumin in human studies, it concludes that, in humans, urinary angiotensinogen is a filtration barrier damage marker with the same predictive power as urinary albumin. In contrast, in animals, tubular angiotensinogen release may occur, although tubulus-specific knockout studies do not support a functional role for such angiotensinogen. Urinary renin levels, relative to albumin, are >200-fold higher and unrelated to albumin. This may reflect release of renin from the urinary tract, but could also be attributed to activation of filtered, plasma-derived prorenin and/or incomplete tubular reabsorption.

Introduction
Renal angiotensin (Ang) II levels are several orders of magnitude higher than circulating Ang II levels, and it is therefore generally believed that renal Ang II originates at renal tissue sites. Indeed, making use of infusions of ^{125}I-labeled Ang I and II we were able to show that, despite significant uptake of circulating ^{125}I-labeled Ang II, >90% of renal Ang II could not be explained on the basis of such uptake, and thus truly is derived from local synthesis.[1] Where in the kidney such generation occurs, and what the effects of locally generated Ang II are, has been discussed elsewhere in detail.[2-4] An obvious question is what determines such generation, i.e., where do the renin, angiotensinogen and angiotensin-converting enzyme (ACE) originate that together generate renal Ang II? A role for non-ACE enzymes (e.g., chymase) seems unlikely given the virtual absence of renal Ang II following ACE knockout.[5]

Renin is made in the juxtaglomerular apparatus, and released into the interstitial space, from where it may reach the circulation via diffusion across the peritubular capillaries. Proximal tubular fluid, however, also contains renin, suggesting that circulating renin is filtered in the kidney.[6] Unexpectedly, renin expression has also been observed in the principal cells of the collecting duct.[7] Kang et al. suggested that such expression is upregulated in the diabetic kidney, and contributes to the elevated levels of prorenin (the inactive precursor of renin) in patients with diabetic nephropathy.[8]

Circulating, liver-derived angiotensinogen diffuses into the interstitium, reaching interstitial fluid levels that are comparable to those in blood.[9] In addition, angiotensinogen mRNA has been demonstrated in the proximal straight tubule, suggesting that angiotensinogen synthesis may also occur locally in the kidney, independently of its synthesis in the liver.[4]

Circulating ACE plays little, if any, role, and thus, renal Ang II generation will depend entirely on locally expressed, membrane-bound ACE in the kidney.[10] Indeed, in the human kidney, ACE is abundant in the brush border of the proximal tubule, and, remarkably, usually absent in endothelial cells of any vessel type.[11] Endothelial neoexpression of ACE comes into play in different diseases, e.g., diabetes mellitus and chronic arterial hypertension.[11]

Recent interest focuses on the occurrence of both renin and angiotensinogen in urine, as markers of renal renin-angiotensin system (RAS) activity, potentially reflecting the disease state.[12, 13] If indeed the renal levels of both proteins reflect their production at renal tissue sites, their measurement would be a simple manner to determine whether the renal RAS is upregulated, thus reinforcing the need for treatment with a RAS blocker. However, before

drawing this conclusion we need to be certain that these urinary proteins, like albumin, do not simply reflect breakdown of the glomerular filtration barrier, i.e., that they are kidney- and not plasma-derived. If plasma-derived, their clinical value on top of a much cheaper urinary albumin measurement needs to be proven.

This review summarizes all current findings on urinary angiotensinogen and renin. It will also discuss the presence of prorenin in urine. Given the less abundant literature on urinary ACE, it does not focus on this RAS component. Moreover, it will also not address urinary aldosterone and Ang II, since these peptides are much smaller and thus highly likely to easily filter from the circulation. Indeed, all urinary aldosterone is plasma-derived,[12] and thus urinary aldosterone is a well-established parameter of changes in circulating aldosterone.

Urinary angiotensinogen

Animal studies

Infusing Ang II (40 ng/min) in rats on top of a high-salt diet (8%) not only raised the renal Ang II content, but also increased the urinary angiotensinogen excretion 4-fold, from 1 to 4 nmol/day.[14] At a rat urinary volume of ~10 mL/day, this corresponds with urinary angiotensinogen levels of 100-400 pmol/mL. Given earlier reports on angiotensinogen expression in proximal tubular cells,[15] and the fact that in this study urinary angiotensinogen excretion correlated with renal Ang II but not plasma Ang II, it was suggested that urinary angiotensinogen reflects renal Ang II production. Surprisingly, Ang II in fact stimulated renal angiotensinogen synthesis, resulting in both elevated renal angiotensinogen levels and increased urinary angiotensinogen excretion, thus, potentially creating a positive feed-forward loop.[16] According to this concept, the rise in renal Ang II content following Ang II infusion involves de novo Ang II formation in the kidney from locally generated angiotensinogen.[17]

When interpreting these results, it should be kept in mind that Ang II also raised blood pressure and is known to stimulate hepatic angiotensinogen production.[18] In combination with the deleterious effects of Ang II on glomerular barrier function, occurring, at least in part, due to the rise in blood pressure, an alternative explanation of these findings is therefore that they reflect increased filtration of circulating, liver-derived angiotensinogen, particularly when its levels are increased.[19] Moreover, infusing ^{125}I-labeled Ang II results, via Ang II type 1 (AT_1) receptor-mediated endocytosis,[20, 21] in renal ^{125}I-Ang II levels that, per gram wet weight, are ~4-5-fold higher than the plasma ^{125}I-Ang II levels per mL at steady state.[22] Thus, the rise in renal Ang II following Ang II infusion in rats could be easily attributed to AT_1 receptor-mediated endocytosis of infused Ang II.

Interestingly, under high-salt conditions, male rats displayed a higher rise in urinary angiotensinogen excretion than female rats during Ang II infusion.[23] Although this could be attributed to the higher renal angiotensinogen mRNA expression in males exposed to Ang II and high salt, an alternative explanation is the higher degree of proteinuria in the male rats of this study, which would be accompanied by increased filtration of circulating angiotensinogen. The urinary angiotensinogen levels in this study ranged from ~0.1 (control) to >30 ng/day (after Ang II infusion) per gram body weight. At a body weight of ~250 g and a urinary volume of ~10 mL/day, this would correspond with urinary angiotensinogen concentrations of 2.5-750 ng/mL (0.04-12 pmol/mL). Thus, the control angiotensinogen levels in this study are >1000-fold lower than those in the above described studies.

Rats exposed to deoxycorticosterone acetate plus high salt display hypertension but not elevated Ang II levels. In this model, urinary angiotensinogen remained in the normal range (1 nmol/day).[24] Yet, the Dahl salt-sensitive rat, when put on a high-salt diet (suppressing the circulating RAS) did display inappropriately elevated renal and urinary angiotensinogen

levels in the face of hypertension.[25] Other models evaluating renal dysfunction in the presence of low-to-normal circulating RAS activity (e.g., mice with diabetic nephropathy[26] or IgA nephritis[27] and spontaneously hypertensive rats[28]) also displayed elevated urinary angiotensinogen levels. This could be suggestive for an activated renal RAS, as is believed to exist in diabetes mellitus.[29] If, however, this involves increased renal angiotensinogen expression and the subsequent release of this angiotensinogen into urine, the question is whether the increased renal angiotensinogen expression is the cause or consequence (feed-forward concept) of the elevated renal Ang II levels. The urinary angiotensinogen excretion in diabetic mice amounted to 250 µg/day (vs. 20 µg/day in controls) at three days after the induction of diabetes. Interestingly, in that same time period, while on insulin treatment, the urinary volume also increased ~10-fold (from 1-1.5 to 10-15 mL/day), implying that the actual urinary angiotensinogen concentration did not change. The urinary angiotensinogen concentration in IgA nephritic mice was 15 ng/mL, threefold higher than in control mice. Assuming a mouse urinary volume of ~1 mL/day, this would imply that these mice excreted 15 ng angiotensinogen/day (vs. 5 ng/day in controls), i.e., >1000-fold less than the mice of the diabetes study.

Taken together, the current animal studies report a wide range (>1000-fold) of urinary angiotensinogen concentrations, even in normal controls, and these levels are, confusingly, presented either per animal per day, per g body weight per day or per mL, and in either grams or moles. Only rarely, plasma and urinary angiotensinogen levels have simultaneously been determined.[27] Unfortunately, the plasma levels were often reported in densitometric units on the basis of Western blot analysis,[25] thus not allowing a comparison with the actual urinary levels of angiotensinogen. This raises the need for a reference protein in urine, preferably of identical size. An obvious candidate is albumin, which has a comparable molecular weight (67 kD vs. 65 kD), and is a well-established marker of nephropathy. Since urinary albumin is generally believed to be entirely plasma-derived, a careful comparison of the urinary albumin and angiotensinogen levels would clarify to what degree urinary angiotensinogen is plasma-derived as well.

Human studies

Unfortunately, data on the urinary albumin levels in the above animal studies are scarce. Yet, ample studies have evaluated the two proteins in urine in humans. In fact, in these studies, without exception, a strong correlation was observed between urinary angiotensinogen and albumin.[12, 13, 30, 31] To study angiotensinogen independently of this relation, Saito et al. selected 28 type 1 diabetes mellitus patients without microalbuminuria, and still observed a 3-fold rise (from 4 to 12 µg angiotensinogen/g creatinine) in urinary angiotensinogen versus 21 controls, although this rise was borderline for significance (P=0.045). In type 2 diabetes mellitus patients, urinary angiotensinogen (10-150 µg angiotensinogen/g creatinine) correlated strongly with the albumin/creatinine ratio, and predicted the annual decline in the estimated glomerular filtration rate (eGFR) over a 10-year period. Not surprisingly, the patients with both albuminuria and high urinary angiotensinogen levels showed the worst decline of eGFR, and a higher incidence of renal and cardiovascular composite endpoints.[30] Unfortunately, no rigorous statistical analysis was performed to determine the independency of the effect of angiotensinogen and albumin on eGFR decline. Such an analysis was performed in 201 patients with chronic kidney disease (eGFR <60 mL/min/1.73 m^2 or presence of albuminuria at ≥30 mg/24 hr).[31] Their urinary angiotensinogen levels were 6-fold elevated vs. 201 controls (26.3 vs. 4.4 µg/g creatinine), and correlated highly significantly with urinary albumin. Moreover, both urinary angiotensinogen and

urinary albumin correlated negatively with eGFR. However, when correcting for albumin, the angiotensinogen-eGFR association remained significant. Thus, elevated urinary angiotensinogen levels on top of elevated urinary albumin levels independently determined eGFR decline. Yet, the analysis did not correct for the elevated plasma angiotensinogen levels that were observed in the patients with chronic kidney disease of this study, and thus it cannot be excluded that the independent effect of urinary angiotensinogen in reality, via glomerular filtration, reflects the activation of the systemic RAS. Yamamoto et al. confirmed the association between urinary angiotensinogen and eGFR decline in patients with chronic kidney disease, and additionally showed that the AT_1 receptor antagonist losartan reduced urinary angiotensinogen.[32] Importantly, losartan also reduced plasma angiotensinogen, albeit to a lesser degree. Unfortunately, no comparison with urinary albumin was made in this study.

In hypertensive patients with a preserved kidney function, the urinary angiotensinogen/creatinine ratio correlated with blood pressure and the urinary albumin/creatine ratio.[13] Treatment with RAS blockers decreased the urinary angiotensinogen/creatinine ratio in these patients to levels that were also observed in normotensive controls (from 25 to 14 µg/g creatinine). This decrease was comparable to that in urinary albumin (from 132 to 29 mg/g creatinine). A correlation between blood pressure and the urinary angiotensinogen/creatinine ratio was even observed after excluding patients with diabetes mellitus or patients who were receiving antihypertensive treatment, in the Bogalusa Heart Study.[33] Lantelme et al. observed a relationship between urinary angiotensinogen and blood pressure only in women with essential hypertension and a low plasma renin/aldosterone ratio, and not in men with this type of hypertension, nor in patients with other types of essential hypertension.[34]

Elevated urinary angiotensinogen/creatinine ratios have been observed in IgA nephropathy in several studies,[35, 36] and kidney biopsies of such patients confirmed upregulated expression of angiotensinogen and enhanced Ang II immunoreactivity.[36] Treatment of patients with IgA nephropathy with an AT_1 receptor antagonist (valsartan) reduced urinary angiotensinogen.[36] Furthermore, the sodium sensitivity index (i.e., the reciprocal of the slope of the pressure-natriuresis curve drawn by linking the datapoints obtained during a normal- and a low-salt diet) in IgA nephropathy patients correlated positively with the urinary angiotensinogen/creatinine ratio (17.5 vs. 7.9 µg/g, normal vs. low-salt), but not with the urinary protein excretion.[37] However, when analyzing the data, protein excretion was neither corrected for creatinine, nor analyzed with a non-parametric approach despite its usual non-normal distribution, thus making a true comparison with angiotensinogen difficult.

Summarizing, the variation in urinary angiotensinogen levels in humans is much smaller than in rodents, and the levels in healthy individuals are in a very close range: usually <10 µg/g creatinine, or, at a creatinine level of ~1 g/L, 10 ng angiotensinogen/mL, i.e., 0.2 pmol/mL. This is several orders of magnitude lower than the urinary levels reported in rodents. Human values were in most cases log-transformed prior to analysis. Studies that also determined urinary albumin/protein usually found a close correlation between the two proteins, including the changes that occurred following RAS blockade. In the rare cases that the associations between urinary angiotensinogen and disease parameters were independent of or different from albumin (or protein), either the albumin (or protein) data were differently expressed (i.e., not corrected for creatinine and non-log-transformed[37]) or the changes in circulating angiotensinogen were not taken into consideration.[31] The latter is of the utmost importance, to rule out that urinary angiotensinogen truly

behaves independently of circulating angiotensinogen. Indeed, when correcting urinary angiotensinogen for plasma angiotensinogen in 101 hypertensive patients with or without diabetes mellitus, we observed that urinary angiotensinogen in all aspects mimicked urinary albumin.[12]

Filtration versus local synthesis

An elegant study in mice by Nakano et al. has recently investigated to what degree infused human angiotensinogen filters into urine, simultaneously making a comparison with albumin.[38] Both proteins were fluorescently labeled and applied via bolus injection into the carotid artery. Immediately thereafter, fluorescence was measured in Bowman's space and the glomerular capillaries to determine glomerular permeability. The data revealed that almost no labeled human angiotensinogen showed up in Bowman's space (four times less than labeled albumin), and that its urinary levels were below the detection limit. Increased sieving of angiotensinogen did occur with an increasing level of glomerulosclerosis, but not to a degree that would explain the rise in urinary angiotensinogen in this condition. Here it should be acknowledged that both Richoux et al. and Pohl et al. have demonstrated that angiotensinogen does occur in the ultrafiltrate, but is largely removed via endocytotic uptake in the tubulus, in a megalin-dependent manner.[19, 39] Endocytosed angiotensinogen is subsequently degraded, and the amount of angiotensinogen in the proximal convoluted tubules correlated closely with the plasma level of angiotensinogen.[39]

Importantly, in the Nakano et al. study, the glomerular permeability of infused human angiotensinogen correlated with the urinary albumin levels, but not with the urinary rodent angiotensinogen levels.[38] The authors therefore proposed that endogenous urinary angiotensinogen originated in the kidney, most likely in the tubules. They detected urinary mouse angiotensinogen levels of ~2 ng/90 minutes versus ~2000 ng/mL in plasma. In rats developing glomerulosclerosis, the urinary angiotensinogen levels were ~30 ng/90 minutes at young age, and these levels increased to ~100 ng/90 minutes at older age. Given the urinary volume of rodents of at most a few mL per day, this implies that in these animals the endogenous angiotensinogen levels in urine, like in all previous rodent studies, were several orders of magnitude above those in humans.

When considering the absence of human angiotensinogen in rodent urine, it should be taken into account that the application of a bolus injection will not result in a stable steady-state level of angiotensinogen in blood. Nakano et al. measured circulating angiotensinogen at one time point (90 minutes after injection), and found it to be equal to the level of endogenous mouse angiotensinogen. However, angiotensinogen, like renin, normally diffuses into the interstitium,[9, 40] reaching interstitial levels that are comparable to those in plasma.[41-43] Consequently, a substantial amount of the infused human angiotensinogen would be expected to diffuse into the interstitial space in the first few hours post-injection, thereby potentially limiting its accumulation in urine. Normal angiotensinogen levels in human urine are ~0.2 pmol/mL (see above), versus ~1200 pmol/mL in plasma, i.e., the urinary levels are at most 0.01% of the plasma levels. It is therefore, likely that, in mice, the urinary levels of human angiotensinogen, in view of its whole-body distribution, remained <0.01% of its post-injection plasma level, i.e., (far) below the detection limit of the applied assay. A similar line of reasoning applies to a study where the authors failed to detect human angiotensinogen in rat urine following its intravenous injection.[24]

A unifying concept, given the >100-fold higher urinary angiotensinogen levels in rodents, might be that in humans urinary angiotensinogen is exclusively plasma-derived, hence, its close correlation with albumin, whereas in rodents it is indeed largely kidney (proximal

tubule)-derived (see *Figure 1*). To investigate the physiological importance of such kidney-derived angiotensinogen, a kidney-specific angiotensinogen knockout mouse has been developed.[44] Unexpectedly, such mice had renal angiotensinogen and Ang II levels that were identical to those in control mice, both under normal conditions and following podocyte injury. Thus, it was concluded that, even if angiotensinogen is synthesized in the kidney, it has no functional role, i.e., it does not contribute to renal angiotensin production. This contradicts the feed-forward loop between Ang II and renal angiotensinogen,[16] but would still imply that (non-functional) angiotensinogen in urine of rodents provides an indication of the amount of Ang II to which the kidney (proximal tubule) is exposed. Clearly, evidence is now needed to what degree this holds true in animal models with severe glomerular damage, resulting in increased filtration of plasma proteins, including albumin and angiotensinogen.

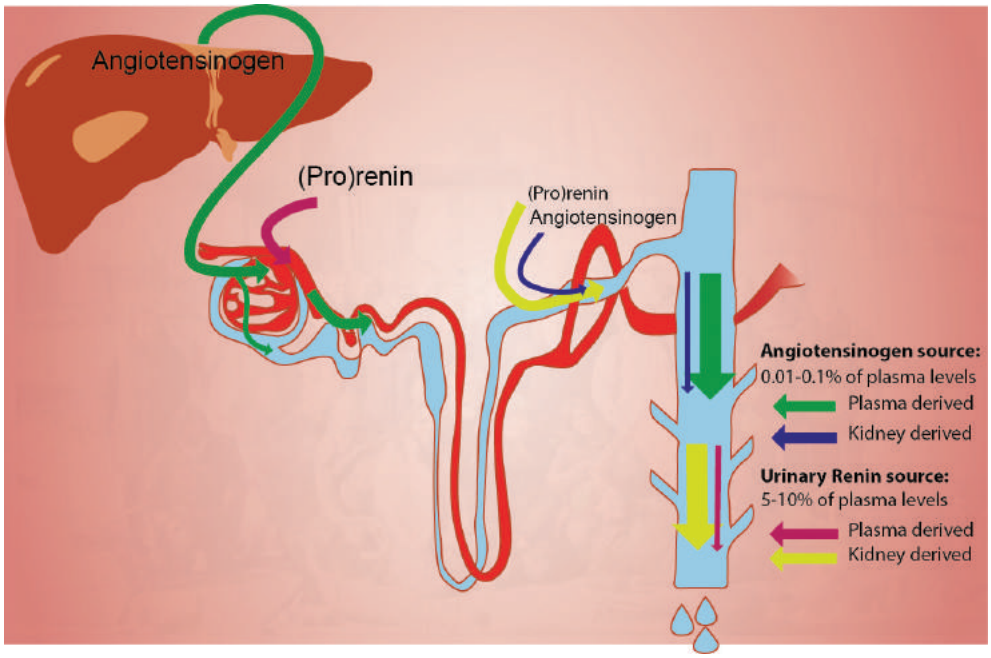


Figure 1. Overview of the origin of urinary renin and angiotensinogen. In humans, angiotensinogen in urine correlates closely with albumin, and thus, like albumin, reaches urine via glomerular filtration from plasma. Levels range from <10-150 µg/g creatinine, or (assuming a creatinine content of ~1 g/L), <10-150 ng/mL (i.e., <0.2-2 pmol/mL). This corresponds with <0.01-0.1% of the plasma angiotensinogen levels in humans. In rodents, urinary angiotensinogen levels are usually much higher, and range from <0.5-4000 pmol/day, or (assuming a urinary volume of ~1 mL/day in mice and ~10 mL/day in rats) <0.1-400 pmol/mL. This implies that the urinary angiotensinogen levels in rodents sometimes are higher than their plasma levels. Such high levels could reflect angiotensinogen release from renal tissue sites, possibly the proximal tubule. Urinary renin levels in humans were 5-10% of the plasma renin levels, i.e., 1-2 orders of magnitude higher than the corresponding urinary levels of albumin and angiotensinogen. Prorenin was undetectable in urine. Most likely, therefore, urinary renin has three sources: filtration from plasma, release from the urinary tract (possibly the collecting duct), and intrarenal conversion of (plasma-derived) prorenin to renin.

Urinary renin and prorenin

Renin was originally described in human urine by Lumbers and Skinner.[45, 46] They found no relationship with urinary creatinine or urinary protein, nor with plasma renin. On average, the urinary renin level was ~7% of that in plasma. Remarkably, although women normally have lower plasma renin levels than men,[47] their urinary renin levels were higher. Natriuretic therapy (spironolactone + chlorothiazide) elevated plasma renin 5-fold, but only modestly increased urinary renin. As a consequence, renin clearance (mL plasma/24 hours) fell by >50%, whereas protein clearance fell by ~20%. The authors concluded that during sodium depletion either increased tubular reabsorption of renin had occurred, or that urinary renin originated from the urinary tract (tubular sites and/or the collecting duct). In case of the latter, such local renin release then apparently remained relatively constant in the face of increased circulating renin levels.

Yukimura et al. confirmed the modest role of urinary excretion in the metabolism of renin, and were unable to demonstrate prorenin in urine of dogs.[48] Unlike Lumbers and Skinner, these authors did see a rise in urinary renin excretion when elevating plasma renin in dogs, although in this case plasma renin was elevated by injecting semipurified kidney renin. Mazanti et al. found that renin in mouse urine was two orders of magnitude lower than in plasma, and also observed an increase in urinary renin following injection of exogenous renin.[49] Importantly, when blocking tubular reabsorption with lysine, urinary renin rose >100-fold, without altering plasma renin.[49] This suggests that normally, ultrafiltered renin is reabsorbed almost completely. Indeed, Pohl et al. confirmed such reabsorption, and suggested that it involves megalin, since immunoreactive renin could not be demonstrated in the proximal tubulus of mice lacking tubular megalin.[19] Nielsen et al. observed very little, if any, prorenin in mouse urine.[50] However, after lysine, prorenin levels in urine became detectable, and the authors were able to estimate that the urinary clearance of prorenin in the absence of tubular reabsorption was ~10-fold lower than that of renin.

In a recent study involving 101 diabetic and non-diabetic patients with or without hypertension, we observed urinary renin levels that were around ~6% of those in plasma,[12] identical to the observations by Lumbers and Skinner.[45, 46] Prorenin was undetectable in urine in our hands, and this was not due to prorenin-renin conversion in urine, since adding prorenin to urine did not result in renin generation. Renin in urine was unrelated to urinary albumin or angiotensinogen, and relative to these proteins >200-fold higher. Urinary renin did not correlate with plasma renin or prorenin. Unexpectedly, urinary renin decreased in patients treated with a RAS blocker (despite their elevated plasma renin levels), and increased in diabetics (despite their lower plasma renin levels). This would be in agreement with the concept that urinary renin is derived from the distal nephron, i.e., the collecting duct, and that Ang II stimulates the release of renin/prorenin from this site, as opposed to its inhibitory role towards renin release from the juxtaglomerular cells. Indeed, several animal studies support the concept of renin synthesis in the principal cells of the collecting duct, and its upregulation by Ang II and diabetes mellitus.[7, 8, 51]

The inability to detect prorenin in human urine may be so for several reasons. First, concentrations may be too low to detect, implying that prorenin is not filtered to the same degree as renin, reabsorbed to a greater degree, and/or not released from the collecting duct. Second, proteolytic cleavage of filtered prorenin may have occurred in the kidney, during the formation of urine. Clearly, such cleavage does not occur in urine per se (given our inability to observe prorenin-renin conversion when adding prorenin to urine ex vivo), although at the same time it is known that the kidneys are the only sites in the body where prorenin-renin conversion occurs.[52, 53]

In summary, it appears that circulating renin is filtered in the glomerulus (given its presence in proximal tubular fluid),[54] but subsequently highly efficiently reabsorbed in the proximal tubule (see *Figure 1*). This resembles the renal handling of circulating angiotensinogen.[19] For unknown reasons, filtration of circulating prorenin tends to occur to a lesser degree, and is likely to be followed by proteolytic cleavage to renin in the urinary system, thus explaining why urinary prorenin is undetectable in most studies. In addition, there may be Ang II-stimulated renin release from the collecting duct. This would explain why urinary renin levels are far above those of albumin and angiotensinogen, and do not run in parallel with changes in plasma renin.

Conclusion

In humans, urinary angiotensinogen correlates closely with urinary albumin, and when carefully correcting for changes in circulating angiotensinogen, there is of yet no convincing evidence that there are conditions where urinary angiotensinogen behaves independently of urinary albumin. Therefore, urinary angiotensinogen, like albumin, clearly is a marker of renal disease, and the measurement of both proteins offers the same information. Interestingly, using a proteomics approach Alge et al. recently identified urinary angiotensinogen and albumin as the two strongest biomarkers in patients with acute kidney injury predicting the need for renal replacement therapy.[55] These investigators subsequently confirmed the prognostic predictive power of urinary angiotensinogen in 97 patients who underwent cardiac surgery for the development of acute kidney injury. Unfortunately, they did not verify the prognostic power of urinary albumin, despite the many studies that support such a role.[56-58]

In rodents, there may be release of non-functional angiotensinogen into urine from proximal tubular synthesis sites. Ang II stimulates such release. Remarkably, this angiotensinogen neither contributes to the renal angiotensinogen content nor to the renal Ang II levels,[44] implying that all functionally renal angiotensinogen is liver-derived. Liver-derived angiotensinogen may reach renal tissue sites via diffusion into the interstitium and/or glomerular filtration.

Urinary renin potentially has three sources: filtration from plasma, release from the collecting duct, and intrarenal conversion of (plasma-derived) prorenin to renin. Normally, filtered renin is highly efficiently reabsorbed, and the same applies to filtered prorenin. Thus, most urinary renin would then be derived from the collecting duct. Only when greatly elevating circulating renin (e.g., by infusing exogenous renin or by blocking the RAS highly efficiently), tubular reabsorption falls short, allowing plasma renin to also accumulate in urine. The same will occur when plasma prorenin levels are elevated, provided that filtered prorenin is converted to renin in the urinary system. Thus, an alternative explanation for the elevated urinary renin levels in diabetics vs. non-diabetics is that it is the consequence of their elevated prorenin levels.[12, 47] Clearly, we need prospective studies evaluating the use of urinary renin as an independent marker, e.g. to establish the renal efficacy of RAS blockers and renal RAS activity in diseases such as diabetes mellitus and CKD.

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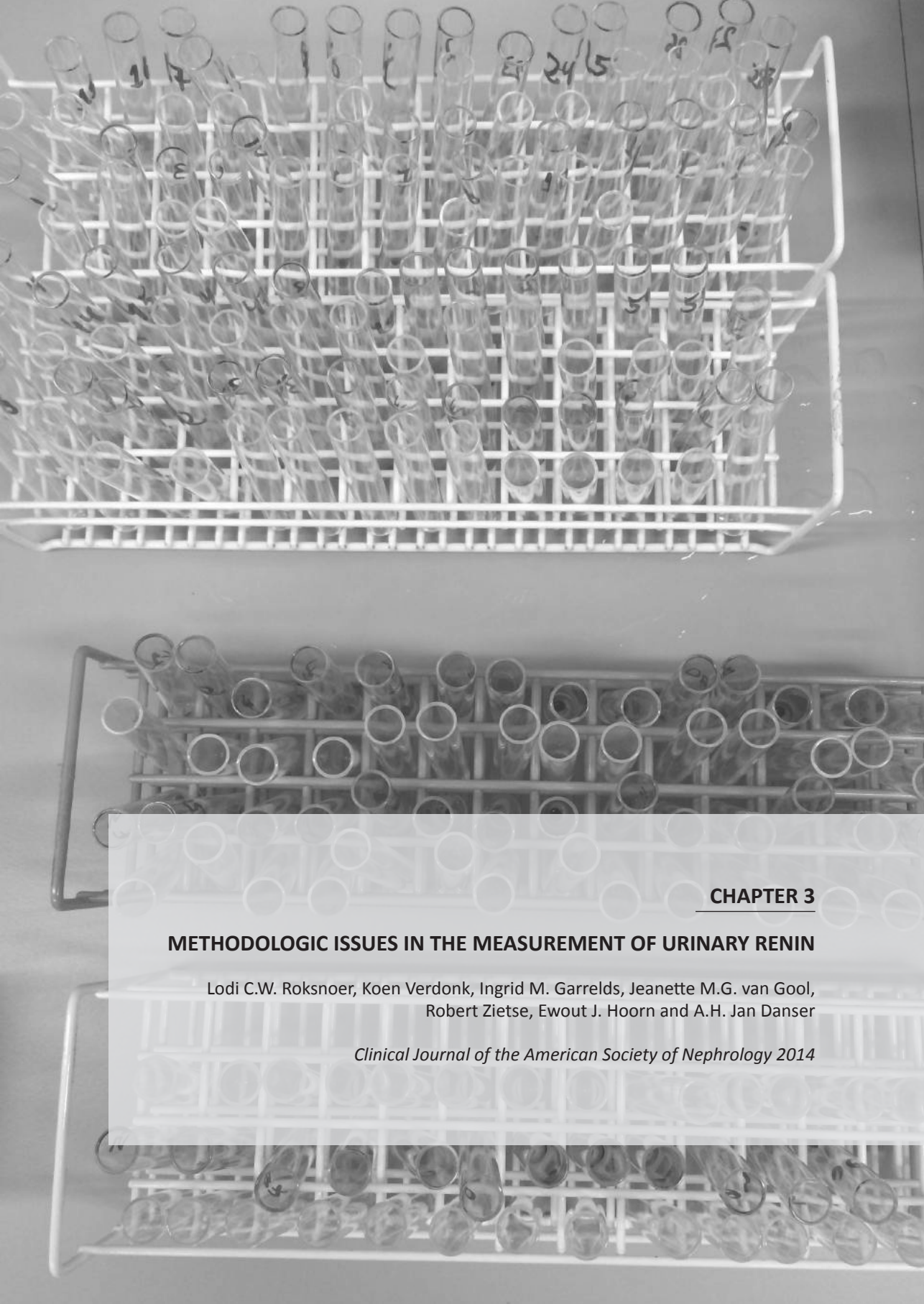

CHAPTER 3

METHODOLOGIC ISSUES IN THE MEASUREMENT OF URINARY RENIN

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Abstract

Background and objectives Alge et al. recently reported that urinary renin may be a prognostic biomarker for AKI after cardiac surgery. However, their urinary renin levels far exceeded published plasma renin levels, whereas normally, urinary renin is <10% of plasma renin. This result raises questions about the specificity of the new Quantikine Renin ELISA kit used in the work by Alge et al., which is claimed to detect total renin (i.e., renin and prorenin). Therefore, this study tested this assay.

Design, setting, participants, and measurements Plasma and urine from 30 patients with hypertension, diabetes, or preeclampsia and 10 healthy pregnant women (randomly selected from sample sets obtained earlier to investigate urinary renin-angiotensin system components) were used to compare the ELISA with a validated renin and an in-house enzyme kinetic assay. Measurements were performed before and after in-vitro prorenin activation, representing renin and total renin, respectively.

Results Total renin measurements by ELISA, were highly correlated. However, ELISA results were consistently ≥10-fold higher. The ELISA standard yielded low to undetectable levels in the immunoradiometric assay and enzyme kinetic assay, except after prorenin activation, when the results were ≥10-fold lower than the ELISA results. In plasma, prorenin activation increased ELISA results by 10%-15%. Urine contained no detectable prorenin.

Conclusions The ELISA renin kit standard is prorenin, and its immunoreactivity and enzymatic activity after conversion to renin do not match the International Reference Preparation of human renin that has been used to validate previous immunoradiometric assays and enzyme kinetic assays; in fact, they are at least 10-fold lower, and thus, any measurements obtained with this ELISA kit yield levels that are at least 10-fold too high. The ELISA antibodies detect both renin and prorenin, with a preference for the former. Given these inconsistencies, urinary renin levels should be measured by established renin assays.

Introduction

Renin-angiotensin system (RAS) components in urine are currently being evaluated as renal disease markers, the underlying concept being that they reflect the activity of the renal RAS independent from the circulating RAS and that the renal RAS contributes to renal disease.[1, 2] For instance, Alge et al. recently suggested that urinary angiotensinogen is a prognostic biomarker for the progression of Acute Kidney Injury (AKI) after cardiac surgery.[3] In a subsequent study, Alge et al. additionally studied urinary renin to improve prognostic power.[4] Remarkably, the urinary renin-to-creatinine ratios in that study, involving patients between 58 and 79 years old, ranged from 114 to 2894 pg/mg. Given the urinary creatinine concentration in such patients (approximately 1 mg/mL), these results would translate to urinary renin levels of 114-2894 pg/mL.[5, 6] Such levels are several orders of magnitude higher than the urinary renin levels reported by others (approximately 1-2 pg/mL; range 0.03-157 pg/mL),[7-9] and even substantially higher than the plasma renin levels in cardiac patients.[10] This finding raises questions about the specificity of the ELISA used in the works by Alge et al. to measure renin (Quantikine ELISA, R&D systems, Abingdon, UK). One reason for this discrepancy might be that the ELISA simultaneously detects prorenin, the inactive precursor of renin (i.e., it actually measures total renin [renin and prorenin]), and not renin. However, although prorenin levels in plasma are generally 10-fold higher than plasma renin levels, urine contains no detectable prorenin,[7] thereby ruling out this possibility.

Classically, renin is measured immunoreactively, making use of antibodies that recognize the active site of renin, or enzyme-kinetically (i.e., based on its capacity to generate

angiotensin I [Ang I]).[11] The latter is more sensitive but can obviously not be used in samples from patients treated with a renin inhibitor. In the present study we compared the new ELISA kit with both an established renin immunoradiometric assay (IRMA) and an in-house enzyme-kinetic assay (EKA). We focused on urine samples obtained from various patient groups but also tested plasma samples.

Materials and methods

Samples

Urine samples were obtained from 10 hypertensive patients (six men and four women; age=58±10 years), 10 patients with diabetes mellitus (eight men and two women; age=59±10 years), 10 healthy pregnant women (age=33±4 years), and 10 women with preeclampsia (age=31±5 years). From the latter two groups, plasma samples were also available. The former two groups have been described previously,[7, 8] and the latter two groups are part of an ongoing study investigating the RAS in pregnancy and preeclampsia. The four patients groups were chosen on the basis of availability and their wide variety in renin and prorenin levels; diabetic patients and preeclamptic women have relatively low renin and high prorenin levels versus hypertensive patients, and pregnant women have relatively high renin and high prorenin levels.[12, 13] The 10 representatives from each group were chosen in a random manner. All studies were approved by the Medical Ethical Review Board and performed in accordance with the Declaration of Helsinki and Good Clinical Practice. Informed consent was obtained from each subject. Blood was collected in EDTA tubes and centrifuged, and plasma was stored at -20°C until analysis. Urine was frozen without inhibitors and also stored at -20°C until analysis. The samples from the healthy pregnant women and the women with preeclampsia had not been thawed before, whereas the samples from the diabetic and hypertensive patients had been thawed once. This is important, because repetitive thawing and freezing of plasma, but not urine, is known to cause prorenin activation (i.e., prorenin-to-renin conversion), without affecting the total amount of renin and prorenin.

Biochemical measurements

- Total renin measurement in plasma and urine by Quantikine ELISA

Total renin in urine and plasma was measured by Quantikine ELISA, which makes use of a 96-wells plate precoated with a (pro)renin antibody (i.e., an antibody that recognizes both renin and prorenin). After binding renin and prorenin, a secondary (pro)renin-specific enzyme-linked monoclonal antibody is added, which after washing, is incubated with a substrate solution that allows a color to develop in proportion to the amount of renin bound in the initial step. The color is subsequently detected making use of a microplate reader (VersaMax ELISA Microplate Reader, Molecular Devices, Sunnyvale, CA, USA). The detection limit is 4.4 pg/mL, and the standard curve ranges from 31.3-2000 pg/mL.

- Renin and total renin measurement in plasma by Cisbio IRMA

Renin in plasma was measured with an IRMA (Renin III; Cisbio, Gif-sur-Yvette, France), making use of an active site-directed radiolabeled antibody.[11] Total renin can also be measured with this assay either after converting prorenin to renin with trypsin (proteolytic activation; i.e., the prosegment is cleaved off) or by incubating the sample for 48 hours with 10 µmol/L aliskiren.[14] The latter procedure induces a conformational change in the prorenin molecule (non-proteolytic activation; involving the removal of the prosegment from the enzymatic cleft), allowing its recognition by the active site-directed antibody. The detection limit of the IRMA is 1 pg/mL, and the standard curve ranges from 2.5 to 320 pg/mL.

- Renin measurement in urine by Cisbio IRMA or EKA

Renin in urine was measured by either IRMA or EKA.[11] The latter involves incubation with excess sheep angiotensinogen and angiotensinase inhibitors, and the subsequent detection of the generated Ang I by radioimmunoassay. The detection limit of the EKA is 0.05 ng Ang I/mL per hour. Ang I-generating activities were converted to renin concentrations based on the fact that 1 ng Ang I/mL per h corresponds with 2.6 pg human renin/mL.[15] Before their application in the IRMA, the urine samples from the patients with hypertension and diabetes were concentrated 5-fold with Amicon Ultra-10 centrifugal filter devices (Millipore, Cork, Ireland). This concentration step was not applied to the samples from pregnant women because of their higher renin content. Prorenin activation did not increase the renin content of the urine samples,[7] suggesting that urine does not contain prorenin.

Statistical analysis

Results are expressed as mean and SD. Levels that were below the detection limit were considered to be equal to the detection limit to allow for statistical evaluation. Differences between levels before and after prorenin activation were assessed by paired t test. Data were logarithmically transformed before analysis in case of non-normal distribution. Correlations between assay results were assessed by linear regression. $P<0.05$ was considered statistically significant. Statistical analyses were performed with SPSS (Version 21; IBM).

Results

Urinary renin in 40 samples, when measured by IRMA or EKA, amounted to 1.9±2.2 pg/mL. Results were identical after prorenin activation (data not shown), suggesting that urine does not contain prorenin. When measuring these same urine samples in the ELISA, which measures renin and prorenin (i.e., total renin), total renin levels were ≥10-fold higher than levels measured by IRMA or EKA (*Figure 1A*). An identical 10-fold higher value was obtained when detecting the IRMA renin standard in the ELISA (*Figure 1B*). Importantly, urinary total renin levels, when measured by ELISA, were below the detection limit in 26 samples, compared with 1 sample when applying the IRMA or EKA. Assuming that the levels in the samples that were below the detection limit equaled the detection limit, the urinary total renin levels on the basis of the ELISA were 20±31 pg/mL.

Plasma renin in 20 samples, when measured by IRMA, was 30±18 pg/mL and increased to 244±100 pg/mL after prorenin activation ($P<0.001$). The latter values represent renin and prorenin (i.e., total renin). The same samples, when measured by ELISA, yielded approximately 10-fold higher total renin levels (2315±810 pg/mL) (*Figure 1C*). Taken together, these data suggest that the ELISA is less sensitive than the other two approaches and yields total renin levels that are ≥10-fold higher.

The most logical explanation of the above findings is that the ELISA standard differs from the IRMA standard. We therefore measured the ELISA standard in both the EKA and IRMA before and after prorenin activation with trypsin. *Figure 1D* shows the comparison between IRMA and ELISA. Before prorenin activation, the ELISA standard yielded levels in the IRMA that were approximately 300-fold lower than expected, whereas after activation, the levels were approximately 30-fold lower than expected. Results obtained by EKA were identical to results by IRMA (data not shown). Given the approximately 10-fold increase in both renin immunoreactivity and enzymatic activity after prorenin activation, it is clear that the ELISA standard is actually prorenin. The standard curve of the ELISA is claimed to run from 31.3 to 2000 pg/mL. In reality, this now appears to be from 1 to 67 pg/mL.

Finally, because the ELISA antibodies detect renin and prorenin equally well, it should not

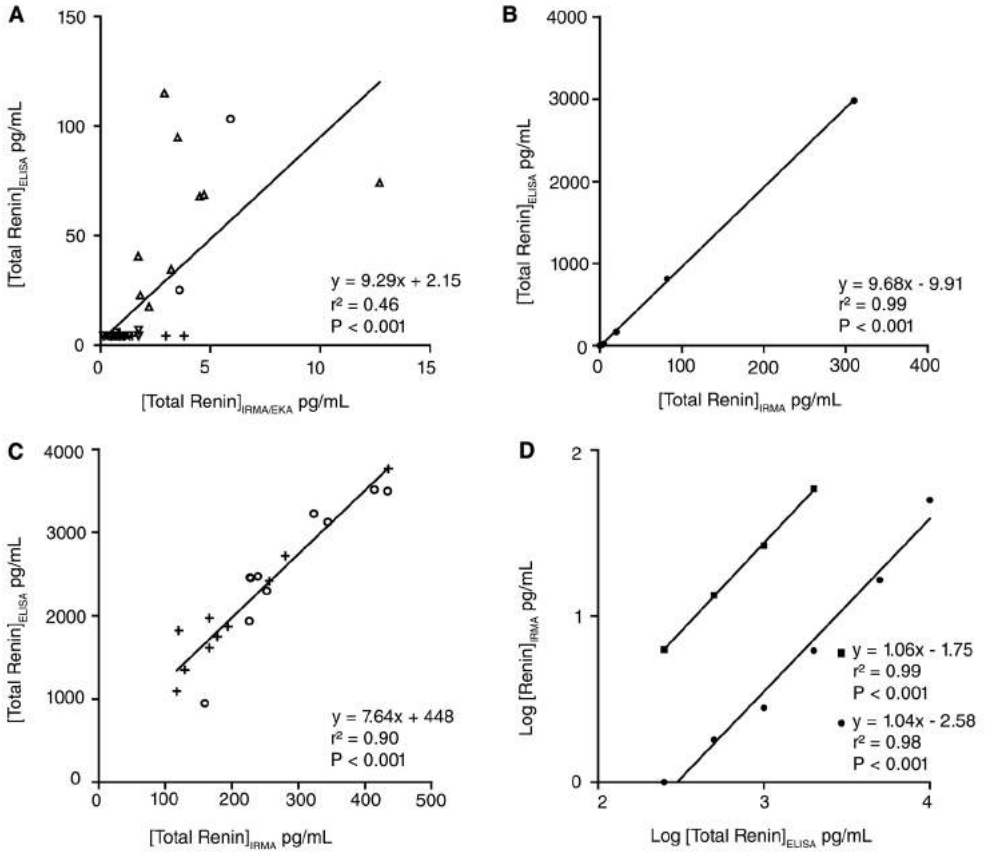

Figure 1. Comparison of immunoradiometric assay (IRMA) and ELISA total renin measurement. (A) Urine samples; (B) IRMA renin standard; and (C) and plasma samples. Samples were obtained from patients with hypertension (ᵛ), diabetes (Δ), or preeclampsia (○) and healthy pregnant women (+). D compares the ELISA standard before (●) and after (■) prorenin activation; not enough standard was available to have the same number of observations after prorenin activation as before prorenin activation. In C, the relationship between the two measurements was not different when limiting it to samples that were above the detection limit.

matter whether samples are measured before or after non-proteolytic prorenin activation by aliskiren. However, when measuring eight individual plasma samples (four samples from healthy pregnant women and four samples from women with preeclampsia randomly chosen from the 10 samples of each group that were used in this study) before and after aliskiren treatment pairwise in the ELISA, we consistently observed that the levels detected after activation (2626±756 pg/mL) were 10%-15% higher than before activation (2361±719 pg/mL; P<0.01). This suggests that at least one of the two antibodies applied in this assay has a preference for renin.

Discussion

Urinary renin is increasingly believed to serve as a prognostic biomarker.[1, 4, 7, 8] It may be filtered from plasma or could be kidney-derived.[1] Normally, urinary renin levels are 5%-10% of plasma renin levels.[7-9] Particularly under conditions where plasma renin levels are high, a

substantial percentage of urinary renin may be plasma-derived.[8] Thus, to what degree urinary renin yields information independent from the circulating RAS remains to be determined. Alge et al. recently reported that urinary renin, together with urinary angiotensinogen, associates with AKI after cardiac surgery.[4] Their urinary renin levels were measured with the Quantikine renin ELISA Kit, and exceeded published plasma renin levels.[11, 12]

However, the present study reveals that this ELISA does not provide reliable renin levels. In fact, the kit simultaneously detects both renin and prorenin, and thus, given that in most body fluids prorenin greatly exceeds renin (up to 100-fold), it rather provides information on prorenin.[11, 12, 16] Remarkably for a renin kit, the ELISA uses recombinant prorenin as standard, and even more concerning, this prorenin has not been calibrated against the International Reference Preparation of human renin. As a consequence, its total renin (renin and prorenin) results are more than one order of magnitude from published total renin levels over the past three decades. Indeed, the normal range claimed by the manufacturer is 201 to 1851 pg/mL in plasma and nondetectable to 96.7 pg/mL in urine (i.e., >10-fold higher than the levels reported earlier by others in normal subjects).[7, 8, 11, 12]

The Quantikine prorenin standard, when converted to renin, yielded ≥10-fold lower levels than expected in the well established Cisbio renin IRMA and our in-house renin EKA. According to the manufacturer's manual, the ELISA kit relies on antibodies that detect both renin and prorenin (i.e., that recognize epitopes on renin's main body, and not on its active site or the prosegment). Nevertheless, our data consistently showed that the ELISA kit results after prorenin activation were 10%-15% higher than before activation, which is suggestive of a renin preference of at least one of the two antibodies. Unfortunately, the manufacturer did not provide information on the precise epitopes recognized by these antibodies, and thus, we cannot explain this renin preference.

Given the non-calibrated standard of this ELISA, it is not surprising that Alge et al., when using this kit, obtained urinary renin levels that are far above the normal range in plasma and,[11] most likely, even above the plasma levels in patients who developed AKI after cardiac surgery.[4] It should be noted that plasma renin values were not reported in their study but that their highest urinary total renin levels (2894 pg/mL) exceed the plasma levels of total renin measured in heart failure patients treated with RAS blockers.[10]

Clearly, the urinary renin levels detected with this kit do not reflect reality, and realistic values would only be obtained when taking into consideration that the ELISA standard contains ≥10 times less prorenin than claimed. Without such correction, the ELISA results do not allow comparison with published urinary renin levels. However, even when corrected for the lower prorenin content of the ELISA standard, the kit does not offer a greater sensitivity than the Cisbio IRMA or the EKA. Nevertheless, in view of the correlation between the levels measured with this kit and the levels measured with validated assays (*Figure 1*), it is still possible that urinary renin is a prognostic biomarker for AKI after cardiac surgery. We suggest that in future studies urinary (and plasma) renin levels should be measured by established renin assays that have been rigorously validated by making use of the International Reference Preparation of human renin.[11] EKAs are most sensitive and can be applied directly to non-concentrated urine samples. However, they depend on the availability of angiotensinogen and require the measurement of Ang I by radioimmunoassay. IRMA kits are easier to handle but display a lower sensitivity. Ideally, urine samples obtained from healthy individuals should, therefore, be concentrated approximately 5-fold before the application of such assays. The concentration step is not required when studying samples from patients with an activated RAS.

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CHAPTER 4

ON THE ORIGIN OF URINARY RENIN. A TRANSLATIONAL APPROACH.

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Abstract
Urinary angiotensinogen excretion parallels albumin excretion, which is not the case for renin, while renin's precursor, prorenin, is undetectable in urine. We hypothesized that renin and prorenin, given their smaller size, are filtered through the glomerulus in larger amounts than albumin and angiotensinogen, and that differences in excretion rate are due to a difference in reabsorption in the proximal tubule. To address this, we determined the glomerular sieving coefficient of renin and prorenin, and measured urinary renin/prorenin 1) after inducing prorenin in Cyp1a1-Ren2 rats, and 2) in patients with Dent disease or Lowe syndrome, disorders characterized by defective proximal tubular reabsorption. Glomerular sieving coefficients followed molecular size (renin>prorenin>albumin). The induction of prorenin in rats resulted in a >300-fold increase in plasma prorenin and doubling of blood pressure but did not lead to the appearance of prorenin in urine. It did cause parallel rises in urinary renin and albumin, which losartan but not hydralazine prevented. Defective proximal tubular reabsorption increased urinary renin and albumin 20- to 40-fold, and allowed prorenin detection in urine, at ~50% of its levels in plasma. Taken together, these data indicate that circulating renin and prorenin are filtered into urine in larger amounts than albumin. All three proteins are subsequently reabsorbed in the proximal tubule. For prorenin such reabsorption is ~100%. Minimal variation in tubular reabsorption (in the order of a few %) is sufficient to explain why urinary renin and albumin excretion do not correlate. Urinary renin does not reflect prorenin that is converted to renin in tubular fluid.

Introduction
Renal clearance of solutes occurs through a combination of glomerular filtration, tubular secretion and tubular reabsorption. The molecular weight (MW) cut-off for glomerular filtration is thought to be 30-50 kD.[1] Urinary albumin (MW 67 kD) is widely accepted as a measure of damage to the glomerular filtration barrier (GFB). Recently, urinary levels of renin-angiotensin system (RAS) components have been suggested to reflect the activity of the intrarenal RAS, independently of GFB damage. This particularly concerns angiotensinogen and renin.[2-4] Indeed, angiotensinogen synthesis has been demonstrated in the proximal tubule,[5] suggesting that renal angiotensin (Ang) II production might occur independently of circulating (hepatic) angiotensinogen. Yet, the urinary excretion pattern of angiotensinogen (MW 55-65 kD) is identical to that of albumin in a wide variety of patients.[4, 6-10] Moreover, two landmark studies, making use of a kidney-specific angiotensinogen knockout mouse, revealed that, both under normal and pathological conditions, renal Ang II production depends entirely on plasma-derived (i.e., hepatic) angiotensinogen.[11, 12] These studies suggested a new concept, namely that it is filtered, plasma-derived angiotensinogen that accumulates in the proximal tubule, rather than locally synthesized angiotensinogen, and that this filtered angiotensinogen contributes most to renal Ang II production.[12] Indeed, filtered angiotensinogen largely, if not completely, is intact angiotensinogen (and not cleaved, des-Ang I-angiotensinogen), truly allowing a contribution to renal angiotensin generation.[8] Pohl et al. have suggested that tubular angiotensinogen uptake occurs via endocytosis, in a megalin-dependent manner.[13]

Renal tubular renin expression is believed to occur more distally, in the collecting duct, in addition to its classical expression in the juxtaglomerular apparatus.[14] Importantly, urinary renin excretion does not correlate with albumin excretion,[4, 15] except when the GFB is damaged and circulating renin levels are greatly elevated (e.g., during preeclampsia).[8] Given its MW (48 kD), circulating renin is likely to pass through the glomerulus. To what degree

this also applies to prorenin (MW 54 kD), the inactive precursor of renin, remains to be determined. Prorenin could be detected in urine of preeclamptic women,[8] but not in urine of hypertensive patients or diabetic subjects, despite the fact that the latter have greatly elevated circulating prorenin levels.[4, 15] One possibility is that prorenin, once filtered, is converted to renin in tubular fluid. If so, this would explain why there is usually no prorenin in urine, and why urinary renin levels do not correlate with urinary albumin levels. Here, it should be kept in mind that renin, like angiotensinogen, is also endocytosed by the proximal tubule in a megalin-dependent manner.[13]

In this study, we set out to delineate the origin of urinary renin. To do so, we used three approaches, based on the hypothesis that renin and prorenin, given their smaller size, are filtered in larger amounts than albumin, and that all three proteins are subsequently reabsorbed in the proximal tubule. First, the glomerular sieving coefficients (GSCs) for renin and prorenin versus albumin were determined, both under normal conditions and after doxorubicin-induced GFB damage. Second, urinary renin levels after the induction of prorenin production in the liver were studied, using Cyp1a1-Ren2 rats, i.e., transgenic rats, which express the mouse Ren2 gene exclusively in the liver in an inducible manner.[16] Renal Ren2 expression did not occur in these animals,[16] and their elevated renal Ang II levels[17] were therefore most likely because of sequestration of circulating Ang II.[18, 19] Third, blood and urine of patients with Dent disease or Lowe syndrome were collected. Such patients have a mutation that disturbs acidification of subapical endosomes in the proximal tubule, thereby disabling reabsorption by megalin.[20] Results in such patients may mimic those in the megalin knockout mice that were used to demonstrate the contribution of megalin to the endocytosis of RAS components in the proximal tubule.[13] Indeed, if so, their urinary renin, prorenin and angiotensinogen levels should be far above normal, supporting the concept that, normally, these levels are low or undetectable due to effective tubular reabsorption.

Methods

Animal and human studies

Glomerular filtration of renin and prorenin was studied in C57/Bl6 mice with or without GFB damage due to doxorubicin administration. Urinary renin and prorenin excretion were quantified after hepatic prorenin induction with indole-3-carbinol (I3C) in Cyp1a1-Ren2 rats, treated with either placebo, the Ang II type 1 receptor antagonist losartan, or hydralazine. Plasma and urine for the determination of RAS components were collected from patients with Dent disease or Lowe syndrome visiting the outpatient clinic. For further details, see the Methods section in the online-only Data Supplement.

Statistical analysis

Data are expressed as mean±SEM when normally distributed, or as geometric mean and range. Differences between I3C and control animals, and I3C animals treated with losartan or hydralazine were assessed by one-way ANOVA with post-hoc Dunnett correction. Data with a non-normal distribution were logarithmically transformed prior to ANOVA. Differences between renin and total renin levels were assessed by Wilcoxon signed ranks tests. Correlations between urine/plasma concentration ratios (*100%) of parameters were assessed by Spearman correlation coefficient. $P<0.05$ was considered statistically significant. All statistical analyses were performed using SPSS (IBM SPSS Statistics 20, Chicago, IL).

Results

Glomerular filtration of renin and prorenin in normal and albuminuric mice

The average GSC of mouse albumin in healthy C57/Bl6 mice was 0.00417±0.00043. The GSC of recombinant human prorenin was significantly greater than that of albumin (*Figure 1*). The GSC of recombinant human renin was significantly greater than that of both albumin and prorenin. In doxorubicin-treated mice, the GSC of each protein was greater than that in normal mice. Both prorenin and renin showed significantly greater GSCs than albumin, whereas the difference in GSC between prorenin and renin was diminished in doxorubicin-treated mice (*Figure 1*).

Urinary renin and prorenin and renal megalin expression after hepatic prorenin induction in Cyp1a1-Ren2 rats

As reported previously,[21] inducing hepatic prorenin synthesis with I3C in Cyp1a1-Ren2 rats increased mean arterial blood pressure from 111±4 to 197±6 mm Hg (P<0.01; n=9-11). Hydralazine partly (150±4 mm Hg; n=4) and losartan fully (121±7 mm Hg; n=7) prevented this rise. I3C exposure increased urinary volume from 10±1 to 57±13 mL/day (P<0.05), and the urinary creatinine concentration decreased accordingly from 5.9±0.3 to 0.6±0.2 mmol/L (P<0.05). Losartan and hydralazine normalized urinary volume to 6±1 and 9±1 mL/day, despite the fact that only losartan normalized mean arterial blood pressure. Unfortunately, we did not monitor drinking behaviour, thus not allowing us to conclude whether the volume normalization during hydralazine was due to a reduction in water intake. The net creatinine excretion per day was identical in all groups (data not shown).

Compared with control (n=4), I3C exposure (n=5) increased plasma renin ~20-fold, and plasma total renin ~200-fold, confirming that its effect predominantly concerned prorenin (*Figure 2A*). On top of I3C, losartan (n=4) and hydralazine (n=4) additionally increased plasma renin and total renin ~2- and ~20-fold, although these changes were significant for hydralazine only (P<0.05 versus I3C). The I3C-induced rise in plasma renin and prorenin was accompanied by a ~200-fold rise in urinary renin levels (P<0.05; *Figure 2B*), and a ~1000-fold rise in urinary renin excretion (P<0.05; *Figure 3A*). Hydralazine partially prevented this

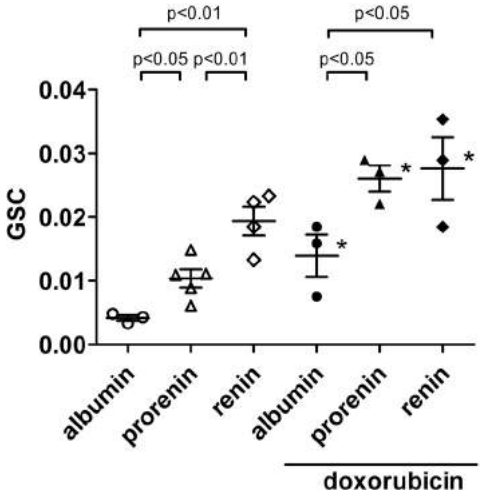

Figure 1. Glomerular sieving coefficient (GSC) of albumin, prorenin, and renin in healthy C57/Bl6 mice (left) and C57/Bl6 mice treated with doxorubicin (right). *p<0.05 versus healthy mice. Data are mean±SEM.

rise (*Figure 3A*), without affecting the urinary renin levels, while losartan fully prevented it. Under all conditions, urinary total renin levels were not significantly different from urinary renin levels, suggesting that urine did not contain prorenin (*Figure 2B*).

As expected based on the rise in renin levels, I3C lowered plasma angiotensinogen levels, particularly in combination with losartan and hydralazine (*Figure 4A*). In fact, after

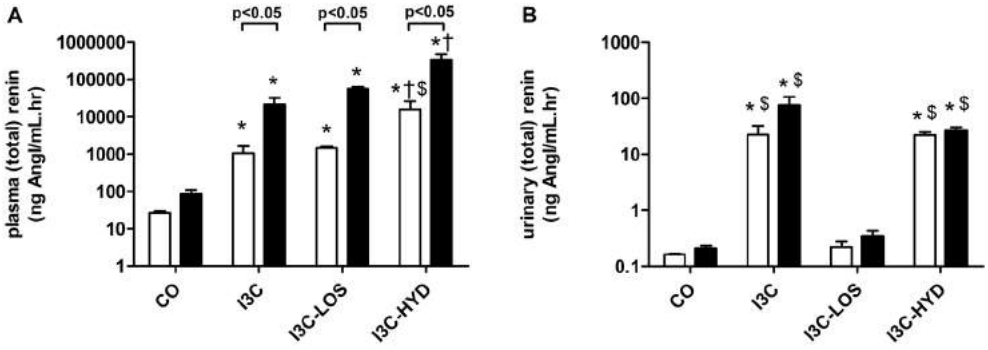

Figure 2. Plasma (A) and urinary (B) renin (white bars) and total renin (black bars) levels in transgenic Cyp1a1-Ren2 rats before (control, CO) and after treatment with indol-3-carbinol (I3C), with or without concomitant exposure to losartan (LOS) or hydralazine (HYD). *P<0.05 versus CO, †P<0.05 versus I3C, and $P<0.05 versus I3C-LOS. Data are mean±SEM of n=4-5.

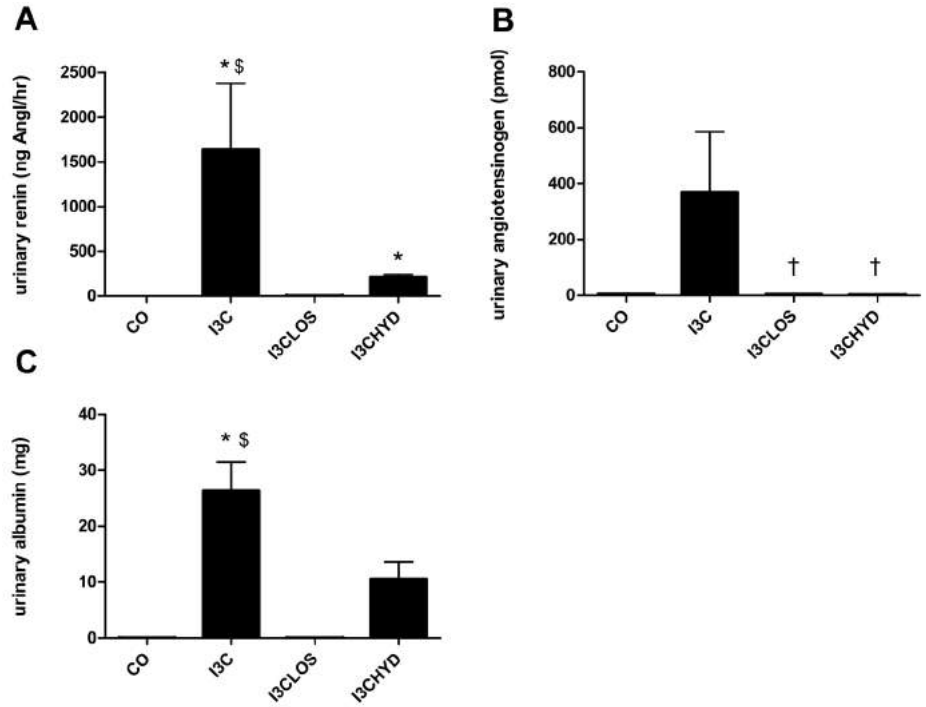

Figure 3. Urinary excretion (per 24 hours) of renin (A), angiotensinogen (B) or albumin (C) before (control, CO) and after treatment with indol-3-carbinol (I3C), with or without concomitant exposure to losartan (LOS) or hydralazine (HYD). *P<0.05 versus CO, †P<0.05 versus I3C, and $P<0.05 versus I3C-LOS. Data are mean±SEM of n=4-5.

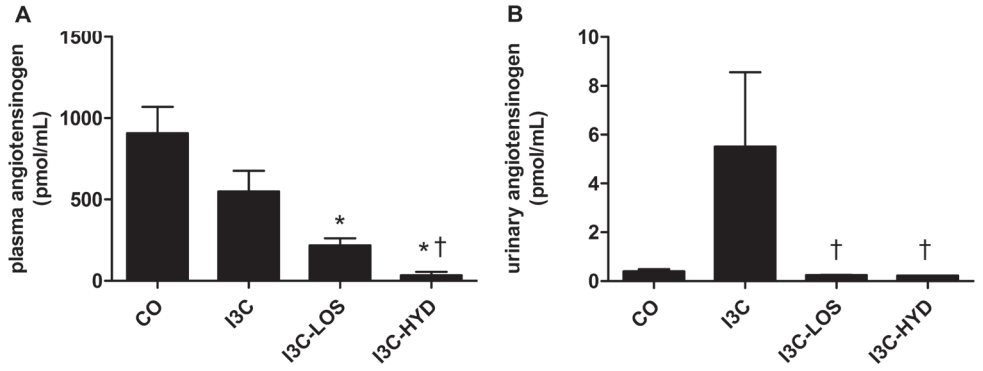

Figure 4. Plasma (A) and urinary (B) angiotensinogen levels in transgenic Cyp1a1-Ren2 rats before (control, CO) and after treatment with indol-3-carbinol (I3C), with or without concomitant exposure to losartan (LOS) or hydralazine (HYD). *P<0.05 versus CO, and †P<0.05 versus I3C. Data are mean±SEM of n=4-5.

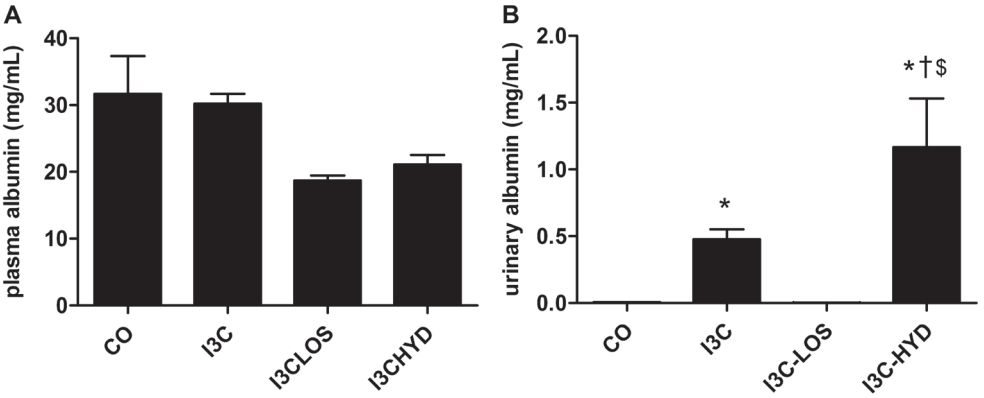

Figure 5. Plasma (A) and urinary (B) albumin levels in transgenic Cyp1a1-Ren2 rats before (control, CO) and after treatment with indol-3-carbinol (I3C), with or without concomitant exposure to losartan (LOS) or hydralazine (HYD). *P<0.05 versus CO, †P<0.05 versus I3C, and $P<0.05 versus I3C-LOS. Data are mean±SEM of n=4-5.

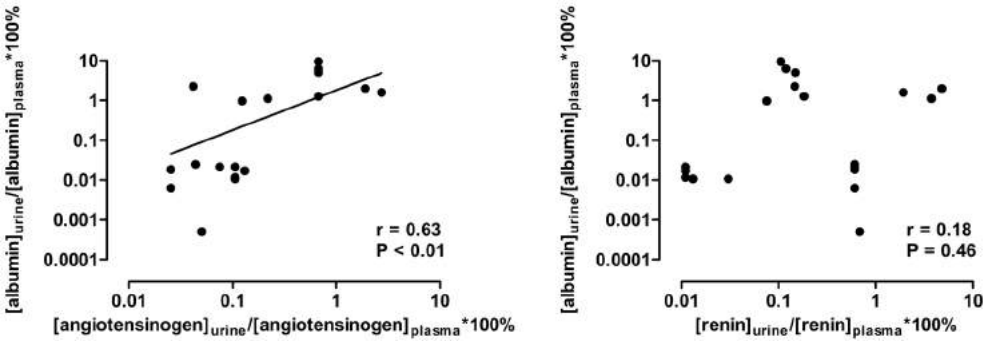

Figure 6. Correlations between the urine/plasma concentration ratios (*100%) of albumin and angiotensinogen (left) or renin (right) in transgenic Cyp1a1-Ren2 rats, at baseline or during treatment with indol-3-carbinol in the absence or presence of losartan or hydralazine.

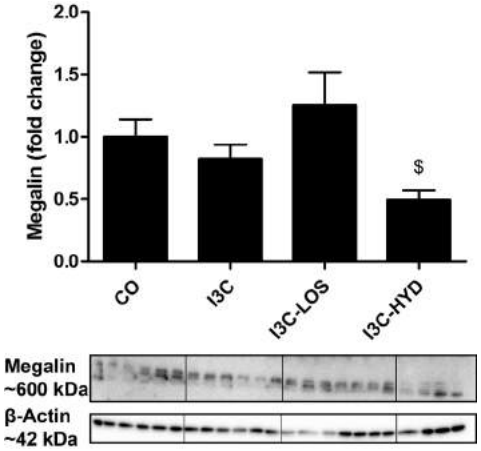

Figure 7. Megalin protein expression in kidneys of transgenic Cyp1a1-Ren2 rats before (control, CO) and after treatment with indol-3-carbinol (I3C), with or without concomitant exposure to losartan (LOS) or hydralazine (HYD). $P<0.05 versus I3C-LOS. Data are mean±SEM of n=4-7.

hydralazine, plasma angiotensinogen was close to zero. The urinary levels of angiotensinogen rose ~10-fold after I3C, although this did not reach significance versus control (*Figure 4B*), and urinary angiotensinogen excretion increased ~100-fold (*Figure 3B*). I3C in combination with losartan or hydralazine reduced urinary angiotensinogen excretion to that of control animals.

I3C with or without antihypertensive treatment did not affect plasma albumin levels (*Figure 5A*). Urinary albumin levels rose ~100-fold after I3C (P<0.05), and urinary albumin excretion rose ~500-fold (P<0.05; *Figure 3C*). Losartan fully, and hydralazine partially prevented this rise (*Figures 3C and 5B*).

The urine/plasma concentration ratio of albumin correlated with that of angiotensinogen (r=0.63, P<0.05), but not with that of renin (r=0.18, P=0.46; *Figure 6*).

I3C treatment tended to reduce renal megalin expression (*Figure 7*), although this decrease became significant only (P<0.05) in the presence of hydralazine. Losartan prevented this phenomenon.

Plasma and urinary RAS components in patients with Dent disease or Lowe syndrome

Plasma samples were obtained from two men with Dent disease (due to a CLC5 mutation) and two men with Lowe syndrome (due to a OCRL mutation, age 24-47 years), and in three of these men one or more urine samples could additionally be obtained (seven urine samples in total). All patients were normotensive, but one patient used an ACE inhibitor for proteinuria.

Urinary total renin levels were higher than urinary renin levels in all seven urine samples, and thus the urine of these patients did contain prorenin. *Figure 8* shows that urinary renin levels were comparable with plasma renin levels (range 68-275%), while urinary prorenin levels were ~2-fold lower than plasma prorenin levels. Angiotensinogen and albumin levels in urine were 1% to 2% of the concomitantly measured plasma levels. Urinary aldosterone levels (6966 pg/mL) were higher than plasma aldosterone levels (485 pg/mL). Plasma creatinine levels averaged 284±72 μmol/L.

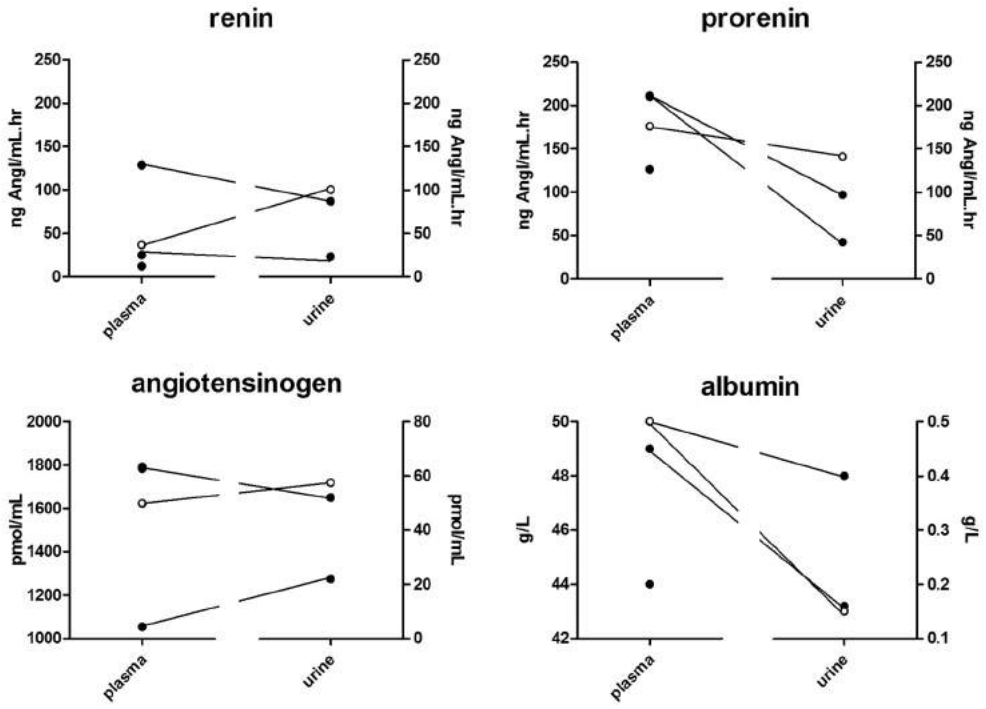

Figure 8. Plasma and urinary levels of renin, prorenin, angiotensinogen, aldosterone, albumin and creatinine in four patients with Dent disease or Lowe syndrome. Urine was collected from three patients. Open symbols resemble the one patient that was treated with an angiotensin-converting enzyme inhibitor.

Discussion

This study shows that the GSCs of renin, prorenin and albumin correlate with their molecular size, albumin displaying the lowest GSC and renin the highest. The GSCs of all three proteins increased after inducing GFB damage with doxorubicin, and under this condition the GSC of prorenin was no longer different from that of renin. This is suggestive of a decrease in glomerular size-selectivity after exposure to doxorubicin. Severe prorenin-induced hypertension also resulted in rises in urinary renin and albumin. Since this was not accompanied by changes in nephrin expression or Wilms tumor staining (both suggestive for GFB damage),[21] these rises were pressure-induced.[22] Indeed, blood pressure lowering prevented or diminished these rises, complete normalization occurring only when blood pressure had been normalized completely (with losartan). Remarkably, even a >200-fold elevation of circulating prorenin, with or without hypertension, did not result in detectable prorenin levels in urine. Only when megalin-dependent tubular prorenin reabsorption was absent, did urinary prorenin become detectable. These data support the concept that urinary renin is plasma-derived, and does not represent activated prorenin of plasma or renal origin. Circulating prorenin apparently does filter into the proximal tubule, but is fully reabsorbed in a megalin-dependent manner.

Multiphoton imaging previously revealed that the GSC of angiotensinogen amounted to 25% to 50% of that of albumin,[23] despite the fact that its MW is comparable with that of albumin. Since there are no reasons why the GSC of albumin would be up to 4-fold higher

than that of angiotensinogen,[23] the authors suggested that this difference is due to the presence of low-molecular weight fragments in their albumin preparation.[24] Such fragments would have a GSC of 100%, and a fragmented fraction of labelled albumin of only 0.19% would have been enough to explain the difference. Thus, in reality, the GSCs of albumin and angiotensinogen are most likely comparable. Our earlier data in humans fully support this view: in hypertensive subjects the albumin and angiotensinogen levels were ~0.05% of their concomitant plasma levels.[4] The present study now reveals that the urinary albumin and angiotensinogen levels in patients with disabled tubular reabsorption are up to 1% to 2% of their plasma levels, i.e., 20- to 40-fold higher than normal. It is important to note that the plasma angiotensinogen levels in these patients were identical to those in hypertensive patients.[4] These data therefore demonstrate that normally >95% of filtered angiotensinogen and albumin is reabsorbed. Given the strong correlation between the urinary/plasma concentration ratios of albumin and angiotensinogen in a wide variety of patients,[4, 6-9] as well as in healthy pregnant women (who display greatly elevated plasma angiotensinogen levels) and women with preeclampsia,[8] it is highly likely that the process underlying tubular reabsorption (megalin-dependent endocytosis) is identical for both proteins.[13] Our data in Cyp1a1-Ren2 rats stress the importance of normalizing the urinary angiotensinogen levels versus plasma angiotensinogen levels. Obviously, this is also true for urinary renin.[25] Indeed, after I3C prorenin induction, particularly in combination with losartan or hydralazine, circulating renin levels became so high that circulating angiotensinogen started to get depleted. This resembles the situation in heart failure patients treated with RAS blockers, or the application of excessive RAS blocker doses.[26, 27] An additional reason for the high renin levels during hydralazine may be that this drug, through an interaction with CYP1A2, induces the generation of autoantibodies,[28] thereby potentially worsening renal pathology. The virtual absence of circulating angiotensinogen during hydralazine explains why the urinary angiotensinogen levels in the hydralazine-treated rats were normal, while simultaneously urinary renin was excessively elevated.

Urinary renin levels in humans amount to ~6% to 7% of the renin levels in plasma, normal values ranging from 0.05-5 ng Ang I/mL.hr (geometric mean 0.2 ng Ang I/mL.hr).[4, 29] This study now reports urinary renin levels in patients with Dent disease or Lowe syndrome that are up to 68% to 275% of plasma renin. This is 20- to 40-fold higher than normal, identical to the rise observed for albumin and angiotensinogen in these patients. Blocking tubular reabsorption with lysine in mice even yielded a 100-fold rise in urinary renin, without affecting plasma renin.[30] Combined with the larger GSC for renin versus albumin, these data indicate that circulating renin is readily filtered into urine, and subsequently reabsorbed for 90% to 98%. Small variations in reabsorption (in the order of a few %) may easily double or triple urinary renin. Such variations, if not exactly paralleled by the variation in albumin reabsorption, could explain why urinary renin excretion does not always correlate with urinary albumin excretion.

An alternative explanation for the different pattern of renin excretion versus albumin excretion is the release of renin into urine from the collecting duct. Yet, synthesis at this site largely concerns prorenin.[31] Thus, such release, if occurring, would require the conversion of prorenin to renin. The same would apply to the possibility that urinary renin is in fact circulating prorenin that has been converted to renin within the tubular fluid. The present study allowed us to address these two scenarios. First, when elevating circulating prorenin several 100-fold in Cyp1a1-Ren2 rats (which is far above the rises observed in human physiology, which are <10-fold, and rather maximally 3- to 5-fold32), urinary renin excretion rose ~1000-fold. A comparable (~500-fold) rise was observed for albumin, while prorenin

remained undetectable in urine. Had conversion of filtered, circulating prorenin contributed to urinary renin, the rise in urinary renin should have been many orders above that of albumin, given the much greater rise in circulating prorenin. Second, and most important, urine of the patients with Dent disease or Lowe syndrome did contain prorenin, albeit in amounts that, when corrected for its plasma concentration, were ~2- to 3-fold lower than those of renin. This difference fits perfectly with the reduced GSC of prorenin versus renin under normal circumstances. Clearly, therefore, prorenin is filtered in the glomerulus, but its tubular reabsorption is virtually 100%, leaving no detectable prorenin in urine under normal circumstances. Early studies in mice, which observed prorenin in urine only when tubular reabsorption was blocked with lysine, complement this view.[33] In summary, these findings argue against prorenin-renin conversion in tubular fluid, and also suggest that urinary renin is not activated prorenin from the collecting duct, at least under the conditions of this study.

The circulating levels of renin and prorenin in patients with disabled proximal tubular reabsorption were 2-to 3-fold above normal,[34] suggestive for RAS activation in these patients. This might relate to the polyuria in patients with Dent disease or Lowe syndrome.[35] RAS activation also resulted in elevated plasma aldosterone.[4] The urinary aldosterone levels in these patients were >1 order of magnitude above the plasma aldosterone levels. This is not different from previous studies,[4, 8] and reflects the well-known fact that aldosterone, as a small molecule, is readily filtered in the kidney, and subsequently concentrated in urine, without being reabsorbed. As a result, aldosterone concentrations in urine correlate with those in plasma, in full agreement with their common (adrenal) origin.[4, 8]

Finally, the partial normalization of the elevated urinary excretion of renin and albumin by hydralazine in the I3C-treated Cyp1a1-Ren2 rats could be due to the fact that it did not fully normalize blood pressure. However, because Ang II suppresses the protein expression of megalin via its type 1 receptor,[36, 37] an alternative explanation is that RAS blockers not only lower urinary protein because they lower blood pressure, but also because they increase reabsorption of proteins via megalin. Conversely, intrarenal RAS activation will lower megalin expression, thereby increasing urinary renin levels. The elevated urinary renin levels in patients with diabetes,[4] in whom such intrarenal RAS activation is well-known to occur,[38, 39] might be the consequence of this phenomenon. Our data on megalin expression are in complete agreement with this concept: expression decreased following RAS stimulation, particularly in the presence of hydralazine, and losartan prevented this.

Perspective

Urinary renin originates in the circulation. Because of their smaller size, both plasma renin and prorenin are filtered into urine in larger amounts than plasma albumin. All three proteins are subsequently reabsorbed by >90% in the proximal tubule. Remarkably, for prorenin such reabsorption is virtually 100%, and there is no evidence for its conversion to urine in tubular fluid. Minor differences in tubular reabsorption (in the order of a few %) are sufficient to explain why urinary renin and albumin excretion, unlike urinary angiotensinogen and albumin excretion, are unrelated. Such differences may relate to the absolute molecular size and charge. An attractive concept would be that the presence of the prosegment, i.e., the 43 aminoacids that represent the difference between renin and prorenin, plays a decisive role. Such a role has also been demonstrated with regard to prorenin binding to the (pro)renin receptor.[40, 41] Glycosylation is less likely to contribute to this phenomenon, since Ren2 prorenin is non-glycosylated (unlike the recombinant human renin and prorenin preparations applied in the present study) and yet it behaved in exactly the same manner

as prorenin in the human and mouse studies: it remained undetectable in urine, despite excessive rises and high blood pressure-induced reninuria and albuminuria.

Sources of funding
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Novelty and significance
What is new?
Renin and prorenin are filtered through the glomerular filtration barrier to a greater extent than albumin, and differences in their tubular reabsorption rate, rather than release of (pro) renin from the collecting duct, are likely to explain the difference in excretion rate.

What is relevant?
Urinary renin is plasma-derived, and does not represent activated prorenin of plasma or renal origin.

Summary
The glomerular sieving coefficients of renin, prorenin and albumin correlate with their molecular size, with albumin displaying the lowest glomerular sieving coefficient and renin the highest. All three proteins are subsequently reabsorbed by >90% in the proximal tubule. For prorenin such reabsorption is virtually 100%, and there is no evidence for its conversion to urine in tubular fluid. Minor differences in tubular reabsorption (in the order of a few %) are sufficient to explain why urinary renin and albumin excretion, unlike urinary angiotensinogen and albumin excretion, are unrelated.

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Supplemental information

Materials and methods

Glomerular filtration of renin and prorenin in normal and albuminuric mice

Experiments with mice were approved by the Institutional Animal Care and Use Committee of the University of Southern California (USC). Animals had free access to food and water throughout the experimental period. Male and female 3- to 5-week-old C57/Bl6 mice from established animal colonies at the University of Southern California were used for experiments (originally purchased from the Jackson laboratory, Bar Harbor, ME). One group of animals served as control group, and one group of animals received an intravenous injection with doxorubicin (25 mg/kg) to induce glomerular filtration barrier damage. At day 4-5 after doxorubicin administration, animals were injected with proteins conjugated with Atto565 maleimide (Sigma-Aldrich, St. Louis, MO) as described before.[1] The dosages of each protein were 6.7 mg/kg for albumin (Sigma-Aldrich), 6.7 mg/kg for recombinant human prorenin, and 0.3 mg/kg for recombinant human renin (the latter two were a kind gift of dr. W. Fischli, Actelion, Allschwil, Switzerland). GSC was measured by multiphoton microscopy as described before.[1]

Urinary renin and prorenin after hepatic prorenin induction in Cyp1a1-Ren2 rats

The rat experiments were performed in accordance with guidelines issued in the 'Guide for the Care and Use of Laboratory Animals' (2010), and approved by the institutional Animal Care and Use Committee of Maastricht University. Animals were housed with free access to food and water throughout the experimental period and were housed under controlled conditions of temperature (21°C). Regular 12-hour diurnal cycles were maintained. Transgenic young Cyp1a1-Ren2 rats were obtained from an internal breeding stock, originally derived from animals supplied by the Centre of Cardiovascular Science, University of Edinburgh, UK. In transgenic Cyp1a1-Ren2 rats, the mouse Ren2 gene is placed under control of a cytochrome P450 promotor, Cyp1a1, on the Y-chromosome. The transgene is expressed primarily in the liver. Transcription of mouse prorenin can be induced by adding the natural xenobiotic indole-3-carbinol (I3C) to the diet. After withdrawal from I3C, the production of mouse prorenin stops.[2, 3] Rats were fed a diet containing 0.3% I3C (Sigma-Aldrich) from 4 to 8 weeks of age. Since this also induces severe hypertension, the rats were treated with either placebo, the angiotensin II type 1 receptor antagonist losartan, or hydralazine (n=4-11/group). Losartan (MSD, Oss, the Netherlands) was dissolved in PBS (GIBCO, Life Technologies, Carlsbad, CA) and administered at a dose of 20 mg/kg per day, via subcutaneously implanted osmotic minipumps (MODEL 2004 Alzet, Durect Corporation, Cupertino, CA). Osmotic minipumps were implanted under isoflurane anesthesia (1% to 4% Forane, Abbott House, Berkshire, UK) and buprenorphine analgesia (0.03 mg/kg s.c.). Hydralazine (Sigma-Aldrich) was administered via drinking water at a dose of 100 mg/kg per day. Rats were weighed, and drinking water, with the appropriate dilution of hydralazine, was prepared every other day. In addition, an osmotic minipump containing hydralazine at a dose of 3 mg/kg per day was implanted, for maximum effect and equal procedures between groups. For comparison, a subset of age-matched transgenic Cyp1a1-Ren 2 rats received normal rat chow (Sniff, Soest, Germany) and was treated with placebo. After 4 weeks of treatment rats were placed in metabolic cages to collect 24-hour urine. Additionally, mean arterial blood pressure was measured in conscious state, as described before.[3] Afterwards, rats were sacrificed by exsanguination via the abdominal aorta to collect blood. Blood was collected in tubes containing EDTA and aprotinin, centrifuged, and stored at -20°C. Kidneys

were cut into eight equal pieces and frozen in liquid nitrogen.

Plasma and urinary RAS components in patients with Dent disease or Lowe syndrome
The experimental protocol of this study was approved by the local research ethics committee. Spot urine samples (n=7) were obtained from three patients with Dent disease or Lowe syndrome visiting the outpatient clinic, frozen and stored at -80°C. Blood (n=5) was obtained from four patients during routine blood sampling, collected in tubes containing EDTA, centrifuged at 3,000 g for 10 minutes, and stored at -80°C. In case of more than one urine or blood sample per patient was collected, the urine or plasma levels measured in these patients were averaged, to obtain one urine and one plasma concentration per person.

Biochemical measurements
Renin concentration was measured by enzyme-kinetic assay, by quantifying angiotensin (Ang) I generation in the presence of excess sheep (human samples) or porcine (rat samples) angiotensinogen.[4, 5] Total renin was measured after incubation with trypsin (which converts prorenin into renin) coupled to Sepharose, and the prorenin concentration was subsequently calculated by subtracting renin from total renin. In the human samples, ng Ang I/mL.hr activities were converted to pg/mL concentration as described before.[6] Angiotensinogen was measured as the maximum quantity of Ang I generated during incubation, at pH 7.4 and 37°C, with recombinant human renin (human samples) or rat kidney renin (rat samples) in the presence of a mixture of ACE, angiotensinase, and serine protease inhibitors.[4, 5] Rat albumin was measured by ELISA (AssayPro, St. Charles, MO). Aldosterone was measured by solid-phase radioimmunoassay (Diagnostic Products Corporation, Los Angeles, CA). Human albumin and creatinine were measured at the clinical chemical laboratory of the Erasmus MC using standard protocols. Megalin expression was assessed by immunoblotting as reported previously.[7] Kidneys were cut into eight equal pieces, and one piece was homogenized on ice in isolation buffer to perform immunoblotting as reported previously.[7] The antibody against megalin was a kind gift of dr. Erik I. Christensen, Aarhus University, Aarhus, Denmark (1:20.000).[8] β-actin (1:50.000; Abcam) was used for normalization of protein levels. Protein was visualized using horseradish peroxidase-conjugated secondary antibodies (1:3000; Bio-Rad, Veenendaal, The Netherlands). Signals were detected by chemiluminescence (Pierce, Rockford, IL) and quantified using ImageQuant LAS 4000 (GE Healthcare, Diegem, Belgium).

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CHAPTER 5

THE INTRARENAL RENIN-ANGIOTENSIN SYSTEM: DOES IT EXIST? IMPLICATIONS FROM A RECENT STUDY IN RENAL ACE KNOCKOUT MICE.

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Nephrology Dialysis Transplantation 2013

Abstract
A large body of evidence supports the presence of local production of angiotensins in the kidney. It is widely believed that renin-angiotensin system (RAS) blockers, through interference with such production and/or the local effects of angiotensin (Ang) II, exert protective renal effects. Yet, whether such production affects blood pressure independently from the circulating RAS is still a matter of debate. To investigate this, a recent study by Gonzalez-Villalobos et al. (J Clin Invest 123: 2011-2023, 2013) has studied the consequences of infusing Ang II or the nitric oxide synthase inhibitor L-NAME in mice lacking renal angiotensin-converting enzyme (ACE). They observed blunted blood pressure and renal responses in the renal ACE knockout mice versus wild-type controls. This review discusses to what degree these findings can be considered as unequivocal evidence for ACE-mediated Ang II formation in the kidney as an independent determinant of hypertension.

Introduction: the origin of renal angiotensin II

Local production of angiotensin (Ang) II in the kidney is well established, and interference with such production or the local effects of this locally generated Ang II is believed to underlie the beneficial renal effects of blockers of the renin-angiotensin system (RAS). Early studies making use of infusions of ^{125}I-labelled angiotensins (i.e., allowing the detection of ^{125}I-angiotensins at levels that do not affect blood pressure) revealed that there is substantial uptake of circulating ^{125}I-Ang II at renal tissue sites: its steady-state tissue levels corresponded to four to five times the steady-state plasma levels of ^{125}I-Ang II.[1] Uptake depended entirely on binding to Ang II type 1 (AT_1) receptors.[2] At the same time, the levels of endogenous Ang II at renal tissue sites were up to 100 times higher than the plasma levels of endogenous Ang II, and thus it could be calculated that, despite significant uptake of circulating Ang II, the majority (>95%) of renal Ang II had been synthesized locally (*Figure 1*). Infusion studies with ^{125}I-labelled Ang I confirmed that this was not due to the conversion of plasma-derived Ang I, in other words, these studies unequivocally proved that both Ang I and Ang II are

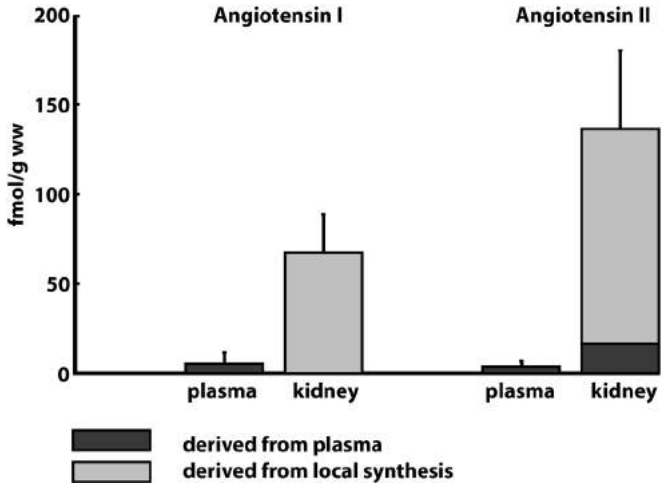

Figure 1. The origin of renal Ang I and II. Uptake from plasma contributes very little, if at all, to the renal angiotensin content. Data are from van Kats et al. [1]

produced locally at renal tissue sites and that renal Ang II is derived from locally generated Ang I and not from plasma-derived Ang I. Furthermore, although most, if not all, renal Ang II was cell-associated (i.e., either bound to membrane AT_1 receptors or located intracellular),[3] this was not due to intracellular Ang generation, but the consequence of AT_1 receptor binding followed by Ang II- AT_1 receptor internalization. Indeed, there was no cell-associated Ang II in AT receptor knockout mice, despite the tremendous rises in renin that occur in such animals.[4] Therefore, renal angiotensin production occurs extracellularly, in the renal interstitial space and/or on the surface of renal cells. If occurring on the cell surface, there may be a role for renin receptors like the (pro)renin receptor. The low affinity of the latter receptor for renin and its precursor prorenin [5, 6] implies that, if it plays a role, this role is limited to sites where (pro)renin levels are sufficiently high, i.e. in (pro)renin-synthesizing tissues like the kidney.

Circulating versus intrarenal RAS

Changes in circulating RAS activity do not always run in parallel with changes in tissue/renal RAS activity (*Figure 2*). For instance, in diabetes mellitus, the leading cause of nephropathy and end-stage renal disease in the Western world, plasma renin is often reduced, whereas the renal plasma flow response of diabetics to RAS blockade is accentuated.[7] This has led to the concept of an activated renal RAS in diabetes mellitus patients. Given the origin of plasma renin in the juxtaglomerular (JG) apparatus, the question arises how renal RAS activity can be upregulated in the face of diminished renin release from the JG cells. The answer may lie in the observation that renin expression also occurs in the principal cells of the collecting duct.[8] Kang et al. suggested that such expression is upregulated in the diabetic kidney, and contributes to the elevated levels of prorenin that have been observed in patients with diabetes.[9] Indeed, elevated prorenin levels provide a strong indication of risk of the microvascular complications of this disease, i.e. nephropathy and retinopathy.[10-12] Unexpectedly, Ang II stimulated the release of renin/prorenin from this site,[8] as opposed to its inhibitory role towards renin release from the JG cells. Given the elevated urinary renin levels in diabetics, combined with the decrease in urinary renin in patients treated with a RAS blocker (despite their elevated plasma renin levels), it seems that urinary renin is derived from the distal nephron, i.e. the collecting duct, and as such might reflect renal RAS activity.[13]

Although circulating, liver-derived angiotensinogen diffuses into the interstitium, reaching interstitial fluid levels that are comparable to those in blood,[14] Kobori et al. have suggested that angiotensinogen synthesis additionally occurs in the proximal straight tubule.[15] Such locally produced angiotensinogen might contribute to renal Ang II production. Interestingly, on the basis of Ang II infusion studies, it was concluded that Ang II in fact stimulates renal angiotensinogen synthesis, resulting in both elevated renal angiotensinogen levels and increased urinary angiotensinogen excretion, thus potentially creating a positive feed-forward loop.[16] According to this concept, the rise in renal Ang II content following Ang II infusion involves de novo Ang II formation in the kidney from locally generated angiotensinogen.[17] Obviously, such local formation also requires angiotensin-converting enzyme (ACE). In the human kidney, ACE is particularly abundant in the brush border of the proximal tubule, and low or absent in endothelial cells of any vessel type.[18] A role for non-ACE enzymes (e.g. chymase) is unlikely given the virtual absence of renal Ang II following ACE knockout.[19]

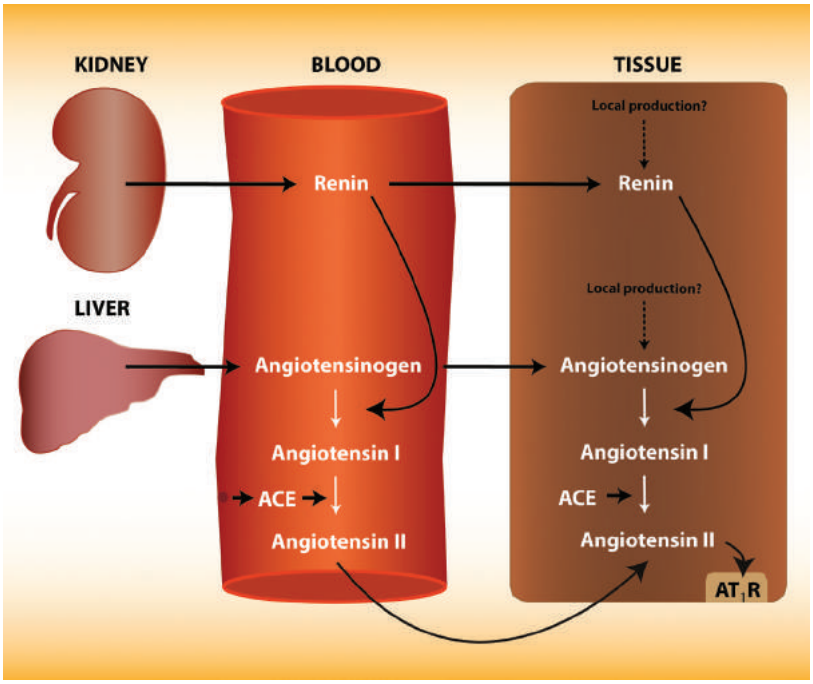

Figure 2. Circulating versus tissue renin-angiotensin system. Circulating renin is kidney-derived, and circulating angiotensinogen originates in the liver. ACE is located on endothelial cells. Ang II generated in the circulation will diffuse to tissues in order to bind to its main receptor (the Ang II type 1 receptor (AT_1 R) to exert effects. In addition, circulating renin and angiotensinogen might also diffuse to tissue sites (e.g., the interstitial space) and generate, with the help of tissue ACE, Ang II locally. In a limited number of tissues (in particular the kidney!) renin and/or its precursor prorenin are produced locally, thus allowing local production of Ang II by locally produced renin. Although this is also claimed for angiotensinogen, current evidence does not support a functional role for kidney-derived angiotensinogen, since the renal Ang II levels in renal angiotensinogen knockout mice are identical to those in wild-type mice.[28]

Hypertension due to selective intrarenal RAS activation?

Given that alterations in the renal and circulating RAS do not always run in parallel, an attractive concept is that hypertension in patients with normal or low circulating RAS activity occurs due to selective renal Ang II overproduction. Such patients may respond well to ACE inhibition despite little or no change in circulating Ang II. To study this phenomenon in detail, a kidney-specific ACE knockout would be useful. However, in view of the many different cells that potentially express ACE in the kidney, this would be technically challenging. Unfortunately, total body ACE knockout mice, like angiotensinogen knockout mice,[20] are hypotensive and display abnormal kidney development.[21] In a recent study, Gonzalez-Villalobos et al. therefore studied mice that, via targeted homologous recombination, expressed ACE only in hepatocytes (ACE 3/3 mice) or in myelomonocytic cells (ACE 10/10 mice).[22] Such mice are phenotypically normal, i.e. they have a normal blood pressure and display no renal abnormalities, possibly due to the fact that such ectopic expression of ACE resulted in sufficiently high circulating Ang II levels to overcome these problems. In fact, according to the authors, the plasma and renal tissue Ang II levels of ACE 10/10 mice were

normal. Given the fact that circulating Ang II normally determines <5% of renal tissue Ang II,[1] it is difficult to understand how unaltered plasma Ang II levels would make up entirely for the lack of Ang II generation at renal tissue sites. One possibility is a substantial increase in renal AT_1 receptor density. Although indeed a ~30% rise was observed, this is far from the >20-fold rise that would be required to explain this discrepancy. Yet, given the recently reported lack of specificity of commercial AT_1 receptor antibodies,[23] a larger rise cannot be entirely excluded. Generation of Ang II by non-ACE enzymes (e.g. chymase) would be a second possibility. However, since the phenotype of ACE - and angiotensinogen knockout mice is identical, a role for chymase in tissue Ang II generation is highly unlikely, even under pathophysiological conditions.

The authors subsequently infused Ang II (400 ng/kg/min subcutaneously) or blocked NO synthase with L-NAME during two weeks. Both procedures significantly increased blood pressure in wild-type controls, while their hypertensive response in the renal ACE knockout mice was blunted. In addition, in the Ang II infusion studies, the authors could demonstrate that all renal responses to high serum Ang II, i.e. intrarenal Ang II accumulation, sodium and water retention, and activation of ion transporters in the loop of Henle and distal nephron as well as the transporter activating kinases, were effectively prevented in mice that lack kidney ACE. Based on these data the authors conclude that renal ACE is a prerequisite to increase renal Ang II, which is needed to stimulate sodium transport in the loop of Henle and the distal nephron, and to induce hypertension. In others words: these data would support a role for renal ACE/Ang II as an independent determinant of hypertension.

Does the absence of intrarenal ACE truly protect against hypertension?

The study by Gonzalez-Villalobos et al. is an impressive technical tour de force, but does it allow us to conclude that activation of the intrarenal RAS can solely induce hypertension? Several caveats need to be considered (*Table 1*).

Kinetics of Ang II

A highly relevant issue is that we do not know whether ACE knockout affects the metabolism of Ang II. Since ACE has many substrates, it is not unreasonable to expect that ACE knockout will result in the upregulation of alternative (amino/carboxy) peptidases, e.g. in the ACE-free kidney, and possibly at all locations that lack ACE. In fact, the authors remark that the plasma Ang II levels in wild-type mice during the first 24 hours were more than double that in the renal ACE knockout mice. They state that it is unclear why this would be so. However, the

Table I. Summary of the main findings of the study by Gonzalez-Villalobos et al.

Normal blood pressure, kidney histology/function and Ang II levels in blood and kidney in mice lacking renal ACE
Yet, their blood pressure responses to Ang II infusion or nitric oxide synthesis (NOS) inhibition are greatly reduced
Moreover, their renal responses to Ang II infusion (i.e., intrarenal Ang II accumulation, sodium and water retention, and activation of ion transporters in the loop of Henle and distal nephron as well as the transporter activating kinases) were also effectively prevented
It is concluded that the absence of intrarenal ACE protects against hypertension, both when circulating Ang II levels are high (Ang II infusion) and low (NOS inhibition)

simplest explanation is that the half-life of Ang II is decreased in the ACE KO mice, due to an upregulation of alternative angiotensinases. If so, all this study shows is that, for a given level of Ang II infusion, ACE KO mice are exposed to lower Ang II levels. Thus, such animals would have a diminished blood pressure response and reduced transporter activation after Ang II infusion simply because their steady-state Ang II levels are lower.

Secondly, the authors report widely varying plasma Ang II levels during the 2-week infusion period. In the wild-type animals, plasma Ang II reaches peak levels on day 1 (> 2000 pmol/L, i.e. many orders of magnitude above the normal levels in humans and mice)[24, 25] and then decreases by ~75%. A similar pattern, albeit at lower levels, is observed in the ACE 10/10 mice. These observations do not support that a steady-state level had been reached, and in fact imply that the subcutaneous application of Ang II may not have resulted in the same Ang II exposure during the entire course of the study. Normally, given the half-life of Ang II (<1 minute in blood versus <1 hour in tissue),[26] a steady state should have been reached within hours and remained constant over the entire 2-week period. That this did not occur suggests that angiotensin-degrading enzymes may have become upregulated to counteract the effects of Ang II infusion.[27] Again, without knowing whether this differed between wild-type and renal ACE knockout mice it is impossible to draw quantitative conclusions on tissue uptake of circulating Ang II versus local production (*Figure 3*). Summarizing, knowledge on the kinetics of Ang II is a prerequisite to fully appreciate the implications from these studies.

Renal angiotensinogen production

The authors propose that the rise in renal Ang II after Ang II infusion is due to local production rather than uptake of infused Ang II. In earlier studies this local production was reported to be the consequence of the rise in renal angiotensinogen expression induced by circulating Ang II, as evidenced by a rise in urinary angiotensinogen.[15] Yet, such a rise in the present study occurred only in wild-type mice. The authors do not consider the lack of such a rise in renal ACE knockout mice surprising, since 'by eliminating renal ACE and local Ang II synthesis, the stimulus for angiotensinogen production is effectively hampered'. This is very difficult to understand, since it seems to imply that an ACE-dependent Ang II upregulation is needed to increase renal angiotensinogen (*Figure 3*). In other words: Ang II infusion results in renal Ang II levels that are apparently sufficiently high to upregulate ACE, but that do somehow not affect renal angiotensinogen expression. At the same time, Ang II infusion did upregulate hepatic angiotensinogen expression in both wild-type and ACE10/10 mice. To add to the confusion, a recent study by Matsusaka et al. reported that renal Ang II production depends entirely on hepatic angiotensinogen, since kidney-specific angiotensinogen knockout did not affect renal Ang II levels.[28] Moreover, in humans, the changes in urinary angiotensinogen occurring in diabetic patients or following RAS blockade closely followed the changes in urinary albumin, suggesting a common origin, i.e. the blood compartment.[13] The latter implies that urinary angiotensinogen in humans originates in the liver. Importantly, in the present study, the renal angiotensinogen levels were actually lower at baseline in the renal ACE knockout mice versus wild-type controls. Yet, their tissue Ang II content was identical. This also supports the idea that sources other than renal angiotensinogen determine renal Ang II levels. Taken together, an increase in renal Ang II production following Ang II infusion, if occurring, is likely to depend entirely on hepatic angiotensinogen, i.e., does not occur independently from the circulating RAS.

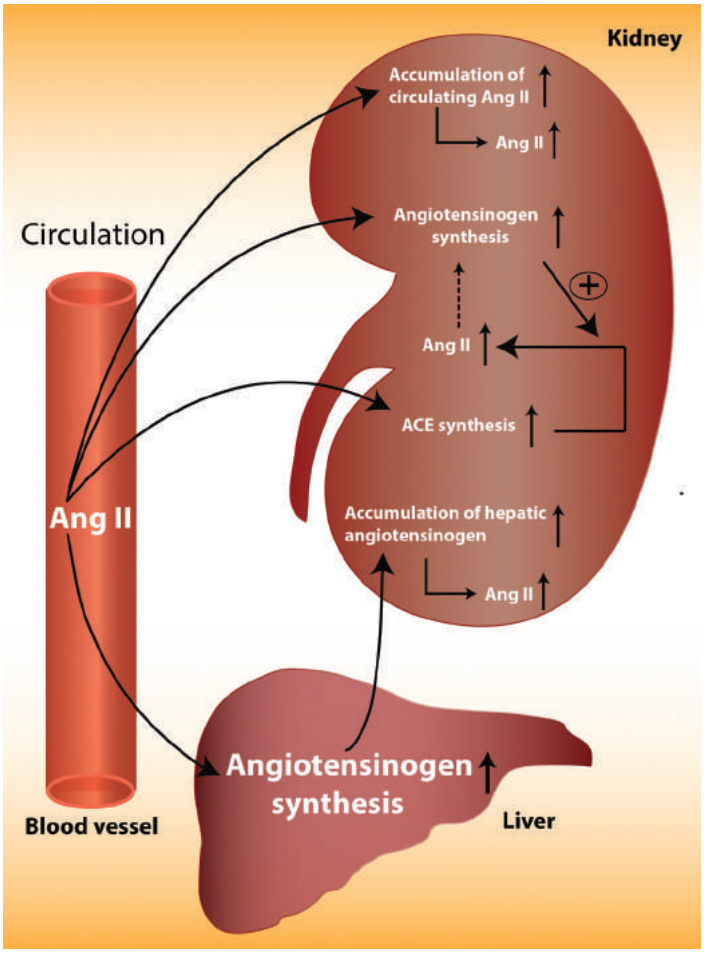

Figure 3. Potential explanations for the rise in renal Ang II during Ang II infusion. See text for further explanation. Not shown is the possibility that high renin levels overcome a substantial (>90%) reduction in tissue ACE.

The two domains of ACE

ACE has two homologous and independent catalytic domains, the so-called N- and C-terminal domains. The C-domain is responsible for Ang I-II conversion.[29, 30] Thus, the consequence of Ang II infusions in ACE C-domain knockout mice should be comparable to those in renal ACE knockout mice, since without the ACE C-domain an increase in renal Ang II production would be impossible. In fact, such studies have been performed by the same investigators. Yet, if anything, Ang II infusion in ACE C-domain knockout mice resulted in larger rises in blood pressure, and the rise in renal Ang II was identical to that in wild-type mice.[24] This is difficult to reconcile with the current study by Gonzalez-Villalobos et al.

Both ACE domains equally contribute to bradykinin degradation,[31] and in the absence of ACE one would expect a rise in renal bradykinin. To what degree this has contributed to the beneficial effect of ACE knockout in the two hypertension models remains to be determined. This would require the actual measurement of bradykinin and/or studies in the presence of a bradykinin type 2 receptor blocker.

Residual ACE in the kidney?
Renal ACE in ACE10/10 mice is mentioned to be 'negligible' (<10% of wild-type), while in the ACE3/3 mice it is 14% of wild-type values. It is important to note that the latter situation (a reduction of 86% in tissue ACE activity) might also occur in humans during ACE inhibition, and can be easily matched by a ~7-fold rise in renin,[32] i.e. a 7-fold rise in renin is sufficient to result in the same Ang II levels at 14% of normal ACE activity (this probably explains the so-called 'Ang II escape' during ACE inhibition). From this point of view, there is no reason why ACE3/3 mice should not respond the same as wild-type mice. Moreover, since even renin rises of >100-fold are feasible,[33] a >90% reduction of ACE in ACE10/10 mice might simply be matched by a >10-fold rise in renin. Thus, we need to know the renin levels in these mice. If greatly elevated, this might be sufficient to explain the identical renal Ang II levels (derived from local production!) in the face of renal ACE levels that are less than 10% of control.

If ACE3/3 mice have more renal ACE than ACE10/10 mice, one would expect their blood pressure response to Ang II, if anything, to be larger than in ACE10/10 mice. Yet, the blood pressure response in ACE3/3 mice to Ang II infusion is less than half that in ACE10/10 mice. Finally, although Ang II infusion would normally increase the filtration of ACE-expressing macrophages (and the authors' data fully confirm this), renal ACE expression in the Ang II-infused kidneys of ACE10/10 mice is unaltered versus non-infused mice. This seems to imply that there already was a substantial 'background' amount of ACE-expressing myelomonocytic cells in the kidney, even at baseline.

Renal RAS upregulation during NO synthase inhibition?
The diminished blood pressure response during L-NAME treatment in renal ACE knockout mice might imply a role for the renal RAS in this condition. However, no data on the renal Ang II content during these studies were obtained, and given the fact that the lack of ACE was not limited to the kidney, the diminished pressor response cannot be unequivocally attributed to the renal RAS. Moreover, L-NAME usually only induces the RAS after renal injury has occurred, and thus the question is whether this has occurred at all in this model.

Conclusion
The studies by Gonzalez-Villalobos et al. are highly original. Nevertheless, even this approach does not provide waterproof evidence for the importance of renal ACE in Ang II- and L-NAME-dependent hypertension. The RAS has ample regulatory mechanisms to allow its return to the same degree of RAS activity during blockade or overexposure. For instance, an upregulation of renin could easily mask a reduction of renal ACE by >90%. In fact, this is well-known to occur in humans during ACE inhibition, and this may explain why the renal Ang II levels in renal ACE knockout mice were unaltered. Furthermore, deleting/reducing ACE may upregulate the metabolism of Ang II by other enzymes, and thus ACE knockout animals would then be exposed to lower Ang II concentrations when infused with a given amount of Ang II. Clearly, their hypertensive and renal responses will then be more modest. Moreover, additional angiotensinases may come into play during Ang II infusion, and this may not necessarily be the same in all knockout models. Finally, an Ang II-induced upregulation of hepatic angiotensinogen synthesis will also affect renal Ang II synthesis, even when no change in renal ACE occurs.

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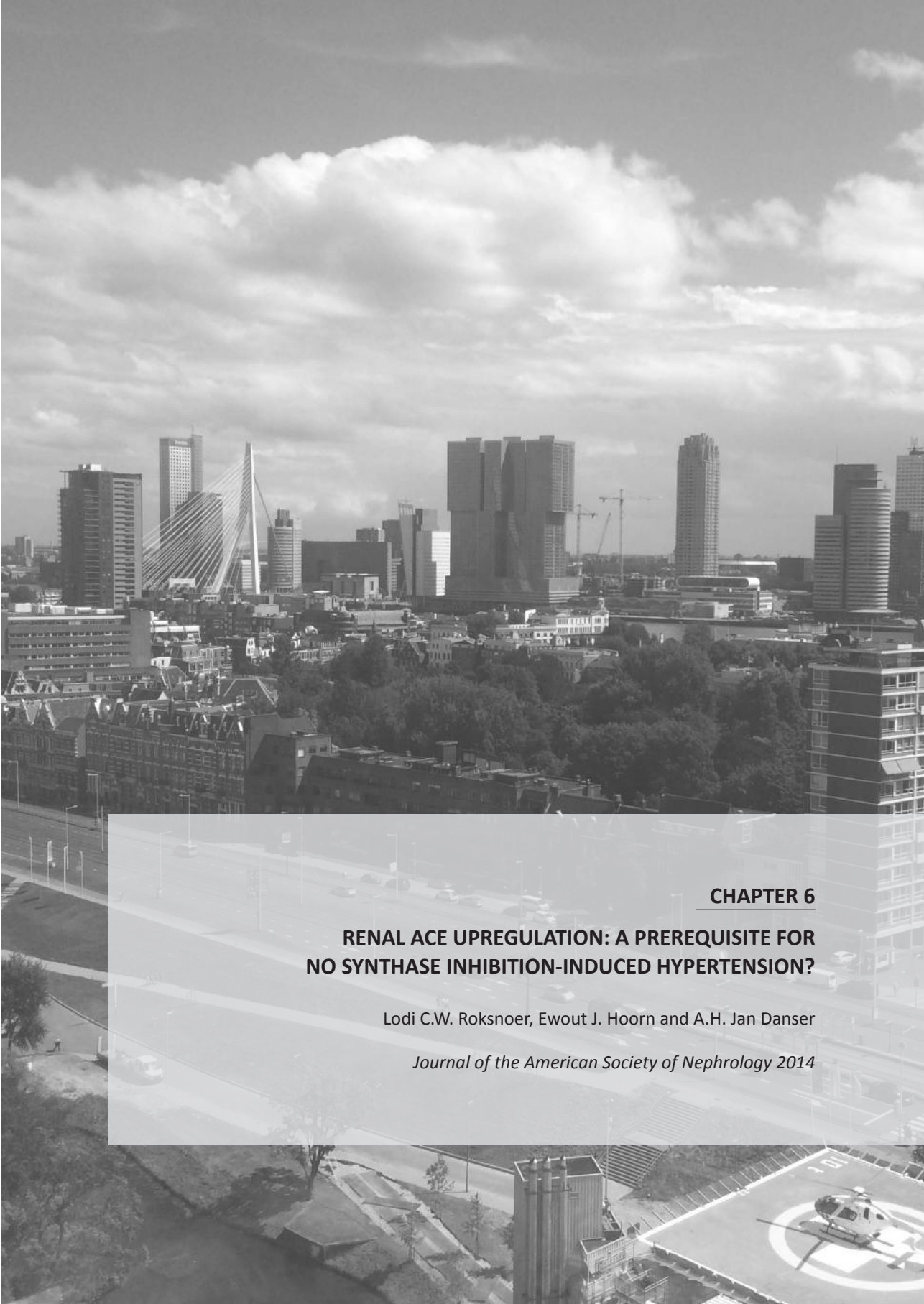

CHAPTER 6

RENAL ACE UPREGULATION: A PREREQUISITE FOR NO SYNTHASE INHIBITION-INDUCED HYPERTENSION?

Lodi C.W. Roksnoer, Ewout J. Hoorn and A.H. Jan Danser

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Angiotensin II (Ang II) production at tissue sites is well-established. Interference with such local generation, rather than with Ang II generation in the circulation, is believed to underlie the beneficial cardiovascular and renal effects of renin-angiotensin system (RAS) blockers. Infusion studies with ^{125}I-Ang I and II, allowing the quantification of tissue uptake of circulating angiotensins, have unequivocally confirmed that the majority of tissue Ang II is not derived from blood but is of local origin.[1] For instance, in the kidney, >95% of tissue Ang II is generated at renal tissue sites from locally synthesized Ang I. This generation depends on renal angiotensin-converting enzyme (ACE), and not chymase, as evidenced by ACE knockout studies and studies with ACE inhibitors.[1, 2] Initially, it was thought that the angiotensinogen required for this local production was also kidney derived because angiotensinogen production had been observed in the proximal straight tubule.[3, 4] However, elegant studies by Matsusaka et al. selectively knocking out angiotensinogen synthesis in the kidney or liver revealed that only hepatic deletion affected renal Ang II, both under normal and pathological conditions.[5, 6] Apparently, therefore, kidney-derived angiotensinogen does not contribute to renal Ang II production and appears unconverted in urine. In humans, urinary angiotensinogen closely follows albumin excretion and is therefore exclusively plasma (i.e., liver-) derived.[7]

Despite their common source of angiotensinogen, circulating and renal Ang II production do not always run in parallel. For instance, in diabetics, plasma renin is low, and yet their renal plasma flow response to RAS blockade is greatly enhanced, suggesting an overactive intrarenal RAS.[8] The opposite occurs after treatment with very high doses of a renin inhibitor.[9] RAS blockers, by interfering with the negative feedback loop between Ang II and renin release, normally upregulate renin synthesis. Particularly after high doses this upregulation may be >100-fold.[9] Renin inhibitors selectively accumulate in renal tissue, and, therefore, after stopping treatment,[10] renal RAS suppression will continue, so that renin release stays high. At the same time the inhibitor starts to disappear from plasma, and thus insufficient renin inhibitor is around to block all renin molecules that continue to be released. As a consequence, plasma renin activity will increase, and extrarenal Ang II and aldosterone levels may even rise to levels above baseline.[9]

The hypertension occurring in animals during inhibition of nitric oxide synthase (NOS) with L-NG-nitroarginine methyl ester (L-NAME) is also believed to involve a discrepancy between the circulating and renal RAS.[11] This concept is based on the observation that RAS blockers lower blood pressure (BP) in this model, despite the fact that circulating renin is suppressed. This renin suppression, however, appears to be transient because chronic L-NAME treatment increases plasma renin levels.[12]

In this issue of JASN, Giani et al. report on the importance of renal ACE in the NOS inhibition model.[13] Their aim was to obtain further evidence for the independency of renal Ang II production (by renal ACE) as a determinant of hypertension. To this end, they used mice that, via targeted homologous recombination, expressed ACE only in myelomonocytic cells (ACE 10/10 mice). Such mice are phenotypically normal (i.e. they have a normal BP and display no renal abnormalities). In fact, according to the authors, the renal Ang II levels of these mice were similar to those of wild-type animals at baseline and remained unchanged during treatment with L-NAME, even though their renal ACE levels were reduced by 90% or more.[13, 14] This is a surprising finding that merits further discussion. The most logical explanation of these findings is that these mice, like humans during ACE inhibitor treatment, display increased renin levels. In humans the return of Ang II to baseline levels, despite ongoing ACE inhibition, is called Ang II escape. In case of 90% ACE inhibition, a 10-fold rise in renin is sufficient to achieve this, and as described above even renin rises of >100-fold are

feasible.[9]

Although the authors emphasize that renal ACE is completely absent in ACE10/10 mice, there appears residual renal ACE staining with immunoblot.[13] In addition, macrophages of ACE 10/10 mice have an upregulated ACE and produce more NO, thus potentially compensating for the absence of renal ACE.[15]

Importantly, Giani et al. demonstrate that NOS inhibition in the low-renal ACE mice does not result in hypertension, cardiac hypertrophy, or proteinuria. In addition, L-NAME did not lead to the acute reduction in GFR or sodium retention that was observed in wild-type mice. If anything, they displayed an acute natriuresis and no change in GFR. The authors meticulously studied all relevant sodium transporters, including the sodium hydrogen exchanger, sodium potassium chloride cotransporter, sodium chloride cotransporter, and the epithelial sodium channel. Although the natriuresis in the ACE 10/10 mice occurred during the first week of L-NAME treatment, most transporters still showed a greater downregulation after 4 weeks of L-NAME. The authors attribute this to fact that the wild-type mice, unlike the ACE 10/10 mice, displayed a rise in renal Ang II after L-NAME. Such a global effect on sodium transporters is uncommon and intriguing, but the proposed model of tubular Ang II affecting sodium transporters via apical AT_1 receptors requires experimental proof.[14] The authors assume the rise in renal Ang II to be due to the approximate 2-fold rises in both renal ACE and angiotensinogen, which did not occur in the ACE 10/10 mice. Surprisingly, however, Giani et al. did not study renal renin expression, which usually displays much larger rises (as discussed above) than the modest rises observed here for ACE and angiotensinogen. On this basis, renin is actually more likely to determine the changes in (renal) Ang II levels. Indeed, a study of the ACE insertion/deletion polymorphism in humans observed that the 60-70% higher tissue ACE levels in DD participants versus II participants had no effect whatsoever on renin or Ang II.[16] This implies that normal ACE levels are non-rate-limiting. The doubling of angiotensinogen in wild-type mice is in full agreement with the doubling of proteinuria after L-NAME and supports the enhanced leakage of circulating angiotensinogen from plasma proposed by Matsusaka et al. as the source of increased renal Ang II generation.[5, 6]

Of interest, renal angiotensinogen in the ACE 10/10 mice was lower than in wild-type mice. This is also suggestive for the upregulation of renin that has most likely occurred in these mice to overcome the consequences of >90% ACE disappearance. Here it should be noted that in humans the levels of angiotensinogen are within the range of its K_m, while in mice they are much lower. Thus, in mice, much more than in humans, fluctuations in angiotensinogen levels have immediate consequences for the degree of angiotensin generation. An alternative explanation of the data is therefore that the ACE 10/10 mice already used their maximum capacity to normalize renal Ang II at the expense of angiotensinogen and were unable to increase Ang II even further after L-NAME. Moreover, NO was recently demonstrated to be of vital importance for the recruitment of renin-expressing cells along preglomerular vessels[17] (i.e., the usual site of renin cell upregulation during ACE inhibition). In other words, L-NAME might have interfered with the delicate balance in the ACE 10/10 mice that allowed the restoration of the renal Ang II levels. From this point of view it would have been no surprise if the ACE 10/10 mice had shown no change in renin or even a renin decrease after treatment with L-NAME. To partially address this point, the authors measured total renin in plasma. Unfortunately, this measurement involved the simultaneous detection of prorenin, the inactive precursor of renin, and thus no clear conclusions can be drawn on the actual changes in plasma renin. The variation in total renin is much larger in the ACE 10/10 mice, and although neither BP nor total renin levels significantly decreased after L-NAME treatment, total renin levels did correlate with delta systolic BP in these animals. This is

difficult to understand and might imply that BP in these animals is more renin-dependent, again supporting the renin upregulation in this model.

In summary, the impressive studies by Giani et al. confirm the importance of renal Ang II upregulation for the hypertensive effects after L-NAME infusion. This obviously depends on ACE, as all Ang II generation does, but to what degree renal ACE - rather than renin upregulation - is the permissive factor cannot yet be said. The ACE 10/10 model most likely is a high-renin model, at least in the kidney, and may thus be less responsive to agents that induce hypertension by inducing renal renin expression. An important question is why NOS inhibition would increase renal Ang II at all. The answer may lie in the complicated consequences of non-selective NOS inhibition, affecting endothelial, inducible and neuronal NOS simultaneously, thereby reducing not only the effect of NO on renin release and renin cell recruitment but also its capacity to suppress the sympathetic nervous system.[11, 12] The sympathetic nervous system interacts at various levels with the RAS, for example by increasing renin release, but also directly with kidney sodium transport.[18] Therefore, to fully understand these issues, we need to know not only the changes in renal and plasma renin but also the degree of sympathetic nervous system activation in this model.

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PART II

CHAPTER 7

OPTIMUM AT_1 RECEPTOR-NEPRILYSIN INHIBITION HAS SUPERIOR CARDIOPROTECTIVE EFFECTS COMPARED WITH AT_1 RECEPTOR BLOCKADE ALONE IN HYPERTENSIVE RATS

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Abstract
Neprilysin inhibitors prevent the breakdown of bradykinin and natriuretic peptides, promoting vasodilation and natriuresis. However, they also increase angiotensin and endothelin-1. Here we studied the effects of a low and a high dose of the neprilysin inhibitor thiorphan on top of AT_1 receptor blockade with irbesartan versus vehicle, in TGR(mREN2)27 rats with high renin hypertension. Mean arterial blood pressure was unaffected by vehicle or thiorphan alone. Irbesartan lowered blood pressure, but after 7 days pressure started to increase again. Low- but not high-dose thiorphan prevented this rise. Only during exposure to low-dose thiorphan plus irbesartan did heart weight/body weight ratio, cardiac atrial natriuretic peptide expression and myocyte size decrease significantly. Circulating endothelin-1 was not affected by low-dose thiorphan with or without irbesartan, but increased after treatment with high-dose thiorphan plus irbesartan. This endothelin-1 rise was accompanied by an increase in renal sodium–hydrogen exchanger 3 protein abundance, and an upregulation of constrictor vascular endothelin type B receptors. Consequently, the endothelin type B receptor antagonist BQ788 no longer enhanced endothelin-1-induced vasoconstriction (indicative of endothelin type B receptor-mediated vasodilation), but prevented it. Thus, optimal neprilysin inhibitor dosing reveals additional cardioprotective effects on top of AT_1 receptor blockade in renin-dependent hypertension.

Introduction
Renin-angiotensin-system (RAS) blockers are the cornerstone in the treatment of hypertension, heart failure and proteinuric chronic kidney disease. Yet, morbidity and mortality of these diseases remain high, despite RAS blocker treatment. Initially, it was thought that this related to incomplete RAS blockade, for example, because of renin upregulation and/or non-ACE-mediated angiotensin II (Ang II) formation. However, trials evaluating dual RAS blockade to achieve near-complete suppression revealed an increased risk of adverse events (including hypotension, hyperkalemia, and acute kidney injury) without additional benefit.[1] Therefore, we need new therapeutic strategies.

The natriuretic peptide system normally counterbalances the RAS, so that enhancing the activity of this system on top of RAS blockade might be beneficial.[2] Neutral endopeptidase (NEP), also known as neprilysin, plays an important role in the degradation of the three currently known natriuretic peptides, that is, atrial, brain and C-type natriuretic peptide (ANP, BNP and CNP), with the least susceptibility to degradation for BNP.[3] These peptides stimulate diuresis, natriuresis, and vasodilation. Their second messenger cyclic guanosine 3'5' monophosphate (cGMP) improves myocardial relaxation and reduces hypertrophy. Yet, NEP additionally degrades the vasodilator bradykinin, and the constrictors endothelin-1 (ET-1), and Ang II, and ET-1 rises have been observed during NEP inhibition in humans.[4, 5] Consequently, NEP inhibition may even lead to an increase in blood pressure. Combined RAS/NEP inhibition is less likely to cause this problem. Indeed, compared with ACE inhibition alone, the combined ACE/NEP inhibitor omapatrilat showed superior effects on blood pressure, left ventricular function, and combined mortality/morbidity endpoints in patients with congestive heart failure.[6, 7] Unfortunately, omapatrilat often caused angioedema, which might have been expected on the basis of the elevated bradykinin levels occurring when the two major bradykinin-degrading enzymes NEP and ACE are being blocked.[8, 9] A better approach would therefore be to combine an Ang II type 1 (AT_1) receptor blocker with a NEP Inhibitor (ARNI). The first ARNI that has been tested clinically, LCZ696, consisted of the NEP inhibitor prodrug AHU377 and the AT_1 receptor blocker valsartan. LCZ696 was superior

to valsartan in the treatment of hypertension,[10] and also to the ACE inhibitor enalapril in the treatment of heart failure.[11] Preclinically, the combination of valsartan and the NEP inhibitor CGS25354 was almost as effective as a dual ACE/NEP inhibitor in lowering blood pressure and improving vascular remodeling in stroke-prone spontaneously hypertensive rats.[12] However, it is currently unknown how much NEP inhibition on top of AT_1 receptor blockade is optimal. This question is especially relevant because NEP inhibition can increase ET-1.[4, 5] ET-1 not only is a potent vasoconstrictor, but also affects kidney sodium handling. ET-1 increases the sodium hydrogen exchanger type 3 (NHE3) in the proximal tubule and decreases the epithelial sodium channel (ENaC) in the distal tubule; both effects are mediated through the ET_B receptor.[13-15]

In the present study, we hypothesized that too much NEP inhibition might be detrimental. We compared single NEP inhibition (thiorphan) and AT_1 receptor blockade (irbesartan) versus the ARNI approach (thiorphan + irbesartan), applying both a low and a high thiorphan dose.[16] Studies were performed in heterozygous TGR(mREN2)27 (Ren2) rats. These rats, by overexpressing the mouse Ren2 gene, display severe Ang II-dependent hypertension, myocardial hypertrophy and vascular remodelling,[17, 18] and have an activated natriuretic peptide system.[19] In view of the potential consequences of NEP inhibition, as described above, we focused on blood pressure, natriuresis, diuresis, cardiac hypertrophy, and vascular reactivity. Additionally, to obtain a better understanding of the biochemical consequences of ARNI, we quantified its effects on the RAS, ANP, cGMP and ET-1. Finally, we evaluated whether ARNI affects well-known Ang II and ET-1 targets, like vascular ET receptors and kidney sodium transporters, as changes therein, on top of changes in RAS, ANP, cGMP, and ET-1, may underlie the hemodynamic and renal effects of ARNI.

Results

Best antihypertensive response with low-dose ARNI

Neither vehicle nor thiorphan alone affected mean arterial pressure (MAP; *Figure 1*). Irbesartan, either alone or combined with the low or high dose of thiorphan, did lower MAP

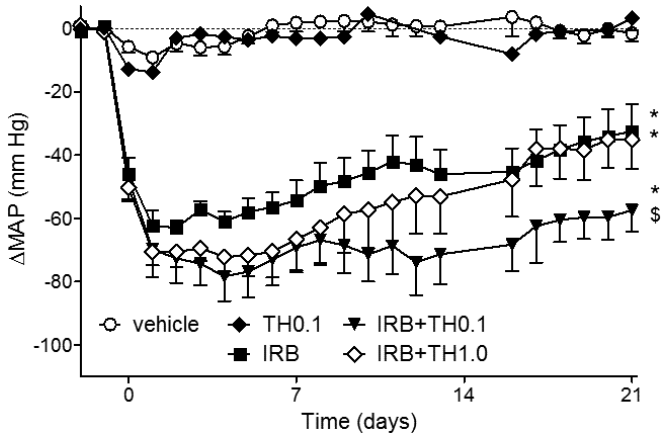

Figure 1. Change in mean arterial pressure (ΔMAP) during a three-week infusion of vehicle, thiorphan 0.1 mg/kg per day (TH0.1), irbesartan (IRB), irbesartan+thiorphan 0.1 mg/kg per day (IRB+TH0.1), or irbesartan+thiorphan 1.0 mg/kg per day (IRB+TH1.0) in Ren2 rats. Baseline MAP was 172 ± 3mmHg. Data are mean ± s.e.m. of eight rats. *P<0.05 vs. vehicle, $P<0.05 vs. irbesartan.

compared with vehicle (*Figure 1*), with no effect on heart rate (363 ± 5, 347 ± 5, 342 ± 3, 348 ± 4 and 348 ± 3 beats per minute after three weeks of treatment with vehicle, thiorphan, irbesartan, irbesartan + low-dose thiorphan, and irbesartan + high-dose thiorphan, respectively). The effects of irbesartan alone on MAP were significant (P<0.01) already on the first treatment day, and reached a maximum around days one to four. Thereafter, MAP slowly started to rise again, although it was still ~30 mm Hg below the MAP of vehicle-treated rats after three weeks. This rise was prevented by the low but not by the high dose of thiorphan.

Urinary volume increased during vehicle treatment, while no such increase was observed during any of the drug treatments (*Table 1*). Changes in urinary volume paralleled those in water intake. Body weight, kidney weight and food intake were not affected by any of the treatments (*Table 1*).

Table 1. Main characteristics in Ren2 rats treated with vehicle, TH0.1, IRB, IRB+TH0.1, or IRB+TH1.0

Abbreviations: BW, body weight; cGMP, cyclic guanosine 3'5' monophosphate; HW, heart weight; IRB, irbesartan; IRB+TH0.1, irbesartan+thiorphan 0.1mg/kg per day; IRB+TH1.0, irbesartan+thiorphan 1.0mg/kg per day; KW, kidney weight; TH0.1, thiorphan 0.1 mg/kg per day. Urine was collected on days 0 and 21 and plasma was collected on day 21. Data are mean ± s.e.m. of n=8. #P<0.05 vs. baseline; *P<0.05 vs. vehicle.

Parameter	Baseline	Vehicle	TH0.1	IRB	IRB+TH0.1	IRB+TH1.0
BW (g)	-	558 ± 12	553 ± 9	554 ± 10	561 ± 21	560 ± 13
HW (g)	-	2.2 ± 0.06	2.1 ± 0.04	2.0 ± 0.08	1.8 ± 0.08*	2.0 ± 0.1
HW/tibia length	-	0.50 ± 0.01	0.49 ± 0.01	0.46 ± 0.02	0.43 ± 0.02*	0.48 ± 0.02
HW/BW (g/kg)	-	4.0 ± 0.1	3.8 ± 0.1	3.6 ± 0.2	3.3 ± 0.1*	3.7 ± 0.2
KW (g)	-	1.6 ± 0.03	1.3 ± 0.04	1.5 ± 0.03	1.5 ± 0.08	1.5 ± 0.04
KW/BW (g/kg)	-	2.9 ± 0.06	2.9 ± 0.04	2.7 ± 0.07	2.7 ± 0.1	2.7 ± 0.04
Water intake (mL per day)	40 ± 2.2	51 ± 7.7	46 ± 1.5	28 ± 3.3*	28 ± 2.5*	34.4 ± 3.5
Food intake (g per day)	22 ± 0.9	21 ± 1.8	22 ± 1.3	21 ± 3.0	18 ± 2.7	23 ± 2.0
Plasma						
Creatinine	-	42 ± 1.8	33 ± 1.7	40 ± 1.9	37 ± 1.8	43 ± 1.6
Creatinine clearance (mL/min)	-	3.0 ± 0.2	3.4 ± 0.2	3.0 ± 0.1	3.4 ± 0.4	2.7 ± 0.2
Na^+ (mmol/L)	-	141 ± 1.0	143 ± 1.0	142 ± 1.1	142 ± 1.3	142 ± 1.3
K^+ (mmol/L)	-	4.7 ± 0.3	4.8 ± 0.3	5.3 ± 0.4	5.0 ± 0.2	5.7 ± 0.2*
Urine						
Volume (mL per day)	23 ± 2	35 ± 5.0#	24 ± 2.3*	14 ± 0.9*	15 ± 0.9*	17 ± 2.3*
Protein (mg per day)	22 ± 2	26 ± 3	19 ± 2	14 ± 2*	12 ± 1*	15 ± 2*
Albumin (mg per day)	14 ± 3	19 ± 5	7.2 ± 1*	5.7 ± 2*	2.8 ± 2*	4.9 ± 2*
Aldosterone (ng per day)	41 ± 5	65 ± 13	27 ± 3*	14 ± 3*	11 ± 3*#	18 ± 6*
Na^+ (mmol per day)	1.6 ± 0.1	1.8 ± 0.2	2.3 ± 0.3#	1.3 ± 0.2	1.3 ± 0.1*	1.5 ± 0.1
K^+ (mmol per day)	3.4 ± 0.2	4.0 ± 0.3	3.3 ± 0.2	3.2 ± 0.2	3.2 ± 0.2	3.5 ± 0.2
Endothelin-1 (pg per day)	12 ± 1	9.8 ± 1.4	5.4 ± 0.8*#	8.5 ± 1.2	10.8 ± 1.1	10.5 ± 1.1
cGMP (nmol per day)	50 ± 3	57 ± 8	69 ± 4#	60 ± 4	53 ± 6	58 ± 10

High-dose ARNI increased endothelin-1

Urinary cGMP, which reflects the renal effects of ANP[20] and may thus serve as a marker of NEP inhibition efficacy, was increased in rats treated with thiorphan alone (*Table 1*). Yet, no increase was observed in the plasma levels of cGMP after thiorphan (*Figure 2A*). Irbesartan alone did not alter cGMP excretion or plasma cGMP, but prevented the rise in urinary cGMP excretion in combination with thiorphan (*Table 1*), and even lowered plasma cGMP in combination with the high thiorphan dose (*Figure 2A*). In agreement with the above concept,[20] changes in plasma ANP levels paralleled those in urinary cGMP excretion (*Figure 2B*).

The generation of ANP from proANP is accompanied by the appearance of proANP's N-terminal end, NT-proANP. Since the latter is not degraded by NEP, its levels should better reflect ANP expression during NEP inhibition than the levels of ANP. NT-proANP levels were lowest during irbesartan + low-dose thiorphan and increased versus this condition when irbesartan was combined with the high dose of thiorphan (*Figure 2C*).

Thiorphan alone decreased urinary ET-1 excretion (*Table 1*), without affecting plasma ET-1 levels (*Figure 2D*). Irbesartan, when given on top of thiorphan, normalized urinary ET-1 excretion, and increased plasma ET-1 when combined with the high thiorphan dose only (*Figure 2D*). Irbesartan alone did not affect circulating or urinary ET-1.

Irbesartan, with or without thiorphan, increased plasma renin two- to three-fold, although significance was reached only for the combination of irbesartan and the high thiorphan dose (*Figure 2E*). Thiorphan alone did not affect plasma renin. Plasma prorenin (1727 ± 117 pmol Ang I per mL per hr in vehicle-treated rats) was unaffected by all treatments

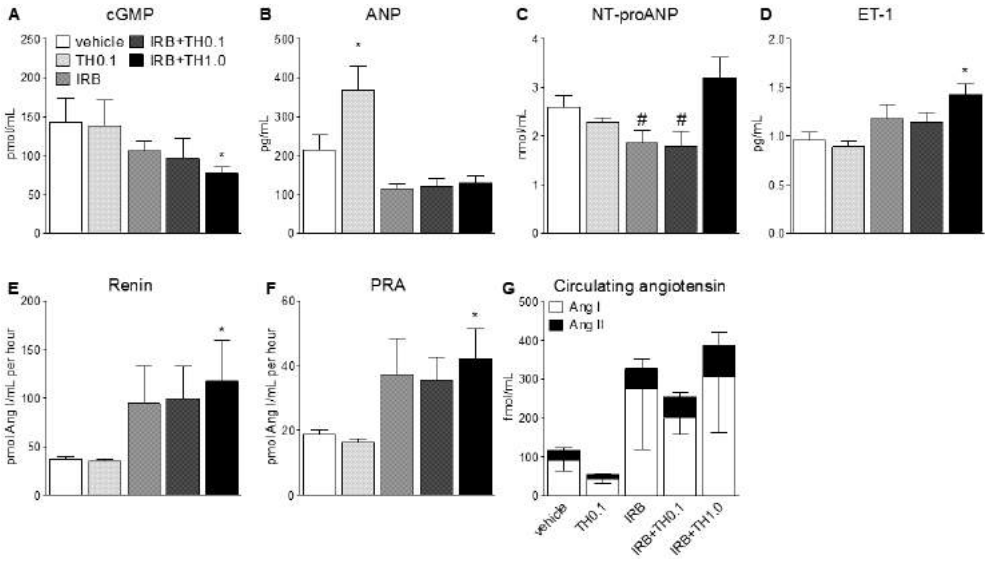

Figure 2. Plasma levels of (a) cyclic guanosine 3'5' monophosphate (cGMP), (b) atrial natriuretic peptide (ANP), (c) proANP N-terminal end (NT-proANP), (d) endothelin-1 (ET-1), (e) renin, and (g) angiotensin I+II (Ang I+II) versus (f) plasma renin activity (PRA) in Ren2 rats treated for three weeks with vehicle, thiorphan 0.1 mg/kg per day (TH0.1), irbesartan (IRB), irbesartan+thiorphan 0.1 mg/kg per day (IRB+TH0.1), or irbesartan+thiorphan 1.0 mg/kg per day (IRB+TH1.0). Data are mean ± s.e.m. of n=8. *P<0.05 vs. vehicle, #P<0.05 vs. IRB+TH1.0.

(2250 ± 176, 1710 ± 170, 1534 ± 145, and 1893 ± 129 pmol Ang I per mL per hr after treatment with thiorphan, irbesartan, irbesartan + low-dose thiorphan, and irbesartan + high-dose thiorphan, respectively). Changes in plasma renin activity (PRA), Ang I, and Ang II paralleled those in plasma renin (*Figures 2F and 2G*), and the same was true for the changes in the renal tissue levels of Ang I, Ang-(1-9), Ang-(1-7), Ang-(1-5), and Ang-(2-10) (*Table 2*). Renal Ang II, Ang-(2-8), and Ang-(3-8). levels were largely unaltered and/or tended to decrease in the irbesartan-treated rats. As a consequence, the renal Ang II/I ratio decreased (in agreement with the concept that, after AT_1 receptor blockade, Ang II can no longer accumulate in renal tissue via AT_1 receptor binding; van Esch et al.[21]), the renal Ang-(1-7)/Ang II ratio increased, whereas the renal Ang-(1-7)/Ang I ratio was unchanged after irbesartan (*Table 2*).

As expected, irbesartan decreased urinary aldosterone excretion versus baseline (*Table 1*), both with and without thiorphan, although significance for this effect was reached only in the low thiorphan group. This decrease in aldosterone was accompanied by a decrease in urinary Na^+ excretion and a rise in plasma K^+ levels (*Table 1*). Plasma Na^+ levels and urinary K^+ excretion were unchanged after irbesartan with or without thiorphan (*Table 1*). Of interest, thiorphan alone increased natriuresis, without altering urinary K^+ or circulating Na^+ and K^+ (*Table 1*). Thiorphan alone did not lower aldosterone versus baseline (*Table 1*), although it did lower aldosterone excretion versus vehicle. The same was true for irbesartan under all conditions, and this is related to an unexpected rise in aldosterone excretion in the vehicle-treated group (*Table 1*).

Vehicle treatment did not alter urinary protein and albumin excretion (*Table 1*). Irbesartan plus the low dose of thiorphan tended to decrease urinary albumin compared with baseline, but this was not significant. Thiorphan and irbesartan, both alone and in combination, lowered urinary albumin excretion compared with vehicle, whereas only irbesartan, independent of its combination with thiorphan, lowered urinary protein excretion (*Table 1*). There were no significant changes in plasma creatinine and creatinine clearance (*Table 1*).

Table 2. Renal levels of angiotensin metabolites and their ratios in kidneys of Ren2 rats treated with vehicle, TH0.1, IRB, IRB+TH0.1, or IRB+TH1.0. Abbreviations: Ang, angiotensin; IRB, irbesartan; IRB+TH0.1, irbesartan+thiorphan 0.1mg/kg per day; IRB+TH1.0, irbesartan+thiorphan 1.0mg/kg per day; TH0.1, thiorphan 0.1mg/kg per day. Data are mean ± s.e.m. of n=5–8.*P<0.05 vs. vehicle.

RAS metabolite (pg/g)	Vehicle	TH0.1	IRB	IRB+TH0.1	IRB+TH1.0
Ang-(1-10) = (Ang I)	163 ± 69	79 ± 16	370 ± 154	568 ± 66	627 ± 206
Ang-(1-8) = (Ang II)	388 ± 36	337 ± 18	288 ± 65	289 ± 44	328 ± 47
Ang-(1-7)	20 ± 3	18 ± 2	48 ± 20	62 ± 22	72 ± 33
Ang-(1-5)	13 ± 4	9 ± 1	16 ± 5	23 ± 4	22 ± 7
Ang-(2-8)	26 ± 3	18 ± 2	30 ± 12	30 ± 10	32 ± 8
Ang-(3-8)	5 ± 0.3	5 ± 0.1	5 ± 0.8	5 ± 0.4	6 ± 0.6
Ang-(2-10)	30 ± 15	16 ± 2.3	89 ± 45	123 ± 23	131 ± 46
Ang-(1-9)	43 ± 12	20 ± 3	38 ± 12	57 ± 16	63 ± 12
Ang-(1-7)/Ang I (x100)	18 ± 3	27 ± 4	16 ± 2	10 ± 3	13 ± 3
Ang-(1-7)/Ang II (x100)	5 ± 1	5 ± 0.5	14 ± 3	20 ± 5*	20 ± 6*
Ang II/Ang I (x100)	437 ± 109	527 ± 87	191 ± 66	51 ± 5*	137 ± 61*

Differential effects of ARNI on NHE3 and ENaC, but no effect on kidney pathology

Irbesartan plus the high thiorphan dose increased the abundance of NHE3 more than twofold (*Figure 3*). In contrast, irbesartan alone or irbesartan plus the low thiorphan dose increased the abundances of α- and γ-ENaC almost twofold, but had no significant effect on NHE3. Aquaporin-2 (AQP2) increased in all rats treated with irbesartan, although this did not reach significance for the group treated with irbesartan plus the low dose of thiorphan. Urine osmolality changes paralleled the AQP2 increases (*Figure 3*). No differences were found in the abundances of the other kidney sodium transporters (Na-K-Cl-cotransporter

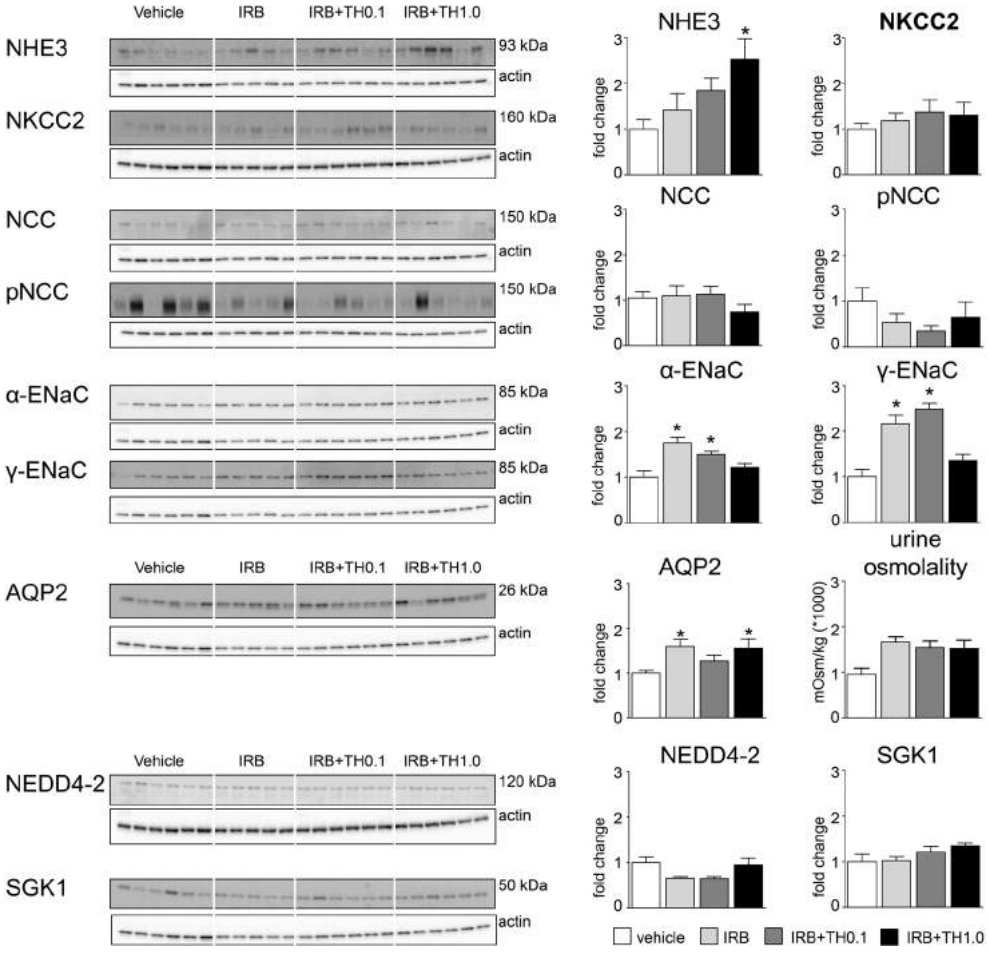

Figure 3. Protein expression of renal sodium transporters (α-epithelial sodium channel (α-ENaC), γ-ENaC, sodium-hydrogen exchanger type 3 (NHE3), Na-Cl cotransporter (NCC), NCC phosphorylated at Thr58 and Na-K-Cl cotransporter (NKCC2)), aquaporin-2 (AQP2), neural precursor cell expressed, developmentally downregulated protein 4-2 (NEDD4-2), and serum- and glucocorticoid-regulated kinase 1 (SGK1) in kidneys and urine osmolality of Ren2 rats treated for three weeks with vehicle, irbesartan (IRB), irbesartan+thiorphan 0.1 mg/kg per day (IRB+TH0.1), or irbesartan+thiorphan 1.0 mg/kg per day (IRB+TH1.0). Data are mean ± s.e.m. of n=5–6. *P<0.05 vs. vehicle.

(NKCC2) and Na-Cl cotransporter (NCC)) or the aldosterone-sensitive proteins ubiquitin ligase NEDD4-2 (neural precursor cell expressed, developmentally downregulated protein 4-2) and SGK1 (serum- and glucocorticoid-regulated kinase 1).
Focal and segmental glomerulosclerosis were only minimally present in the kidneys of the Ren2 rats, and the same was true for tubular injury (*Supplementary Figure S1*). No significant effects of the various drug combinations on these parameters were seen.

High-dose ARNI impairs vasodilation
In vehicle-treated Ren2 rats, acetylcholine (ACh) fully relaxed preconstricted mesenteric arteries. This relaxation is due to NO generation by NO synthase and intermediate+small conductance Ca^{2+}-activated K^{+}-channel by endothelium-derived hyperpolarizing factor(s) (EDHF). Indeed, the NO synthase inhibitor L-NAME, but not combined intermediate+small conductance Ca^{2+}-activated K^{+}-channel inhibition with TRAM34+apamin, partially blocked this effect, whereas all inhibitors together fully blocked the effect of ACh (*Figure 4A*). Results in all thiorphan-treated groups were identical to those in vehicle-treated rats, whereas only in the rats treated with irbesartan alone, TRAM34+apamin significantly shifted the ACh concentration-response curve (CRC) to the right and decreased Emax compared to vehicle-treated rats (pEC50 (the negative logarithm of the half-maximal effective concentration) 7.6 ± 0.2 versus 8.4 ± 0.2 and Emax 73 ± 8.0% versus 91 ± 1.4%, respectively; $P<0.05$ for both). Thus, irbesartan upregulated the EDHF component of the ACh-induced relaxation, and thiorphan reversed this effect. The endothelium-independent vasodilator S-nitroso-N-penicillamine, like ACh, fully relaxed preconstricted mesenteric arteries obtained from vehicle-treated rats, and this effect was unchanged in al treatment groups (*Supplementary Figure S2*).

ET-1 potently constricted mesenteric arteries of vehicle-treated Ren2 rats (pEC50 8.4 ± 0.2; *Figure 4B*), and the ET_A receptor antagonist BQ123 (pEC50 7.8 ± 0.1) prevented this effect. Similar results were obtained after all drug treatments. Simultaneously, the ET_B receptor antagonist BQ788 marginally shifted the ET-1 CRC to the left in all treatment groups, except in the rats exposed to irbesartan plus the high dose of thiorphan, where the Emax after BQ788 was significantly lower than in the vehicle-treated group (142±8.4% versus 202 ± 18.4%, respectively; $P<0.05$). These data suggest that NEP inhibition, at its highest dose, abolished the vasodilatory effect of the ET_B receptor.

Thiorphan alone, but none of the other treatments, upregulated vascular ET_A receptor mRNA expression (*Figure 5A*). A similar upregulation was observed for the vascular ET_B receptor mRNA expression in all thiorphan-exposed rats, although this was significant only in the rats receiving irbesartan plus the highest thiorphan dose (*Figure 5B*). Combined with the absence of ET_B receptor-induced vasodilation in this treatment group, these data suggest that the upregulation concerns constrictor ET_B receptors.

Low-dose ARNI reduces heart weight
Only Ren2 rats treated with irbesartan plus the low thiorphan dose displayed a reduced heart weight (HW), HW/body weight ratio, and HW/tibia length ratio (*Table 1*). Cardiac ANP expression and cardiomyocyte size paralleled these changes (*Figures 5C and 5E*, see *Supplementary Figure S3* for representative images), whereas cardiac β-MHC expression was unaltered (*Figure 5D*). Unexpectedly, irbesartan increased the degree of cardiac fibrosis, while cardiac fibrosis in all other treatment groups was unaltered (*Figure 5F*, see *Supplementary Figure S3* for representative images). HW correlated positively with MAP (*Supplementary Figure S4*).

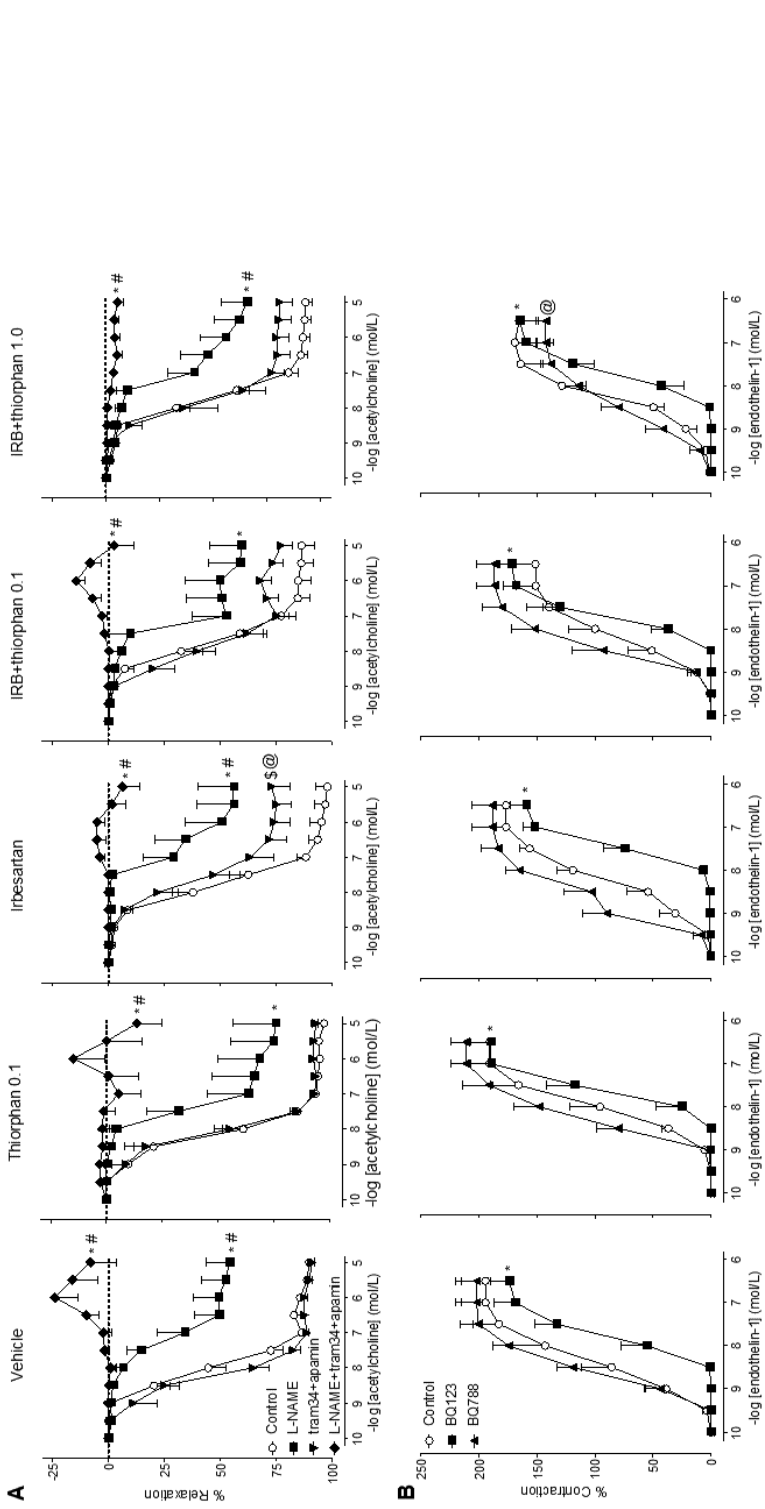

Figure 4. Vascular responses to acetylcholine and endothelin-1. (a) Relaxations to acetylcholine and (b) contractions to endothelin-1 in mesenteric arteries from Ren2 rats treated for three weeks with vehicle, thiorphan 0.1 mg/kg per day (TH0.1), irbesartan (IRB), irbesartan+thiorphan 0.1 mg/kg per day (IRB+TH0.1), or irbesartan+thiorphan 1.0 mg/kg per day (IRB+TH1.0). Relaxations to acetylcholine were studied in the absence (control) or presence of L-NAME (Nω-nitro-L-arginine methyl ester hydrochloride), TRAM34+apamin, or L-NAME+TRAM34+apamin. Contractions to endothelin-1 were studied in the absence (control) or presence of BQ123 or BQ788. Relaxations are expressed as % reduction of preconstriction (with U46619). Contractions are expressed as a percentage of the response to 100 mmol/l K^+. Data are mean ± s.e.m. of n=8. *P<0.05 pEC50 (the negative logarithm of the half-maximal effective concentration) vs. control, $P<0.05 pEC50 vs. vehicle, #P<0.05 Emax vs. control, and @P<0.05 Emax vs. vehicle.

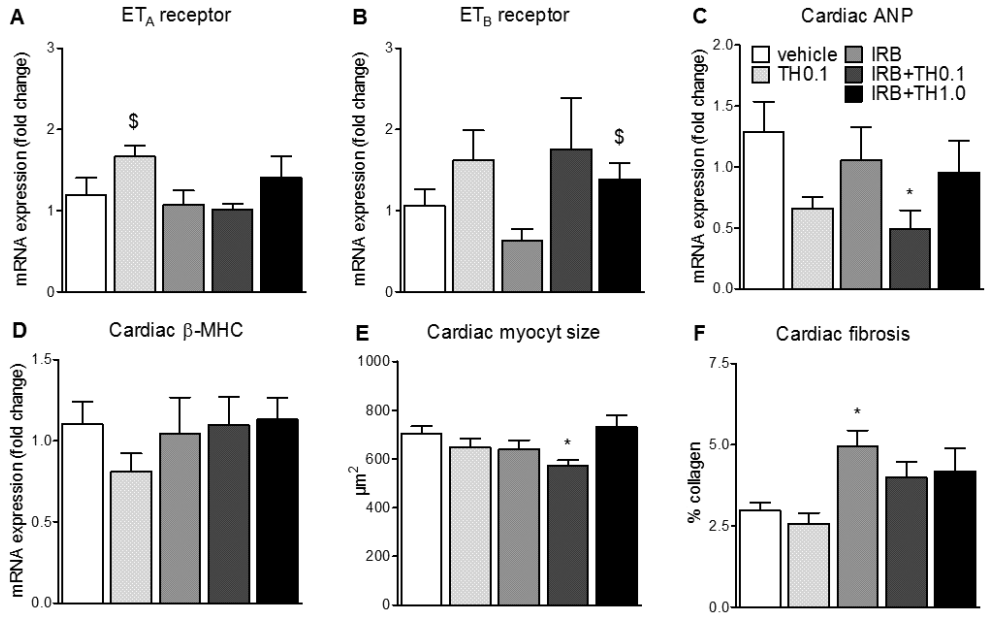

Figure 5. Vascular and cardiac gene expression and cardiac myocyte size and cardiac fibrosis. (a) Endothelin type A (ET_A) and (b) endothelin type B (ET_B) receptor mRNA expression in mesenteric artery segments versus (c) cardiac atrial natriuretic peptide (ANP) and (d) β-myosin heavy chain (β-MHC) expression, (e) cardiomyocyte size, and (f) cardiac fibrotic area in left ventricular tissue from Ren2 rats treated for three weeks with vehicle, thiorphan 0.1 mg/kg per day (TH0.1), irbesartan (IRB), irbesartan+thiorphan 0.1 mg/kg per day (IRB+TH0.1), or irbesartan+thiorphan 1.0 mg/kg per day (IRB+TH1.0). Data are mean ± s.e.m. of n=8. *P<0.05 vs. vehicle and $P<0.05 vs. irbesartan.

Discussion

This study shows that NEP inhibition has favorable effects on top of AT_1 receptor blockade, but that this approach has a limit, that is, these effects disappear when increasing the degree of NEP inhibition. Indeed, a low dose of the NEP inhibitor thiorphan (0.1 mg/kg.day) potentiated the blood pressure-lowering effects of irbesartan in Ren2 rats, and this was accompanied by a significant reduction in cardiac hypertrophy, cardiomyocyte size, cardiac ANP expression, and vascular EDHF release. Increasing the thiorphan dose 10-fold abolished these effects. This most likely relates to the rise in circulating ET-1 with subsequent effects on kidney NHE3 abundance and vascular ET_B receptor expression that occurred at such high doses, in agreement with previous literature.[22, 23] *Figure 6* summarizes these observations.

Thiorphan, when given alone at a dose of 0.1 mg/kg.min, did not affect blood pressure or cardiac hypertrophy. This is in full agreement with previous studies showing no such effects of NEP inhibition in human hypertension.[24] Nevertheless, at this dose, thiorphan did increase circulating ANP, urinary cGMP, and natriuresis, without affecting circulating cGMP. These data confirm that urinary cGMP is largely of renal cellular origin, and correlates with ANP-induced natriuresis.[20] The latter effects of thiorphan were not seen on top of irbesartan, most likely because irbesartan, via its blood pressure-lowering effects, reduced atrial stretch, and thus diminished ANP release. Indeed, in combination with the high thiorphan dose, this even resulted in a significant decrease in circulating cGMP. Given the physiological antagonism between cGMP and ET-1, for example, with regard to cardiac hypertrophy,[25]

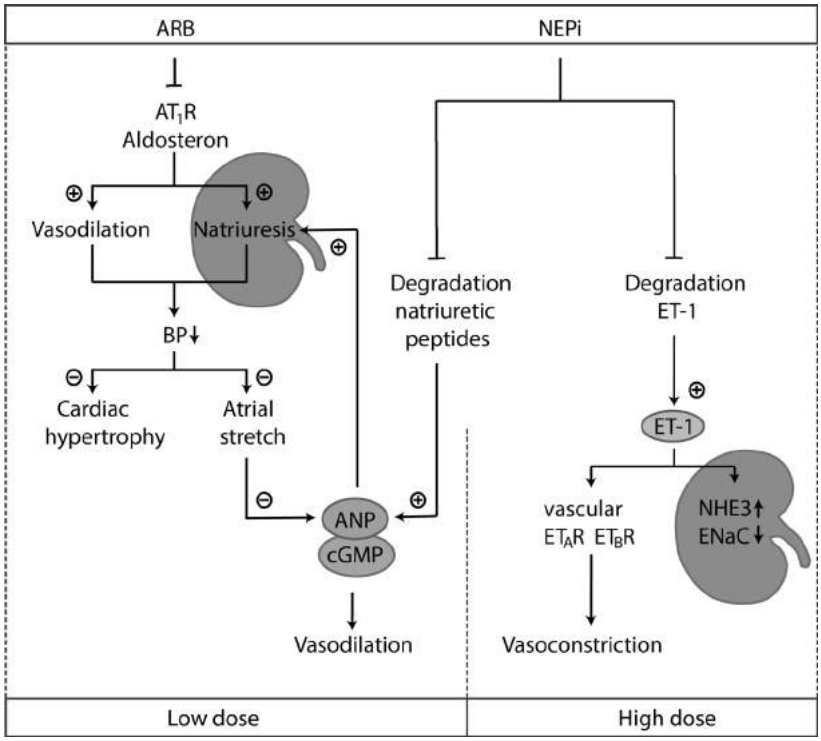

Figure 6. Schematic overview of the main findings on Ang II type 1 receptor (AT_1R) blocker (ARB) with an NEP inhibitor (ARNI) treatment in Ren2 rats with the differential responses to the combination of the ARB irbesartan with either a low or a high dose of the neutral endopeptidase (NEP) inhibitor (NEPi) thiorphan. See text for further explanation; red represents blocking, and black the consequences (of such blocking). ANP, atrial natriuretic peptide; BP, blood pressure; cGMP, cyclic guanosine 3'5' monophosphate; ET-1, endothelin-1; ET_AR, endothelin type A receptor; ET_BR, endothelin type B receptor; ENaC, epithelial sodium channel; NHE3, sodium-hydrogen exchanger type 3.

this phenomenon may underlie the absence of favorable cardiac effects when combining irbesartan with the high thiorphan dose.

Clinical trials evaluating the effects of the ARNI approach in heart failure (making use of LCZ696) observed a reduction in the plasma levels of NT-proBNP and a rise in BNP, despite the fact that both peptides are generated in equal amounts from proBNP.[26] The authors speculated that the decrease in NT-proBNP (which is not degraded by neprilysin) reflects reduced cardiac wall stress, whereas the rise in BNP reflects NEP inhibition. In the present study, we focused on ANP, given its greater susceptibility to degradation by NEP as compared with BNP.[3] In agreement with the above concept, plasma NT-proANP levels better reflected cardiac ANP expression than plasma ANP levels (see *Figures 2C and 5C*): rats receiving the high dose of thiorphan displayed higher ANP expression (due to their higher blood pressure and cardiac wall stress) and NT-proANP levels than rats receiving the low dose. To explain why plasma ANP levels did not increase in parallel with NT-proANP in the high-dose group, where in fact an even higher increase was expected because not only blood pressure increased but also NEP was inhibited, it must be kept in mind that ANP is

not only degraded by neprilysin but also cleared via binding to natriuretic receptors, and via metabolism by alternative enzymes such as dipeptidyl peptidase-4.[3] As such, changes in ANP levels are the net consequence of alterations in release and clearance, and cannot be linked directly to cardiac ANP expression.

Beneficial cardiac effects of NEP inhibition are in agreement with the exaggerated cardiac hypertrophy in ANP-knockout mice exposed to volume or pressure overload.[27, 28] On the basis of our study, it cannot be said to what degree these effects were entirely blood pressure-independent. Cardiac β-MHC mRNA expression, a well-known indicator of pathological hypertrophy, was unaffected by treatment. However, whether β-MHC truly is an obligatory marker of cellular hypertrophy was recently questioned, its re-expression possibly being limited to specific cells.[29] Our approach of measuring β-MHC mRNA expression in whole left ventricular homogenates may therefore not have allowed the detection of regional changes. The degree of cardiac fibrosis in our model was still modest, and thus we were unable to demonstrate significant decreases of this parameter. If anything, irbesartan increased cardiac fibrosis, although in combination with thiorphan no such effects occurred. These data contrast with those obtained by Pu et al. in stroke-prone spontaneously hypertensive rats after ARNI treatment.[12] The fact that the latter authors did see anti-fibrotic effects most likely relates to the much higher baseline percentages of fibrosis in their model (up to 10%, as opposed to ~2% in this study).

NEP is one of many angiotensin-degrading enzymes.[30] Simultaneously, ANP, via its second messenger cGMP, inhibits renal renin release.[31] Thus, NEP inhibition, by increasing Ang II and ANP/cGMP, would be expected to suppress renin activity. However, no such decrease was seen when thiorphan was given alone, nor did thiorphan affect the irbesartan-induced RAS activation. This is most likely due to the fact that in our Ren2 model renin is not exclusively of renal origin. Moreover, in the kidney, thiorphan did not significantly alter the changes in angiotensin metabolites after irbesartan. This argues against a major role for NEP in renal angiotensin metabolism. As expected, irbesartan decreased urinary aldosterone, but only in the rats given irbesartan plus the high dose of thiorphan did this result in a rise in plasma potassium.

The irbesartan-induced decrease in aldosterone was accompanied by a paradoxical increase in the abundance of the aldosterone-sensitive sodium transporter ENaC. This suggests that aldosterone-independent, ENaC-regulating factors such as Ang II or vasopressin may have come into play.[32] Indeed, the increase in AQP2 and urine osmolality in the groups receiving irbesartan is suggestive for higher levels of vasopressin. The high dose of thiorphan abolished this rise in ENaC, most likely due to an overriding stimulatory effect on ET-1, which is known to suppress ENaC abundance via the ET_B receptor.[14] Similarly, the high ET-1 levels may explain the rise in kidney NHE3 abundance observed with the high thiorphan dose.[13] The latter would unfavorably affect kidney sodium handling, and may thus have contributed to the rise in blood pressure.[33] A cumulative sodium balance would have been necessary to analyse whether more sodium retention indeed occurred in the animals receiving the high thiorphan dose. Instead, we measured urinary sodium excretion after three weeks (new steady state) and found that it was lower in the groups with lower blood pressure, which likely reflects differences in pressure natriuresis. Increased sodium reabsorption by the proximal tubule in the high thiorphan group may also have reduced distal sodium delivery, thereby reducing kaliuresis and increasing plasma potassium. In contrast to ENaC, the aldosterone regulation of NCC appeared intact, because the abundances of pNCC and its regulatory protein NEDD4-2 followed a similar pattern as urinary aldosterone.[34]

Vascular endothelial function was still intact in our Ren2 rats. ACh-induced relaxation

involved both NO and EDHF, as illustrated by the partial blockade by the NO synthase inhibitor L-NAME, and the full blockade by L-NAME and TRAM34+apamin, the latter two being inhibitors of the intermediate and small conductance Ca^{2+}-activated K^+-channel, respectively. Nevertheless, in the absence of L-NAME, TRAM34+apamin were without effect, suggesting that NO can normally fully compensate for the absence of EDHF. This phenomenon is well-known.[35, 36] Irbesartan abolished this mechanism, confirming that AT_1 receptor blockade upregulates the EDHF component, as demonstrated before in hypertensive rats.[37, 38] Thiorphan reversed this effect.

The ET_A receptor blocker identically inhibited the ET-1-mediated constrictor responses in all treatment groups, demonstrating a major role for constrictor ET_A receptors under all conditions. The contribution of the ET_B receptor was less uniform. Normally, this receptor is located on the endothelium and induces relaxant effects. Indeed, in most cases a modest (non-significant) leftward shift of the ET-1 CRC was observed. Yet, after the high dose of thiorphan, this no longer occurred, and the ET_B receptor antagonist BQ788 now decreased the effect of ET-1, so that its Emax was significantly smaller than in mesenteric arteries obtained from vehicle-treated animals after BQ788 preincubation. Since this coincided with a rise in vascular ET_B receptor expression, it can be concluded that the upregulation concerned constrictor ET_B receptors, possibly located on vascular smooth muscle cells. A similar observation has been made in a cardiac ischemia-reperfusion model,[39] and it is likely the consequence of the elevated ET-1 levels. Of interest, this change in phenotype is reminiscent of the change in phenotype of the Ang II type 2 receptor from dilator (when located on the endothelium) to constrictor (when located on smooth muscle cells) in hypertensive animals.[40, 41] Clearly, the elevated ET-1 levels, together with an upregulation of constrictor ET_B receptors, may have contributed to the rise in blood pressure in the animals treated with a high NEP inhibitor dose.

Conclusion

The overall effect of ARNI treatment is critically dependent on the dose of the NEP inhibitor, and more is not necessarily better. Similar conclusions have been reached with regard to renin inhibition.[42] Our study reveals that the mechanisms by which high NEP inhibitor doses increase blood pressure are of both renal and vascular origin, and most likely involve ET-1 and its receptors. Additional studies with selective ET receptor antagonists are required to fully establish the causal role of ET-1. Moreover, future ARNI should either apply NEP inhibitor doses that do not affect ET-1, or be accompanied by an endothelin-converting enzyme inhibitor/ET receptor antagonist. The beneficial effects of NEP inhibition on top of AT_1 receptor blockade do not seem to be due to RAS suppression, for example, by preventing the rise in renin release that normally accompanies RAS blockade. Indeed, Demerath et al., in the isolated perfused mouse kidney,[43] were unable to demonstrate any suppressant effect of either ANP or BNP on renin secretion, despite earlier reports suggesting that natriuretic peptides inhibit renin release.[31] They therefore concluded that natriuretic peptides buffer renin-dependent hypertension, for example, by directly attenuating Ang II-mediated constriction. Our study, showing that thiorphan counterbalances the slow rise in blood pressure during prolonged RAS blockade, rather than enhancing the immediate hypotensive effect of irbesartan, concurs with this view. Importantly, our data have been obtained with the NEP inhibitor thiorphan in a high-renin transgenic model displaying natriuretic peptide activation.[19] Clearly, given the success of LCZ696 in heart failure and hypertension,[10, 11] the next step should be to test this drug in our model, and to extend its use to hypertension models beyond the Ren2 rat.

Methods

Animal studies

Heterozygous Ren2 rats (age 10 weeks) were obtained by crossing homozygous Ren2 rats (a kind gift of dr. M. Bader, Berlin, Germany) with Sprague-Dawley rats. All studies were performed under the regulation and permission of the Animal Care Committee of the Erasmus MC; protocol number 2739 (118-12-19). Rats were housed in individual cages and maintained on a 12-hour light/dark cycle, having access to standard laboratory rat chow and water ad libitum. Radiotelemetry transmitters were implanted as described before[44] for continuous measurement of heart rate, blood pressure and activity. After a recovery period of two weeks, osmotic minipumps (2ML4 ALZET, Cupertino, USA) were implanted subcutaneously under isoflurane anesthesia to infuse (1) vehicle (saline containing 0.2% DMSO; n=8), irbesartan (a kind gift of Sanofi-Aventis, Chilly-Mazarin, France, 15 mg/kg per day; n=8), (2) thiorphan (Sigma-Aldrich, 0.1 mg/kg per day diluted in DMSO; n=8), (3) irbesartan (15 mg/kg per day; n=8), (4) irbesartan plus thiorphan (0.1 mg/kg per day; n=8), or (5) irbesartan plus a 10-fold higher dose of thiorphan (1.0 mg/kg per day; n=8). During the study, rats were placed in metabolic cages at day 0 (pre-treatment, baseline measurement) and day 21 (end of treatment) to collect 24-hour urine, for the measurement of total protein, albumin, creatinine, Na^+, K^+, osmolality, cGMP, endothelin-1 and aldosterone. For each rat, baseline urine at day 0 was compared to the urinary data at day 21. Urine was frozen at -80°C until analysis. After three weeks of treatment, animals were anaesthetized by intraperitoneal pentobarbital injection (200-240 mg/kg), and the hepatic portal vein was cannulated to collect blood for the measurement of plasma renin activity (PRA), and the plasma concentrations of renin, prorenin, Ang I, Ang II, ET-1, cGMP, ANP, NT-proANP, Na^+, K^+ and creatinine. Kidneys and heart were harvested and weighed. Hearts and kidneys were divided into transverse segments, and fixated in 4% paraformaldehyde for histological analysis, or frozen in liquid nitrogen for gene expression analysis. Mesenteric arteries were isolated and either used directly in myograph studies to quantify vascular reactivity, or frozen in liquid nitrogen for gene expression analysis.

Biochemical measurements

ET-1 was assessed by chemiluminescent ELISA (QuantiGlo, R&D Systems), albumin by enzyme immunoassay (Spi-Bio, Montigny-Le-Bretonneux, France), ANP by radio-immunoassay (Phoenix Europe, Karlsruhe, Germany), NT-proANP by ELISA (Bioconnect, Huissen, The Netherlands), cGMP by ELISA (Enzo Life Sciences, Raamsdonksveer, The Netherlands) and aldosterone by radioimmunoassay (Coat-a-Count, Siemens Medical Solutions Diagnostics, Los Angeles, CA). Creatinine, K^+, Na^+, osmolality and total protein were measured at the clinical chemical laboratory of the Erasmus MC. PRA, renin, prorenin, Ang I and Ang II (after SepPak extraction and HPLC separation) were measured by in-house assays as described before.[45, 46] The limited amount of plasma did not allow the simultaneous measurement of plasma aldosterone.

Angiotensin metabolites in renal tissue were measured after tissue homogenization and extraction. Briefly, frozen kidneys (>50 mg) were homogenized using pestle and mortar under liquid nitrogen. The frozen tissue powder was dissolved at 100 mg/mL in 6 mol/L aqueous guanidinium chloride supplemented with 1% (v/v) trifluoroacetic acid (both from Sigma-Aldrich) by cooled sonication using a 2 mm microtip (Sonics and Materials Inc., Newton, USA). Stable-isotope-labeled internal standards for individual angiotensin metabolites were added to tissue homogenates at 200 pg/mL and stored at -80°C until LC-MS/MS analysis. LC-MS/MS analysis was performed as described by Kovarik et al.[47] A minimal signal-to-noise

ratio of 10 resulted in lower limits of quantification for individual peptides of 8 pg/g (Ang I), 5 pg/g (Ang II), 16 pg/g (Ang-(1-7)), 3 pg/g (Ang-(1-5)), 7 pg/g (Ang-(2-8)), 5 pg/g (Ang-(3-8)), 16 pg/g (Ang-(2-10)) and 11 pg/g (Ang-(1-9)). In the rare cases that levels were at or below this limit, this limit was applied to allow statistical analysis.

Histology

After fixation, heart and kidney sections were dehydrated and paraffin-embedded. Gomori silver staining was applied to sections (5 µm) of the left ventricle of the heart to visualize individual cardiomyocytes. Sirius red staining was applied to visualize collagen as a measure of cardiac fibrosis. Cardiomyocyte size and the amount of collagen was measured using Qwin (Leica, Cambridge, UK).

Transversely sliced kidney sections (deparaffinized, 2 µm) were stained with periodic acid Schiff –diastase (PAS-D) to asses kidney pathology. In the sections, the presence of focal and segmental glomerulosclerosis was assessed in all glomeruli of one kidney section per animal. All sections were semi-quantitatively scored by a renal pathologist in a blinded manner. Furthermore, each kidney section (100x magnification) was analysed for tubular damage. The damaged tubules were scored using a 5-point scale according to the following criteria: tubular dilatation, cast deposition, brush border loss and necrosis.[48] Each parameter was graded in 10 fields with a score of 0-5 in which grade 0 meant no changes in pathology; grade 1, mild, involvement of less than 10% of the cortex; grade 2, moderate, involvement of 10-25% of the cortex; grade 3, severe, involvement of 25-50% of the cortex; grade 4, very severe, involvement of 50-75% of the cortex; grade 5, extensive damage, involvement of >75% of the cortex.

Quantitative real-time reverse transcription-PCR

Total RNA was isolated from snap-frozen rat heart (left ventricular tissue) and mesenteric arteries using Trizol (Life Technologies, Grand Island, NY) and reverse transcribed into cDNA using the QuantiTect Reverse Transcription Kit (Qiagen, Venlo, The Netherlands). The resulting cDNA was amplified in 40 cycles (denaturation at 95°C for 10 min; thermal cycling at 95°C for 15 sec, annealing/extension at 60°C for 1 min) with a Step-One cycler (NYSE, Life Technologies) using the SYBR® Green PCR Master Mix (Life Technologies). The intron-spanning oligonucleotide primers for qPCR were designed with NCBI (Primer-BLAST; *Supplementary Table S1*). The comparative cycle time method (ΔΔCT) was used for relative quantification of gene expression, using the geometric mean of the housekeeping genes hypoxanthine phosphoribosyl transferase-1, β_2-microglobulin and β-actin for normalization. In the heart, gene expression of ANP and β-MHC was measured, and in the arteries gene expression of the ET_A and ET_B receptor.

Immunoblotting of kidney transporters

Kidneys were cut into four equal pieces, and one piece was homogenized on ice in isolation buffer to perform immunoblotting as reported previously.[49] Antibodies against the following proteins were used: NHE3 (dilution 1:5000), NKCC2 (1:1000), the α- and γ-subunits of ENaC (both 1:1000), and AQP2 (1:1000, all from StressMarq, Victoria, BC, Canada); NCC (1:500) and SGK1 (1:2000) (both Millipore, Temecula, CA); NEDD4-2 (1:4000, Abcam, Cambridge, UK) and NCC phosphorylated at threonine-58 (pNCC-Thr58, 1:500, kind gift of Dr. R.A. Fenton, Aarhus, Denmark). β-actin (1:50.000; Abcam) was used for normalization of protein levels. Protein was visualized using horseradish peroxidase-conjugated secondary antibodies (1:3000; Bio-Rad, Veenendaal, The Netherlands). Signals were detected by

chemiluminescence (Pierce, Rockford, IL) and quantified using ImageQuant LAS 4000 (GE Healthcare, Diegem, Belgium). Kidneys of rats treated with thiorphan alone were not evaluated in this analysis.

Myograph studies

Following isolation, mesenteric arteries were cut into segments of ~2 mm length and mounted in a Mulvany myograph (Danish Myo Technology, Aarhus, Denmark) with separated 6-mL organ baths containing Krebs bicarbonate solution, aerated with 95% O2 and 5% CO2, and maintained at 37°C.[50] Tissue responses were measured as changes in isometric force, using Powerlab with Labchart software. Following a 30-minutes stabilization period, the optimal internal diameter was set to a tension equivalent of 0.9 times the estimated diameter at 100 mm Hg effective transmural pressure, as described by Mulvany and Halpern.[51] Subsequently, to determine the maximum contractile response, the tissue was exposed to 100 mmol/L KCl. The segments were then allowed to equilibrate in fresh organ bath fluid for 30 minutes. Next, segments were pre-incubated for 30 minutes with the NO synthase inhibitor L-NAME (100 µmol/L), the small conductance Ca^{2+}-activated K^+-channel inhibitor apamin (100 nmol/L), the intermediate conductance Ca^{2+}-activated K^+-channel inhibitor TRAM34 (10 µmol/L), the ET_A receptor antagonist BQ123 (1 µmol/L), and the ET_B receptor antagonist BQ788 (1 µmol/L). Thereafter, CRCs were constructed to ET-1. To construct CRCs to the endothelium-dependent vasodilator ACh or SNAP, mesenteric arteries were preconstricted with U46619 (0.1-0.3 µmol/L). All drugs were from Sigma-Aldrich.

Statistical analysis

Data are provided as mean ± SEM. Relaxant responses to either ACh or S-nitroso-N-penicillamine are expressed as a percentage of the contraction to U46619. Contractile responses to ET-1 are expressed as a percentage of the contraction to 100 mmol/L KCl. CRCs were analyzed as described.[52] To obtain pEC50 ($-^{10}$logEC50) values. Data were analyzed by Student's t-test or one-way ANOVA, followed by post-hoc evaluation according to Bonferroni (for comparisons between ≥2 groups). $P < 0.05$ was considered significant.

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Supplemental information

Table S1. Real-time qPCR primers. Abbreviations: HPRT-1, hypoxanthine phosphoribosyltransferase; ANP, atrial natriuretic peptide; β-MHC, β-myosin heavy chain; ET_AR, endothelin-1 type A receptor; ET_BR, endothelin-1 type B receptor.

Gene	Forward primer 5'-3'	Reverse primer 5'-3'
HPRT-1	TGGACAGGACTGAAAGACTTGCTCG	CTTCAGCACACAGAGGGCCACA
β-actin	GGGAAATCGTGCGTGACATT	GCGGCAGTGGCCATCTC
β_2-microglobulin	ATGGCTCGCTCGGTGACCG	TGGGGAGTTTTCTGAATGGCAAGCA
ANP	ATGGGCTCCTTCTCCATCAC	TCTACCGGCATCTTCTCCTC
β-MHC	ATGGACCTGGAGCGAGCAAA	GTCCTTCTTTTTGAGTCGCTCATCC
ET_AR	ATGAGGAACGGCCCCAATG	CCGCCAACAGCTTAAACACA
ET_BR	TGACCACTTAAAGCAGAGACGA	GAAGCCAACAGAGGGCAAAC

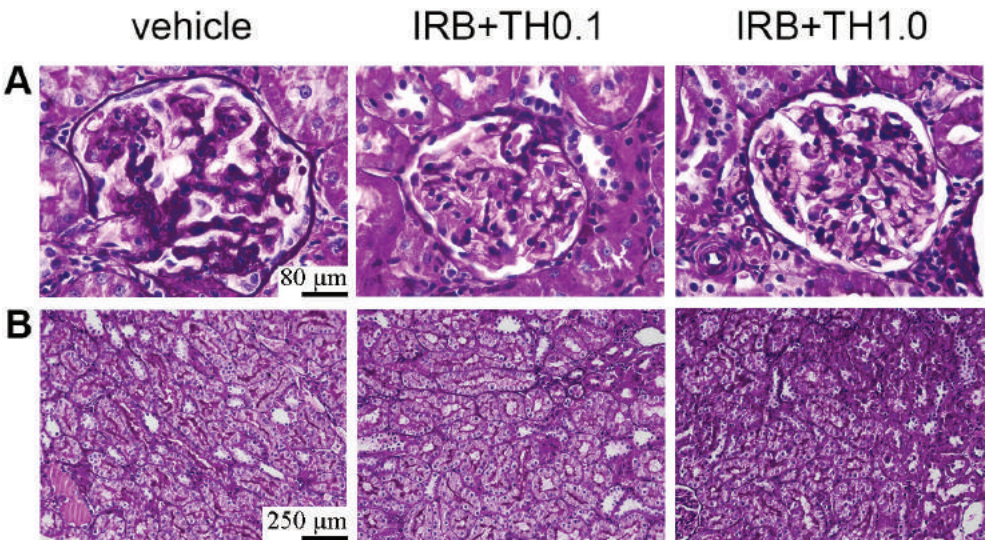

Figure S1. Representative pictures of a glomerulus (A) and tubuli (B) from PAS-stained kidney sections obtained from Ren2 rats treated for 3 weeks with either vehicle, irbesartan+thiorphan 0.1 mg/kg.day (IRB+TH0.1) or irbesartan+thiorphan 1.0 mg/kg.day (IRB+TH1.0). The tubular injury score (TIS) in the corticomedullary junction, as assessed by quantification of tubular dilatation, cast deposition, brush border loss and necrosis, was present, although no significance difference was reached between vehicle and both ARNI treatments (TIS of 1.88±0.3, 1.43±0.2, 1.63±0.3 for respectively vehicle, IRB+TH0.1 and IRB+TH1.0).

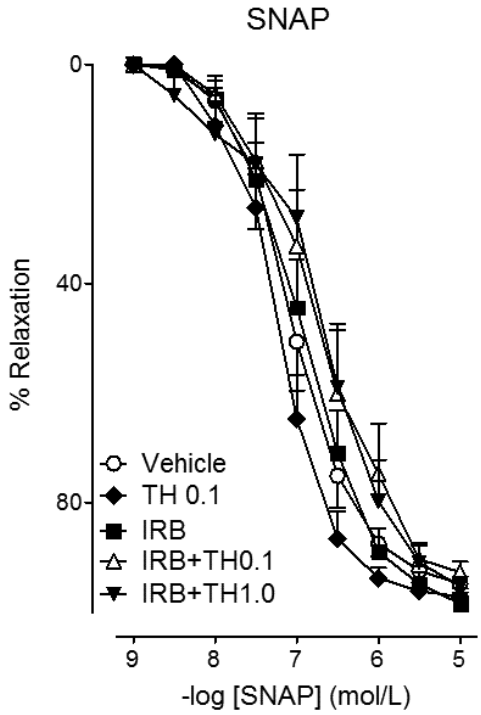

Figure S2. Relaxations to S-nitroso-N-penicillamine (SNAP) in mesenteric arteries from Ren2 rats treated for 3 weeks with vehicle, thiorphan 0.1 mg/kg.day (TH0.1), irbesartan 15 mg/kg.day (IRB), irbesartan+thiorphan 0.1 mg/kg.day (IRB+TH0.1) or irbesartan+thiorphan 1.0 mg/kg.day (IRB+TH1.0). Relaxations are expressed as % reduction of preconstriction (with U46619). Data are mean + s.e.m.of n=8.

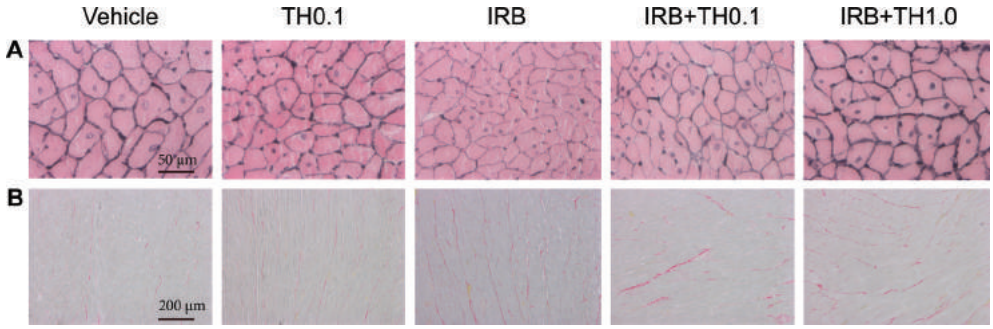

Figure S3. Representative pictures of Gomori-stained cardiomyocytes (A) and Sirius red-stained left ventricular tissue (B) of hearts obtained from Ren2 rats treated for 3 weeks with either vehicle, thiorphan 0.1 mg/kg.day (TH0.1), irbesartan 15 mg/kg.day (IRB), irbesartan+thiorphan 0.1 mg/kg.day (IRB+TH0.1) or irbesartan+thiorphan 1.0 mg/kg.day (IRB+TH1.0).

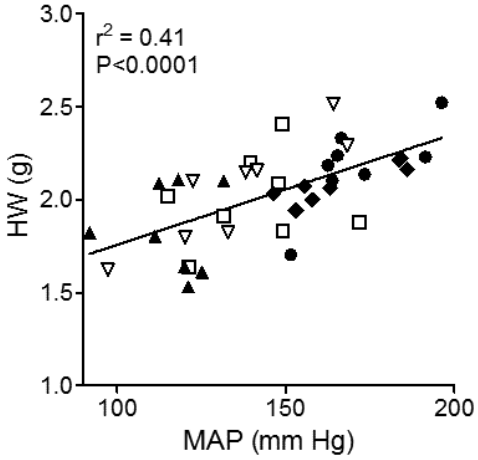

Figure S4. Mean arterial pressure (MAP) versus heart weight (HW) in Ren2 rats treated for 3 weeks with either vehicle (●), thiorphan 0.1 mg/kg.day (♦), irbesartan 15 mg/kg.day (□), irbesartan+thiorphan 0.1 mg/kg.day (▲), or irbesartan+thiorphan 1.0 mg/kg.day (▽).

CHAPTER 8

BLOOD PRESSURE INDEPENDENT RENOPROTECTION IN DIABETIC RATS TREATED WITH AT_1 RECEPTOR-NEPRILYSIN INHIBITION VERSUS AT_1 RECEPTOR BLOCKADE ALONE

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Abstract
ARNI [dual AT_1 (angiotensin II type 1) receptor-neprilysin inhibition] exerts beneficial effects on blood pressure and kidney function in heart failure, compared with ARB (AT_1 receptor blockade) alone. We hypothesized that ARNI improves cardiac and kidney parameters in diabetic TGR(mREN2)27 rats, an angiotensin II-dependent hypertension model. Rats were made diabetic with streptozotocin for 5 or 12 weeks. In the final 3 weeks rats were treated with vehicle, irbesartan (ARB) or irbesartan+thiorphan (ARNI). Blood pressure, measured by telemetry in the 5-week group, was lowered identically by ARB and ARNI. Heart weight/tibia length ratio in 12-week diabetic animals was lower after ARNI compared with after ARB. Proteinuria and albuminuria were observed from 8 weeks of diabetes onwards. ARNI reduced proteinuria more strongly than ARB, and a similar trend was seen for albuminuria. Kidneys of ARNI-treated animals showed less severe segmental glomerulosclerosis than those of ARB-treated animals. After 12 weeks, no differences between ARNI- and ARB-treated animals were found regarding diuresis, natriuresis, plasma endothelin-1, vascular reactivity (acetylcholine response) or kidney sodium transporters. Only ARNI-treated rats displayed endothelin type B receptor-mediated vasodilation. In conclusion, ARNI reduces proteinuria, glomerulosclerosis, and heart weight in diabetic TGR(mREN2)27 rats more strongly than ARB, and this occurs independently of blood pressure.

Introduction
NEP (neutral endopeptidase), or neprilysin, is critical for the processing and catabolism of natriuretic peptides [ANP (atrial natriuretic peptide), BNP (brain natriuretic peptide) and CNP (C-type natriuretic peptide)], angiotensin II, and ET-1 (endothelin-1). NEP is widely expressed, its highest concentrations occurring in the kidney.[1] NIs (NEP inhibitors) induce beneficial effects by increasing natriuretic peptide levels.[2, 3] However, simultaneous NI-induced angiotensin II and ET-1 rises may counteract these effects. To avoid this, ARB [AT_1 (angiotensin II type 1) receptor blockade] might be combined with NI ('ARNI'). The first ARNI, LCZ696, was more effective than single RAS blockade in hypertension and heart failure.[4-6] LCZ696 particularly decreased atrial wall stress in diabetic patients, as was evident by reduced levels of proBNP's N-terminal end (NT-proBNP).[5] The latter peptide is generated from proBNP, together with BNP, but, unlike BNP, cannot be metabolized by NEP. Consequently, a decrease in its levels suggests less cardiac proBNP synthesis. Atrial wall stress increases proBNP synthesis, and elevated NT-proBNP levels therefore are a well-known marker for heart failure severity. NIs also improve vascular function in DM (diabetes mellitus).[7]

Currently, RAS blockade is the first-line treatment for diabetic patients with proteinuria. Yet RAS blockers cannot reverse or halt the progression of diabetic nephropathy. Importantly, LCZ696-treated heart failure patients with an eGFR (estimated glomerular filtration rate) ≥30 mL/min per 1.73m² displayed a lower blood pressure and a better preserved eGFR than valsartan-treated controls.[8] Yet their urinary albumin/creatinine ratio increased more, possibly because elevated ANP levels induce relaxing and proliferating effects on renal mesangial cells,[9, 10] and/or inhibit tubular protein reabsorption.[11] Data on natriuretic peptide plasma levels in diabetes are conflicting: both higher ANP and BNP levels[12] and lower NT-proANP levels have been reported.[13, 14] Higher levels are indicative for (renal) insensitivity to ANP.

In the present study we set out to compare the effects of ARNI and AT_1 receptor blockade in diabetic TGR(mREN2)27 (Ren2) rats. These rats, by overexpressing the mouse Ren2 gene, display severe angiotensin II-dependent hypertension,[15, 16] have an activated natriuretic

peptide system,[17] and, when made diabetic, develop diabetic nephropathy.[18] In non-diabetic Ren2 rats, the NI thiorphan, when dosed optimally, displayed favourable blood pressure and cardiac effects in addition to irbesartan. Yet, when dosed too high, it increased plasma ET-1 and blood pressure.[19] We hypothesized that with an optimal thiorphan dose, ARNI would be superior to ARB not only with regard to blood pressure and cardiac hypertrophy in diabetic Ren2 rats, but also with regard to renoprotection. To gain a better understanding of the underlying mechanisms, we quantified the effects of irbesartan with (ARNI) or without (ARB) thiorphan, on RAS activity, ANP, BNP, and ET-1, as well as their second messengers (cGMP) and renal and vascular targets (kidney sodium transporters, vascular ET receptors). Three-week treatment with ARB, ARNI or vehicle was started at either 2 or 9 weeks after the induction of DM, to distinguish early kidney damage and chronic kidney injury.

Materials and methods

Animal studies

Heterozygous Ren2 rats (age 10 weeks, weight 300-500 g) were obtained by crossing homozygous Ren2 and Sprague-Dawley rats. Studies were performed under the regulation and permission of the Erasmus MC Animal Care Committee. DM was induced by administering streptozotocin [55 mg/kg i.p. (intraperitoneal); Merck Millipore], and DM animals were studied for 5 or 12 weeks.

In the 5-week DM group, heart rate and BP were measured by radiotelemetry transmitters, implanted 2 weeks before DM induction.[20] Rats were checked daily for non-fasting blood glucose and β-ketone levels until day 3 after streptozotocin injection, and thereafter once-weekly or every other week (5- and 12-week groups respectively) (Precision Xceed, Abbott). Only rats with glucose >15 mmol/L were considered diabetic; they received 2-4 units of insulin per day (Levemir®, Novo Nordisk). Rats in both groups were treated during the final 3 weeks of the study (i.e. during weeks 2-5 or 9-12) with vehicle (saline containing 0.2% DMSO), irbesartan (15 mg/kg per day; Sanofi-Aventis), or irbesartan+thiorphan (0.1 mg/kg per day diluted in DMSO; Sigma-Aldrich) making use of osmotic minipumps (2ML4 ALZET). Rats were placed in metabolic cages at the week before DM induction (baseline), and at weeks 2, 5 or 8 after DM induction to collect 24-hour urine. After 3 weeks of treatment, animals were anaesthetized by i.p. pentobarbital injection (200-240 mg/kg), and the hepatic portal vein was cannulated to collect blood. Kidneys and heart were harvested, weighed, divided into transverse segments, and fixated in 4% paraformaldehyde for histological analysis, or snap frozen in liquid nitrogen for gene expression analysis. Mesenteric arteries were isolated and either used directly in myograph studies, or frozen in liquid nitrogen for gene expression analysis.

Biochemical measurements

ET-1 was assessed by chemiluminescent ELISA (QuantiGlo, R&D Systems), albumin by enzyme immunoassay (Spi-Bio), ANP and BNP by radio-immunoassay (Phoenix Europe), cGMP by ELISA (Enzo Life Sciences), and aldosterone by radioimmunoassay (Demeditec Diagnostics). Creatinine, cystatin C, K^+, Na^+ and total protein were measured at the clinical chemical laboratory of the Erasmus MC. PRA (plasma renin activity), PRC (plasma renin concentration) and plasma prorenin concentration were measured by in-house assays.[21, 22]

Histology

After fixation, sections were dehydrated and paraffin-embedded. Gomori silver staining was applied to sections (5 µm) of the cardiac left ventricle to visualize cardiomyocytes. Sirius

red staining was applied to visualize collagen. Quantification occurred using Qwin software (Leica). Transversely sliced kidney sections (deparaffinised, 2 µm) were stained with periodic acid Schiff -diastase. FSGS (focal segmental glomerulosclerosis) was assessed in all glomeruli of one kidney section per animal (199 ± 4 glomeruli/section). All sections were semi-quantitatively scored by a pathologist (M.C.C. v.G.) in a blinded manner,[23] using an arbitrary scale with grade 0 (n0), 1 (n1), 2 (n2), 3 (n3), and 4 (n4) indicating that, respectively, 0, <25%, 25-50%, 50-75%, and >75% of the glomerulus showed sclerosis. The GSI (glomerulosclerosis index) was calculated for each rat with the formula: [(1xn1)+(2xn2)+(3xn3)+(4xn4)]/[n0+n1+n2+n3+n4]. Furthermore, ten images of each kidney section were analysed for arterial hyalinosis, intima fibrosis and media hypertrophy, as well as tubular atrophy, interstitial fibrosis and renal inflammation according to the Banff 97 working classification.[23] Each parameter was graded in ten sequential fields with a score of 0-3 in which 0 meant no changes; grade 1, <25% change; grade 2, 25-50% change; grade 3, >50% of tissue affected. From these data, the TIS (tubulointerstitial score) was calculated by summing the scores of tubular atrophy, interstitial fibrosis and renal inflammation.

Quantitative real-time RT-PCR

Total RNA was isolated from snap-frozen left ventricular tissue, kidney cortex and kidney medulla using TRIzol (Life Technologies) and reverse-transcribed into cDNA using the QuantiTect Reverse Transcription Kit (Qiagen). The resulting cDNA was amplified in 40 cycles (denaturation at 95°C for 10 min, thermal cycling at 95°C for 15 sec, annealing/extension at 60°C for 1 min) with a Step-One cycler (NYSE, Life Technologies) using the SYBR® Green PCR Master Mix (Life Technologies). The intron-spanning oligonucleotide primers for qPCR were designed with NCBI (Primer-BLAST) (see *Supplementary Table S1*). The ΔΔCT method was used for relative quantification of mRNA expression levels, using the geometric mean of the housekeeping genes hypoxanthine phosphoribosyl transferase-1, β2-microglobulin and β-actin for normalization.

Western blotting of kidney transporters

Kidneys were cut into four equal pieces, and one piece was homogenized on ice in isolation buffer to perform immunoblotting as reported previously.[24] Antibodies against the following proteins were used: NHE3 (Na^+/H^+ exchanger) (1:5000 dilution), NKCC2 (Na^+-K^+-Cl^--cotransporter) (1:1000 dilution), and the α- and γ-subunits of ENaC (epithelial Na^+ channel (both 1:1000 dilution) (all from StressMarq); NCC (Na^+-Cl^--cotransporter) (1:500 dilution) and SGK1 (serum- and glucocorticoid-regulated kinase 1) (1:2000 dilution) (both from Millipore); the aldosterone-sensitive proteins ubiquitin ligase NEDD4-2 (neural-precursor-cell-expressed developmentally down-regulated 4-2) (1:4000 dilution (Abcam) and NCC phosphorylated at Thr58 (1:500 dilution) (a gift from Dr. Robert Fenton, Aarhus, Denmark). Anti β-actin (1:50.000 dilution) (Abcam) was used for normalization of protein levels. Protein was visualized using horseradish peroxidase-conjugated secondary antibodies (1:3000 dilution) (Bio-Rad Laboratories). Signals were detected by chemiluminescence (Pierce) and quantified using ImageQuant LAS 4000 (GE Healthcare).

Myograph studies

Mesenteric arteries were cut into ~2 mm-segments and mounted in Mulvany myographs (Danish Myo Technology) with 6-mL organ baths. Tissue response was measured as changes in isometric force, using Powerlab with Labchart software. Following a 30-min stabilization period, the optimal internal diameter was set as described.[25] Subsequently, to determine

the maximum contractile response, segments were exposed to 100 mmol/L KCl. They were then allowed to equilibrate in fresh organ bath fluid for 30 min. Next, segments were pre-incubated for 30 min with the NOS (nitric oxide synthase) inhibitor L-NAME (NG-nitro-L-arginine methyl ester) (100 µmol/L), the SKCa and IKCa (small- and intermediate-conductance Ca^{2+}-activated K^+-channel) inhibitors apamin (100 nmol/L) and TRAM34 (10 µmol/L), the ET_A (endothelin type A) receptor antagonist BQ123 (1 µmol/L), or the ET_B receptor antagonist BQ788 (1 µmol/L). Thereafter, concentration-response curves (CRCs) were constructed for ET-1. To construct CRCs to the endothelium-dependent dilator Ach (acetylcholine) or the endothelium-independent dilator SNAP (S-nitroso-N-penicillamine), mesenteric arteries were preconstricted with U46619 (0.1-0.3 µmol/L). All drugs were obtained from Sigma-Aldrich.

Statistical analysis

Data are expressed as means ± S.E.M. Relaxant responses to either ACh or SNAP are expressed as a percentage of the contraction to U46619. Contractile responses to ET-1 are expressed as a percentage of the contraction to 100 mmol/L KCl. CRCs were analyzed as described previously[26] to obtain pEC50 ($^{-10}$logEC50) and Emax values. Data were analysed by Student's t test or one-way ANOVA (for comparisons between two or more groups), followed by post-hoc evaluation according to Bonferroni. $P < 0.05$ was considered statistically significant.

Results

Comparable effects of ARNI and ARB on blood pressure and RAS

Vehicle treatment did not affect MAP (mean arterial pressure) in the 5-week DM group (*Figure 1*). ARB lowered MAP already on the first treatment day, and a maximum effect was reached between days 5 and 7, which remained unaltered over the remainder of the treatment period. Adding thiorphan as well as irbesartan (ARNI) did not change this response, and no treatment affected heart rate (306 ± 5 beats/min at baseline compared with 279 ± 11, 282 ± 9 and 282 ± 14 beats/min, respectively, after 3 weeks of treatment with vehicle, ARB or ARNI.

ARB increased PRA and PRC both in the 5-week and the 12-week group 2-3-fold (*Figures 2A and 2B*), although significance was reached in the former group only. No significant

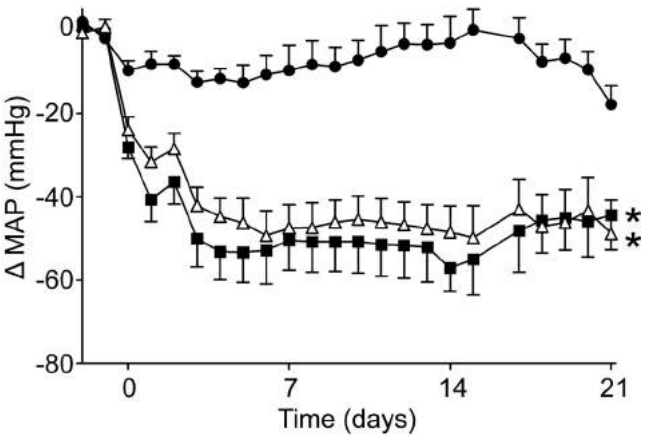

Figure 1. Change in MAP (ΔMAP) during vehicle (●), ARB (■) or ARNI (Δ) infusion. Baseline MAP was 157 ± 5.4 mmHg. Results are means ± S.E.M. (n=8) *P<0.05 compared with vehicle.

increases in plasma prorenin were observed (*Figure 2C*), and adding thiorphan to irbesartan (ARNI) did not alter any of these changes, although there was a tendency for plasma prorenin to decrease in the presence of thiorphan. DM increased urinary aldosterone excretion at 5 and 12 weeks (*Figure 2G*), and this was prevented by ARNI at 5 weeks and by ARB at 12 weeks.

Food intake increased over both treatment periods compared with baseline (*Table 1*). However, after 12 weeks of DM, food intake was reduced in comparison with 5 weeks. ARNI prevented this reduction in food intake at 12 weeks. No treatment affected body weight (*Table 1*). DM increased water intake and urinary volume 4-6-fold, and ARNI suppressed this at 5 but not at 12 weeks (*Table 1*). The increased food intake in the DM animals was accompanied by an increase in urinary Na^+ and K^+ secretion (results not shown). Plasma Na^+ and K^+ levels (133 ± 1 and 6.5 ± 0.7 mmol/L in the vehicle group) were unaltered by treatment (results not shown), and the same was true for blood glucose (*Table 1*). Plasma and urinary creatinine were not affected by any condition, and creatinine clearance did not differ between the groups (*Table 1*).

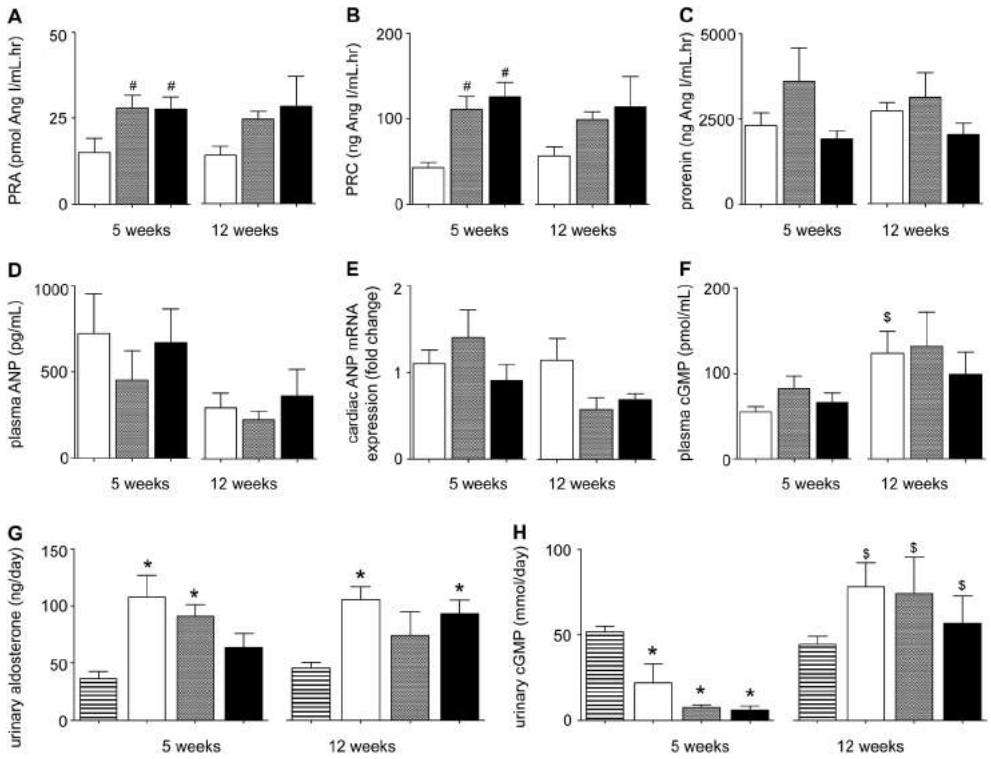

Figure 2. Effects of vehicle, ARB or ARNI on RAS components, ANP and cGMP in plasma and urine. (A) PRA, (B), PRC, (C) plasma prorenin, (D) plasma ANP, (E) cardiac ANP mRNA expression, (F) plasma cGMP, (G) urinary aldosterone and (H) urinary cGMP at baseline (striped bars) and after vehicle (white bars), ARB (dotted bars), or ARNI (black bars) treatment for 3 weeks, started at 2 ('5 weeks') or 9 ('12 weeks') weeks after DM induction. Results are means ± S.E.M. (n=7-12). *P<0.05 compared with baseline; #P<0.05 compared with vehicle; $P<0.05 compared with 5 weeks.

Table 1. Main characteristics of diabetic (DM) Ren2 rats at baseline (BL), and after 3 weeks of treatment with vehicle, ARB or ARNI. Treatment was started either 2 weeks ('5 weeks of DM') or 9 weeks ('12 weeks of DM') after the induction of DM. Results are means ± S.E.M. of 7-12 rats. *P<0.05 compared with baseline; # P<0.05 compared with vehicle; $ P<0.05 compared with 5 weeks; † P<0.05 compared with ARB.

	5 weeks of DM				12 weeks of DM			
Parameter	**BL**	**Vehicle**	**ARB**	**ARNI**	**BL**	**Vehicle**	**ARB**	**ARNI**
Body weight (BW; g)	-	448 ± 13	452 ± 16	450 ± 11	-	448 ± 9	482 ± 20	430 ± 23
Heart weight (HW; g)	-	1.7 ± 0.06	1.5 ± 0.04#	1.5 ± 0.03#	-	1.9 ± 0.08$	2.0 ± 0.10$	1.6 ± 0.12†
Tibia length (cm)	-	4.29 ± 0.05	4.32 ± 0.05	4.31 ± 0.04	-	4.26 ± 0.05	4.19 ± 0.04	4.20 ± 0.05
Kidney weight (KW; g)	-	2.0 ± 0.07	2.0 ± 0.1	2.0 ± 0.06	-	2.3 ± 0.08	2.2 ± 0.08	2.1 ± 0.18
KW/tibia length (g/cm)	-	0.48 ± 0.02	0.46 ± 0.02	0.46 ± 0.01	-	0.53 ± 0.02	0.52 ± 0.02	0.50 ± 0.04
Food intake (g/day)	22 ± 0.9	36 ± 1.1*	35 ± 1.4*	33 ± 1.2*	23 ± 1.3	29 ± 1.5*$	28 ± 2.2$	34 ± 1.3*†
Water intake (mL/day)	38 ± 2	157 ± 13*	143 ± 14*	112 ± 14*#	43 ± 2	124 ± 12*	111 ± 15*	131 ± 19*
Urinary volume (mL/day)	24 ± 2	157 ± 13*	140 ± 14*	100 ± 18*#	28 ± 2	113 ± 14*	102 ± 16*	121 ± 18*
Plasma creatinine (nmol/mL)	-	33 ± 1	32 ± 2	31 ± 1	-	28 ± 1	32 ± 3	28 ± 2
Creatinine clearance (mL/min)	-	2.7 ± 0.2	3.3 ± 0.3	3.2 ± 0.6	-	2.8 ± 0.2	3.1 ± 0.5	3.0 ± 0.3
Blood glucose (mmol/L)	-	27 ± 0.6	27 ± 0.7	27 ± 0.5	-	27 ± 0.3	25 ± 1.9	26 ± 0.7
Plasma Cystatin C (mg/L)	-	2.3 ± 0.2	1.9 ± 0.2	2.2 ± 0.3	-	2.1 ± 0.2	2.5 ± 0.1	2.3 ± 0.1
Na^+ excretion (mmol/day)	2.1 ± 0.1	4.0 ± 0.3*	4.0 ± 0.3*	3.5 ± 0.4*	1.9 ± 0.2	3.1 ± 0.2*	3.8 ± 1.1*	3.3 ± 0.3*

ARNI reduces heart weight

In the 5-week group, irbesartan, with (ARNI) or without (ARB) thiorphan, reduced heart weight (*Figure 3A*). Heart weight increased in the 12-week DM group compared with the 5-week DM group, but at 12 weeks, only dual treatment prevented the increase in heart weight. Results were identical with and without correction for tibia length or body weight. The changes in cardiac fibrosis at 12 weeks, although non-significant, mimicked those in heart weight (*Figure 3B*). Cardiomyocyte size was similar under all conditions (*Figure 3C*).

ARNI up-regulates renal preproET-1 but does not affect circulating ANP

ARB reduced plasma ANP, both at 5 and 12 weeks, and adding thiorphan reversed this effect (*Figure 2D*). However, none of these changes were significant. No significant changes in cardiac ANP expression levels were observed under any condition, although at 12 weeks irbesartan, both with (ARNI) and without (ARB) thiorphan, tended to decrease cardiac ANP expression (*Figure 2E*). NPR (natriuretic peptide receptor)-A and NPR-B expression in the renal medulla and cortex were below the detection limit. NPR-C expression in the medulla was unaffected under all conditions, and increased in the cortex by ARB at 5 weeks ($P<0.05$), but not at 12 weeks (results not shown). DM increased plasma cGMP levels at 12 weeks compared with 5 weeks, and a concurrent rise in urinary cGMP excretion was observed (*Figures 2F and 2H*). In fact, at 5 weeks, urinary cGMP excretion was reduced in comparison with baseline ($P<0.05$). Treatment did not affect this outcome. Plasma BNP was unaltered by treatment both after 5 weeks of DM (132 ± 10, 132 ± 15, and 133 ± 16 pg/mL, for vehicle, ARB and ARNI treatment respectively), and after 12 weeks of DM (160 ± 16, 151 ± 28, and 157 ± 22 pg/mL, respectively).

DM, with or without treatment, did not significantly affect urinary ET-1, and treatment also did not alter plasma ET-1 (*Table 2*). In the 5-week group, ARNI reduced cardiac ET_A receptor expression, and in the 12-week group this was also the case for irbesartan

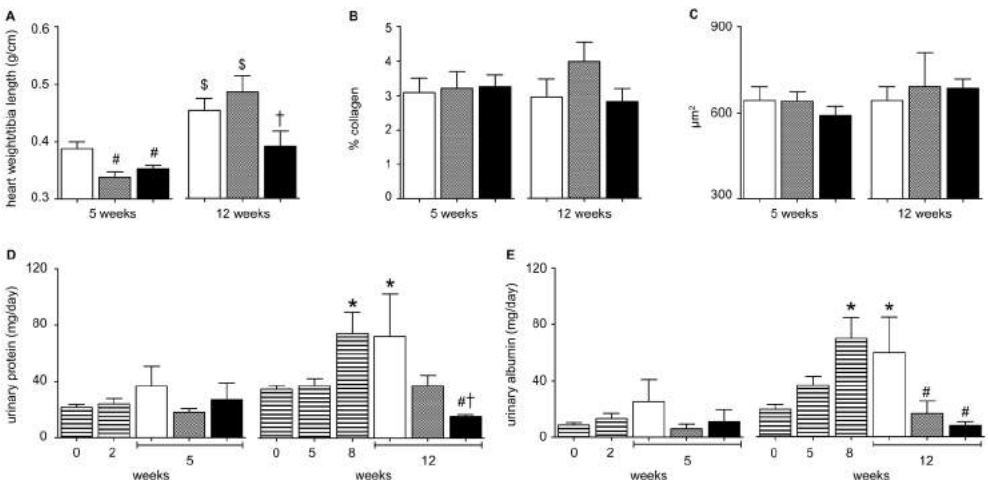

Figure 3. Cardiac and renal effects of vehicle, ARB or ARNI. (A) Cardiac hypertrophy, (B) cardiac fibrosis, (C) cardiomyocyte size, (D) proteinuria, and (E) albuminuria before treatment (striped bars: 0, 2, 5 or 8 weeks of DM), or after vehicle (white bars), ARB (dotted bars) or ARNI (black bars) treatment for 3 weeks, started at 2 ('5 weeks') or 9 ('12 weeks') weeks after DM induction. Results are means ± S.E.M. (n=7-12). *$P<0.05$ compared with baseline (0weeks); #$P<0.05$ compared with vehicle; †$P<0.05$ compared with ARB.

Table 2. Parameters of the ET-1 system, including the ET_A receptor, and the ET_B receptor in diabetic (DM) Ren2 rats at baseline (BL), and after 3 weeks of treatment with vehicle, ARB or ARNI. Treatment was started either 2 weeks ('5 weeks of DM') or 9 weeks ('12 weeks of DM') after the induction of DM. Results are means ± S.E.M. of 4-12 rats. # P<0.05 compared with vehicle; † P<0.05 compared with ARB.

	5 weeks of DM				12 weeks of DM			
Parameter	**BL**	**Vehicle**	**ARB**	**ARNI**	**BL**	**Vehicle**	**ARB**	**ARNI**
Plasma ET-1 (pg/mL)	-	0.82 ± 0.05	0.85 ± 0.08	0.95 ± 0.13	-	0.83 ± 0.09	0.80 ± 0.08	0.62 ± 0.07
Urinary ET-1 (pg/day)	9.0 ± 0.9	5.3 ± 2.4	6.6 ± 2.3	4.9 ± 1.5	9.4 ± 0.6	10.0 ± 5.1	7.2 ± 2.9	4.6 ± 1.8
Relative mRNA expression levels								
PreproET-1 renal cortex		1.03 ± 0.08	1.04 ± 0.13	1.05 ± 0.10		1.00 ± 0.04	1.19 ± 0.12	1.07 ± 0.13
PreproET-1 renal medulla		1.04 ± 0.09	1.05 ± 0.07	1.58 ± 0.13#†		1.03 ± 0.10	1.62 ± 0.10#	1.96 ± 0.23#
ET_AR heart		1.13 ± 0.17	1.01 ± 0.17	0.59 ± 0.09#		1.01 ± 0.06	0.75 ± 0.08#	0.75 ± 0.05#
ET_AR renal cortex		1.08 ± 0.08	0.98 ± 0.08	1.00 ± 0.15		1.07 ± 0.16	1.08 ± 0.12	1.09 ± 0.13
ET_AR renal medulla		1.05 ± 0.16	1.47 ± 0.25	2.17 ± 0.26#		1.05 ± 0.14	0.81 ± 0.09	1.14 ± 0.24
ET_BR heart		1.02 ± 0.07	1.00 ± 0.13	0.93 ± 0.13		1.01 ± 0.04	0.81 ± 0.05#	1.07 ± 0.06
ET_BR renal cortex		1.05 ± 0.09	1.02 ± 0.10	0.97 ± 0.09		1.02 ± 0.08	0.96 ± 0.17	1.30 ± 0.26
ET_BR renal medulla		0.83 ± 0.09	0.97 ± 0.16	1.75 ± 0.19#†		1.08 ± 0.17	1.30 ± 0.26	1.94 ± 0.51

alone (*Table 2*). No significant changes in cardiac ET_B receptor expression occurred after treatment, except for a modest reduction in the 12-week group treated with ARB (*Table 2*). Renal cortical ET_A and ET_B receptor expression were not affected by treatment. In the renal medulla, ET_A and ET_B receptor expression rose significantly in the 5-week group treated with ARNI. In the 12-week group the same upregulation was present, although not statistically significant (*Table 2*). Combined treatment increased prepro-ET-1 expression in renal medulla in both the 5- and 12-week groups, and ARB alone also increased medullary prepro-ET-1 expression in the 12-week group (*Table 2*). No such changes were observed for prepro-ET-1 in the renal cortex.

ARNI ameliorates diabetic nephropathy

DM time-dependently increased urinary protein and albumin excretion, significance being reached (P< 0.05 compared with baseline) from 8 weeks onwards (*Figures 3D and 3E*). In the 12-week group, ARNI fully normalized both parameters, whereas ARB alone only reduced albuminuria. The renal pathology scores FSGS, GSI and TIS followed a similar pattern as

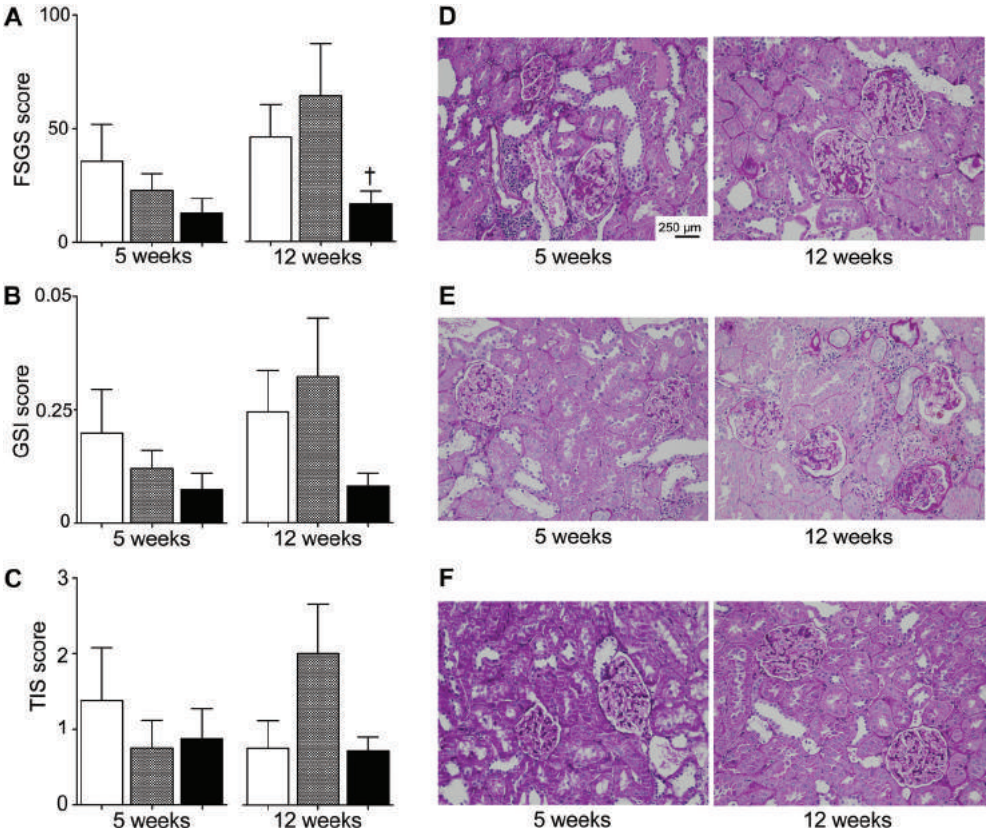

Figure 4. Effects of vehicle, ARB or ARNI on renal histology. (A) FSGS, (B) GSI, and (C) TIS after vehicle (white bars), ARB (dotted bars) or ARNI (black bars) treatment for 3 weeks, started at 2 ('5 weeks') or 9 ('12 weeks') weeks after DM induction. Representative pictures are shown in (D; vehicle), (E; ARB), and (F; ARNI) respectively. Results are mean ± S.E.M. (n=7-8). †P<0.05 compared with ARB.

proteinuria, i.e. the chronic lesions were most prominent at 12 weeks, and fewer lesions were observed after dual treatment (*Figure 4*). However, owing to variability, statistical significance was only achieved for the FSGS score (P<0.05), whereas a trend was seen for the GSI score (P=0.063). Neutrophil gelatinase-associated lipocalin mRNA expression in the renal medulla of the 5-week group was significantly lower only in the ARB group. This effect disappeared in the 12-week group (results not shown).

ARNI reduces NCC

The major apical kidney sodium transporters were analysed in the 5-week and 12-week groups by immunoblotting (*Figures 5A and 5B* respectively). Both in the 5-week and 12-week groups, ARNI consistently reduced total and phosphorylated NCC. In the 12-week group, ARB also significantly reduced both NCC forms. In the 5-week group, ARNI additionally reduced the abundance of NHE3, but increased the abundance of the uncleaved γ-subunit of ENaC. The latter change was probably mediated by an increase, and decrease respectively in its well-known regulators SGK1 and NEDD4-2.[27] The abundance of the cleaved γ-subunit of ENaC was not affected by ARB or ARNI treatment at any timepoint.

ARNI upregulates vasodilator pathways

ACh fully relaxed pre-constricted mesenteric arteries in all treatment groups, both at 5 and 12 weeks (*Supplementary Figure S1*; pEC50=7.9±0.04). Blocking NO with L-NAME partially suppressed the effect of ACh, and the same was true for blocking the EDHF (endothelium-derived hyperpolarizing factor) pathway with TRAM34+apamin. Only when inhibiting both pathways was full blockade of the ACh response obtained. Treatment did not affect this outcome. The effect of L-NAME was generally more modest in the 12-week groups compared with the 5-week groups, and the opposite was true for the effect of TRAM34+apamin. Taken together, these data are suggestive for EDHF upregulation during long-term DM. SNAP, like ACh, fully relaxed pre-constricted mesenteric arteries at both 5 and 12 weeks (pEC50=6.8 ± 0.07), and this was unaffected by treatment, although ARNI slightly increased its potency in the 5-week group (*Supplementary Figure S2*; P<0.05).

ET-1 constricted mesenteric arteries equally in all treatment groups, both at 5 and 12 weeks (*Figure 6*; pEC50=8.2 ± 0.05). The ET_A receptor antagonist BQ123 consistently shifted the ET-1 CRC 5-10-fold to the right under all conditions. A leftward shift (P<0.05) was observed in the presence of the ET_B receptor antagonist BQ788 in the dual treatment group at 5 weeks only. The latter is suggestive for upregulation of ET_B receptor-mediated vasodilation.

Discussion

The present study shows that NI in addition to ARB ameliorates glomerular damage and cardiac hypertrophy in diabetic Ren2 rats. These effects appear to occur in a blood pressure-independent manner, since the antihypertensive effects of ARNI were similar to those of ARB alone, at least between 2 and 5 weeks after DM induction. This contrasts with our recent studies in non-diabetic Ren2 rats,[19] where irbesartan's blood pressure-lowering effect diminished over time, most likely due to the up-regulation of counterregulatory mechanisms such as renin release. This increase in blood pressure was prevented by adding thiorphan at a dose of 0.1 mg/kg per day.[19] The fact that the blood pressure-lowering effect of irbesartan remained constant in the diabetic animals suggests that their capacity to activate counterregulatory mechanisms is diminished. In agreement with this concept, the renin rise induced by RAS blockade with ARB (an indication of the degree of RAS suppression)[28]

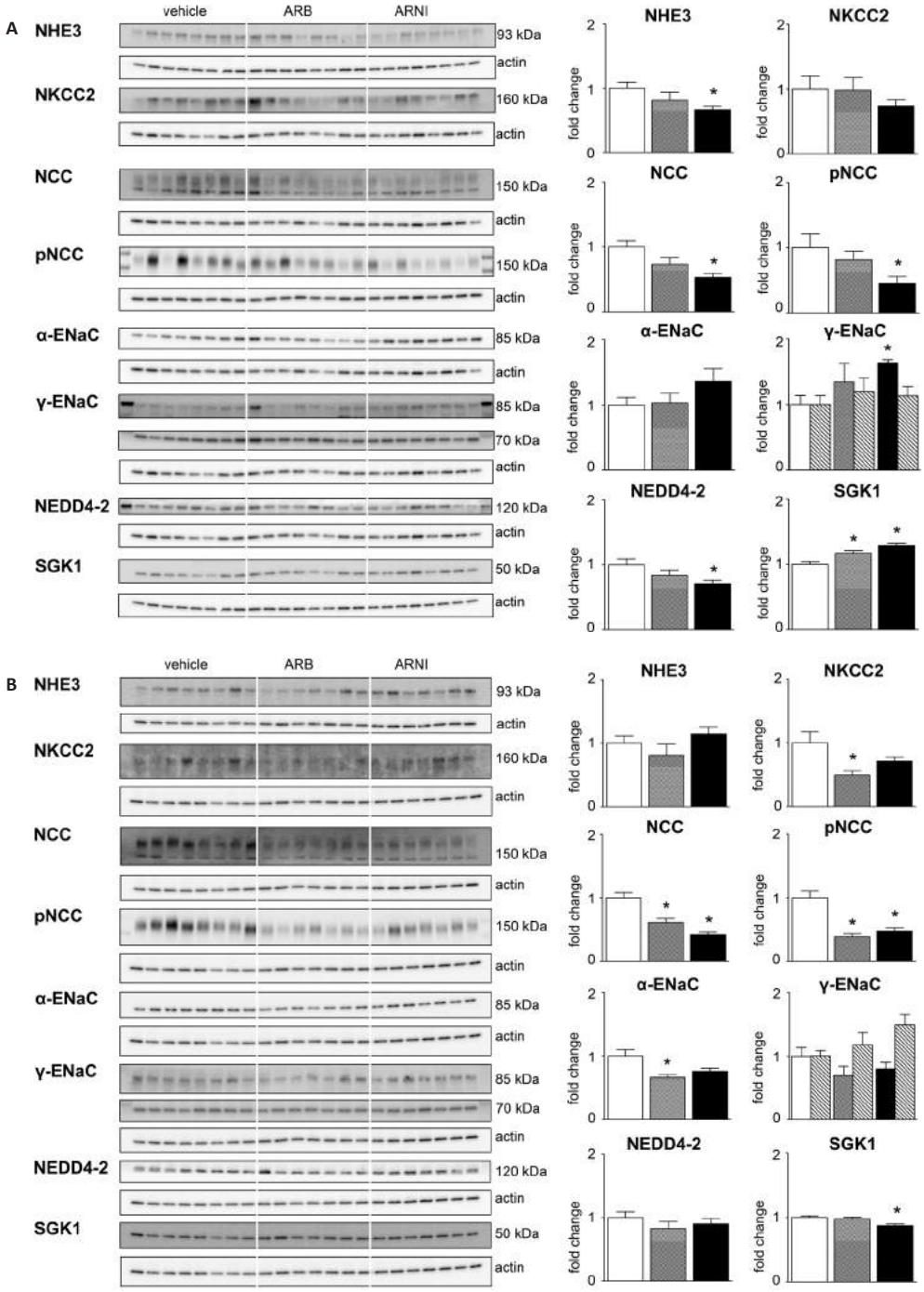

Figure 5. Effects of vehicle, ARB or ARNI on renal sodium transporters. Protein expression of renal sodium transporters, NEDD4-2 and SGK1 after vehicle (white bars), ARB (grey bars) or ARNI (black bars) treatment for 3 weeks, started at 2 (A) or 9 (B) weeks after DM induction. Left, representative blots, right fold change vs. vehicle.

The γ-ENaC graph displays 2 bars for each condition, the white, grey and black bars representing the non-cleaved subunit (85 kDa), and the hatched bar to the right of these 3 bars representing the cleaved form of the γ-subunit (70 kDa). Results are means ± S.E.M. (n=7-8). *P<0.05 compared with vehicle.

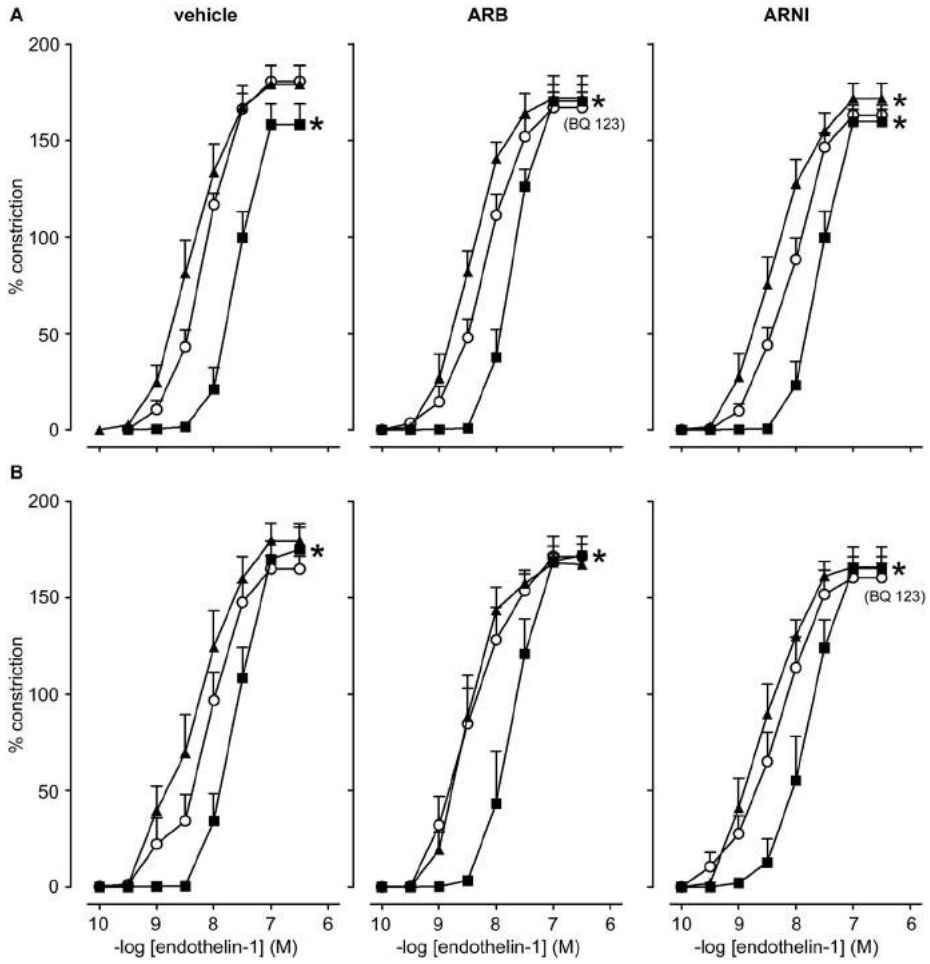

Figure 6. Effects of vehicle, ARB or ARNI on ET-1-induced constriction of mesenteric arteries. Contractions to ET-1 in mesenteric arteries obtained after vehicle, ARB or ARNI treatment for 3 weeks, started at 2 (A) or 9 (B) weeks after DM induction. Contractions were studied in the absence (control; ○) or presence of BQ123 (■) or BQ788 (▲). Results are means ± S.E.M. percentages of the contraction to 100 mmol/L KCl. (n=7-12) *P<0.05 compared with control (pEC50).

was modest at 5 weeks and virtually absent at 12 weeks. Similarly, in diabetic patients, renin levels are low.[29] Yet their levels of renin's precursor, prorenin, are enhanced, possibly reflecting activation of the intrarenal RAS.[29] The diabetic Ren2 rat closely mimics these alterations, and therefore appears a valid model to study the effects of ARNI in diabetes.

When dosed at 1.0 mg/kg per day, thiorphan increased circulating ET-1 in non-diabetic Ren2 rats, which subsequently raised blood pressure and increased renal NHE3 expression.[19] This is because neprilysin also degrades ET-1.[30] The present study, applying a 10-fold lower dose, did not detect any effect of thiorphan on circulating or urinary ET-1 levels. If anything, thiorphan in addition to irbesartan (ARNI) decreased NHE3 expression, a sodium transporter known to be regulated by ET-1.[31] Although this is reassuring with regard to the application of NI in DM, at least when appropriately dosed, we did observe a rise in renal medullary expression of prepro-ET-1 during ARNI treatment, at both 5 and 12 weeks. Since this also occurred during treatment with ARB at 12 weeks, it is not necessarily due to NI. Moreover, it did not result in increased urinary ET-1 levels, nor did it prevent the ARNI-induced renoprotection. Of interest, ARNI treatment up-regulated mesenteric ET_B receptor-dependent vasodilation at 5 weeks. This corresponds with the upregulation of ET_B receptors in the medulla, which are also known to induce vasodilation.[32] Clearly, ARNI has both positive and negative effects on the endothelin system, and the degree of NI will determine the delicate balance between these two.

LCZ696 preserved kidney function in heart failure patients with renal impairment, but simultaneously increased albuminuria.[8] This is not entirely unexpected, as an NI-induced rise in ANP may increase the permeability of the glomerular filtration barrier,[33] thereby annihilating the protective glomerular effects of LCZ696. In the diabetic Ren2 rat, ANP levels were only modestly and not significantly increased during ARNI treatment. It should be realized that plasma ANP levels reflect the net result of synthesis and metabolism. In the present study, its synthesis in the heart will have decreased due to the fall in blood pressure, resulting in diminished atrial stretch. This was evidenced by the reduced cardiac ANP expression at 12 weeks during irbesartan treatment, with (ARNI) and without (ARB) thiorphan. Simultaneously, ANP's half-life will have increased during NI. Yet, given the reduced synthesis, this is unlikely to result in a significant rise of ANP. A similar line of reasoning might apply to BNP. These findings could explain why we did not see a rise in urinary albumin excretion. In fact, thiorphan further reduced albuminuria and, particularly, proteinuria at 12 weeks, and reduced the severity of glomerulosclerosis. Since these effects occurred independently of blood pressure and changes in kidney function (as measured by creatinine or cystatin C), they most likely relate to the beneficial effects of ARNI on intraglomerular hemodynamics. In view of the fact that ARNI did not alter the DM-induced changes in urinary cGMP, it seems that the latter is unrelated to NO-induced cGMP generation. Nevertheless, our data in isolated arteries (albeit non-renal), did show an enhanced response to the NO donor SNAP. This supports an alternative explanation, namely the upregulation of post-cGMP pathways, e.g. at the level of phosphodiesterase and/or cGMP-dependent protein kinase I.[34] DM itself biphasically affected cGMP excretion: after an initial decrease at 5 weeks, a compensatory rise occurred at 12 weeks. The former most likely reflects the immediate consequences of DM on endothelial function, resulting in diminished NO bioavailability due to increased formation of advanced glycation end-products and reactive oxygen species,[35] whereas the latter mimics the increased renal, but not systemic, NO bioactivity known to occur in diabetic patients with renal hyperfiltration.[36] RAS blockers, by inhibiting efferent vasoconstriction, will typically prevent hyperfiltration.

At 5 weeks, ARNI, but not ARB, down-regulated NCC, a highly regulated Na^+-transporter

in the distal convoluted tubule.[37] Although NCC inhibition usually lowers blood pressure, e.g. during the use of thiazide diuretics, ARNI and ARB induced identical blood pressure decreases. The counterregulatory increase in uncleaved ENaC at this time point was probably mediated by NEDD4-2 and SGK1.[27] Despite this increase in uncleaved ENaC, the cleaved form of the γ-subunit of ENaC was not significantly different between the treatment groups at 5 weeks. Since it is the latter form that is associated with a higher open probability of the channel,[38] these data indicate that ENaC alterations did not contribute to the blood pressure-lowering effect of either ARNI or ARB. Because both NCC and ENaC are regulated by the same hormones, it is unclear why opposite effects on NCC and uncleaved ENaC might have occurred. Previously, we observed that losartan reduced NCC but not ENaC.[39] In the present study, at least at 12 weeks, ARB and ARNI identically reduced NCC, without affecting cleaved or uncleaved ENaC. To what degree a natriuretic effect of both treatments contributed to their antihypertensive effect cannot be said. Urinary sodium excretion was not significantly higher in the treatment groups compared with vehicle. Yet, this concerned a single measurement, and, given the new steady state after chronic treatment, cumulative sodium balances would be required to address this question.

ARNI did not affect kidney weight, but reduced heart weight. A parallel, albeit non-significant, reduction in cardiac fibrosis was observed, whereas no effects were seen with regard to cardiomyocyte size. The modest effect on fibrosis suggests that the reduction in heart weight may additionally involve other factors, such as a diminished (interstitial) fluid content, and a reduced deposition of advanced glycation end-products, lipids, or other toxic products of fatty acid metabolism.[40]

In conclusion, the favourable, blood pressure-independent effects of ARNI compared with ARB on the microvascular complications of diabetes, support the application of NI in ARB-treated diabetic patients in future clinical trials.

Funding
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Clinical prespectives

- Dual angiotensin II type 1 (AT_1) receptor-neprilysin inhibition (ARNI) exerts beneficial effects on blood pressure and kidney function in heart failure, compared with AT_1 receptor blockade (ARB) alone. We hypothesized that ARNI improves cardiac and kidney parameters in diabetic TGR(mREN2)27 rats, an angiotensin II-dependent hypertension model.
- ARNI reduced proteinuria, glomerulosclerosis, and heart weight in diabetic TGR(mREN2)27 rats more strongly than ARB, and this occurred independently of blood pressure.
- The favorable blood pressure-independent effects of ARNI versus ARB on the microvascular complications of diabetes support the investigation of NI in ARB-treated diabetic patients in future clinical trials.

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Supplemental information

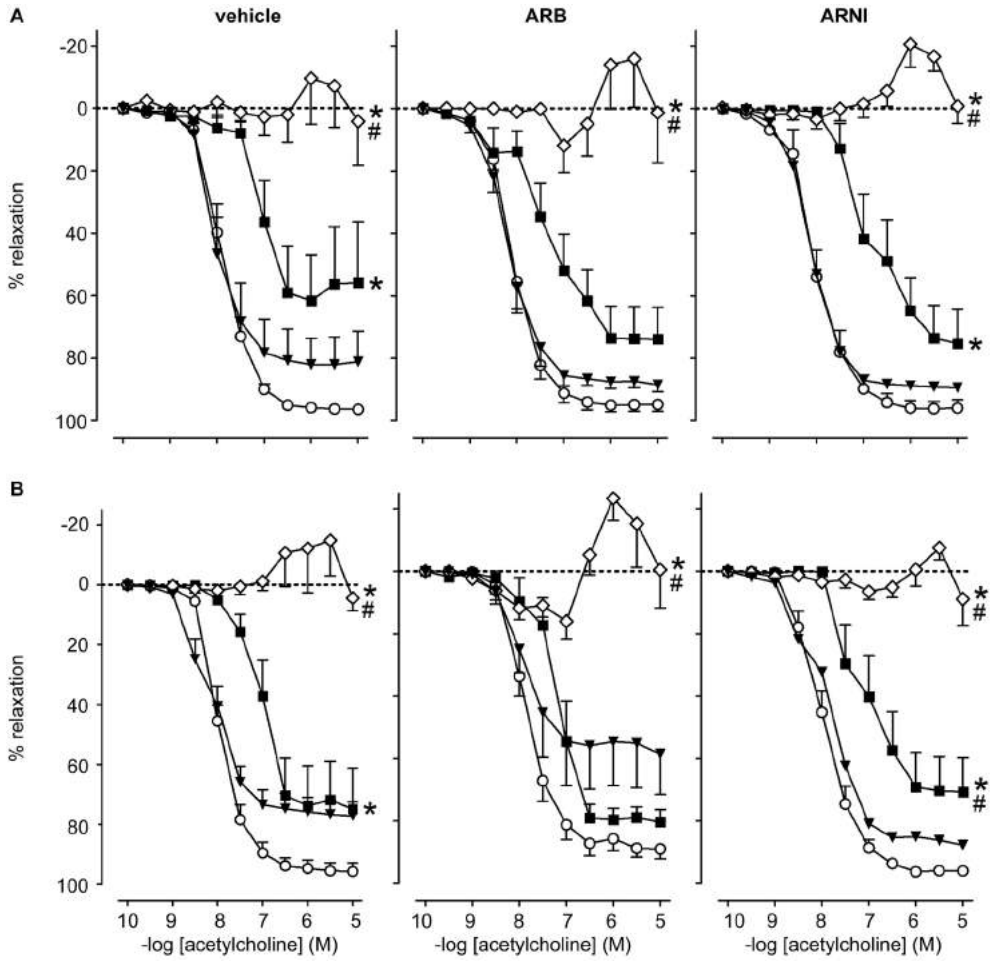

Figure S1. Relaxations to Ach in mesenteric arteries from diabetic (DM) Ren2 rats treated for 3 weeks with vehicle, ARB or ARNI. Treatment was started either 2 weeks (A) or 9 weeks (B) after the induction of DM. Relaxations were studied in the absence (control;○) or presence of L-NAME (■), TRAM34+apamin (▲), or L-NAME+TRAM34+apamin (◊), and are expressed as percentages of preconstriction (with U46619). Results are means ± S.E.M. (n=7-12), *P<0.05 compared with control (pEC50), #P<0.05 compared with control (Emax)

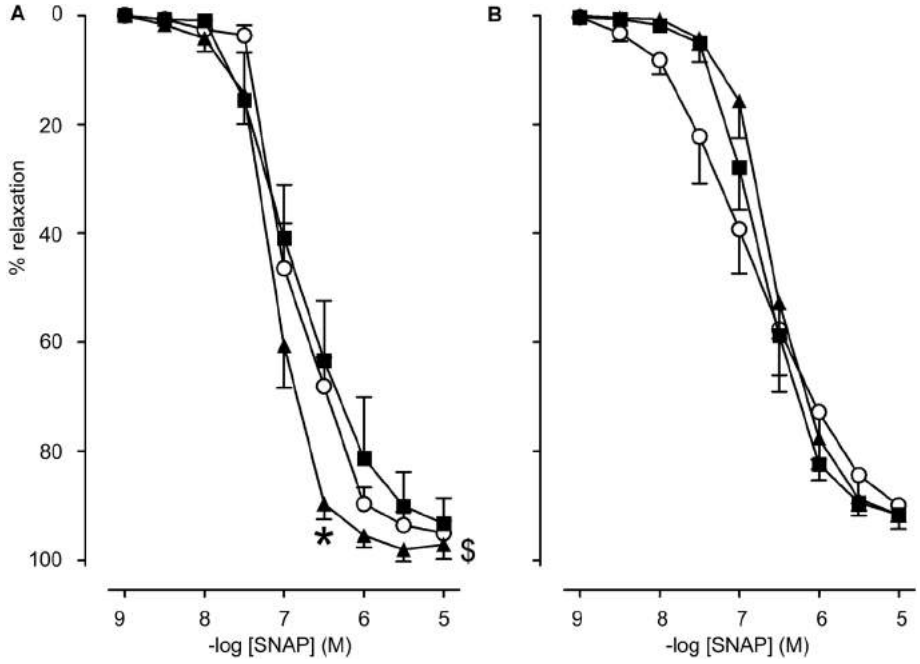

Figure S2. Relaxations to SNAP in mesenteric arteries from diabetic (DM) Ren2 rats treated for 3 weeks with vehicle (○), ARB (■) or ARNI (▲). Treatment was started either (A) 2 weeks or (B) 9 weeks after the induction of DM. Relaxations are expressed as percentages of preconstriction (with U46619). Results are means ± S.E.M. (n=7-12), *P<0.05 compared with control (pEC50), $P<0.05 copared with 12 weeks DM (pEC50).

Table S1. Real-time qPCR primers. Abbreviations: HPRT-1, hypoxanthine phosphoribosyltransferase; ANP, atrial natriuretic peptide; ET_AR, endothelin-1 type A receptor; ET_BR, endothelin-1 type B receptor; prepro-ET-1, prepro-endothelin-1; NPR-A, natriuretic peptide receptor-A; NPR-B, natriuretic peptide receptor-B; NPR-C, natriuretic peptide receptor-C; NGAL, Neutrophil gelatinase-associated lipocalin.

Gene	Forward primer 5'-3'	Reverse primer 5'-3'
HPRT-1	TGGACAGGACTGAAAGACTTGCTCG	CTTCAGCACACAGAGGGCCACA
β-actin	GGGAAATCGTGCGTGACATT	GCGGCAGTGGCCATCTC
$β_2$-microglobulin	ATGGCTCGCTCGGTGACCG	TGGGGAGTTTTCTGAATGGCAAGCA
ANP	ATGGGCTCCTTCTCCATCAC	TCTACCGGCATCTTCTCCTC
ET_AR	ATGAGGAACGGCCCCAATG	CCGCCAACAGCTTAAACACA
ET_BR	TGACCACTTAAAGCAGAGACGA	GAAGCCAACAGAGGGCAAAC
Prepro-ET-1	GACAAAGAACTCCGAGCCCA	AGCTTGGGACAGGGTTTTCC
NPR-A	CTGCTTCGGGGGTAGGATTG	TTCGGTACCGGAAGCTGTTG
NPR-B	GTTCAAGCTGCTTTGGGCAG	TAAAACAACCTCAGCCCGTCA
NPR-C	CCCTGGCGGCGGATTG	TTGCGTTGGAGGTGTTTCCA
NGAL	CAAGTGGCCGACACTGACTA	CCCCTTGGTTCTTCCGTACA

CHAPTER 9

BENEFICIAL EFFECTS OF COMBINED AT_1 RECEPTOR/NEPRILYSIN INHIBITION (ARNI) VERSUS AT_1 RECEPTOR BLOCKADE ALONE IN THE DIABETIC EYE

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Submitted

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Abstract
Aim Dysfunction of the renin-angiotensin system (RAS) contributes to the pathogenesis of diabetic retinopathy (DR). Yet RAS blockers have only limited beneficial effects on the progression of DR in clinical trials. The natriuretic peptide system offsets the RAS, so that enhancing the activity of this system on top of RAS blockade might be beneficial. Neprilysin has an important role in the degradation of natriuretic peptides. We have previously shown that dual angiotensin receptor-neprilysin inhibition (ARNI) has superior renoprotective effects compared with angiotensin receptor blockade (ARB) alone in diabetic hypertensive rats. We therefore hypothesize that ARNI may also outperform ARB in protection against DR. We tested this hypothesis in streptozotocin (STZ)-induced diabetic transgenic (mRen2)27 rats, a well-known model of DR.
Methods Adult (mRen2)27 rats were made diabetic with streptozotocin and followed for 5 or 12 weeks. Treatment with vehicle, irbesartan (ARB) or irbesartan combined with the neprilysin inhibitor thiorphan [irbesartan+thiorphan (ARNI)] occurred during the final 3 weeks. Retinal apoptotic cell death and gliosis were determined by TUNEL assay and immunofluorescence using specific antibodies against cell death markers, glial fibrillary acidic protein and microglial markers. Retinal capillary loss was evaluated from trypsin-digested retinal vascular preparation. Western blot and real-time PCR analysis were performed to quantify the level of inflammatory cell markers.
Results Both ARB- and ARNI-treated groups showed similarly reduced retinal apoptotic cell death, gliosis and capillary loss compared to the vehicle-treated group in the 5-week study. ARNI treatment reduced the expression of inflammatory markers ICAM1, MCP1, VEGF, and TNFα more than ARB treatment in the 5-week study. In the 12-week study, ARNI treatment showed significantly more reduction in apoptotic cell death (51% vs 25% reduction), and capillary loss (68% vs 43% reduction) than ARB treatment. In the 12-week study ARB- and ARNI-treatment reduced the expression of inflammatory markers equally.
Conclusions ARNI provides better protection against DR diabetic (mRen2)27 transgenic rats, compared with ARB alone. This approach may be a promising treatment option for patients with DR.

Introduction

Diabetes affects approximately 387 million people worldwide. Diabetic retinopathy (DR) is the most common complication of diabetes and the major cause of vision loss in middle-aged subjects.[1] The development of DR is strongly associated with the development of diabetic kidney disease, suggesting that the same pathogenic pathway underlies these complications.[2] The renal renin-angiotensin system (RAS) is upregulated in diabetes, evidenced by increased tissue levels of angiotensin II, the main effector peptide of the RAS. Elevated levels of angiotensin II contribute to pathogenic processes such as inflammation, vascular remodeling, and oxidative stress[3] that are associated with various renal and cardiovascular disorders like heart failure, hypertension, diabetes and other metabolic disorders.[4, 5] DR is associated with activation of the local RAS in the eye, evidenced by an increase in retinal angiotensin II levels.[6, 7] Blockers of the RAS, like angiotensin-converting enzyme inhibitors (ACEi) and angiotensin II type 1 receptor blockers (ARBs) provide protection against the progression of cardiovascular disorders, diabetic kidney disease and diabetic retinopathy.[8] Still, these drugs are unable to completely reverse or halt the progression of these complications. The natriuretic peptide system regulates diuresis, natriuresis and vasodilatory functions of the cardiovascular system, and it offsets the RAS, so that enhancing the activity of this

system on top of RAS blockade might be beneficial.[9] Neprilysin (NEP) is a critical enzyme for the breakdown of a variety of substrates, including vasodilators, vasoconstrictors, and natriuretic peptides. The individual use of NEP inhibitors however did not prove to be very beneficial for the treatment of heart failure and other cardiovascular disorders, mainly due to its broad action on different vasodilators and vasoconstrictors. When a NEP inhibitor is combined with an angiotensin receptor blocker (the combination is referred to as 'ARNI'), the balance shifts to the positive, blood pressure-lowering side. Indeed, ARNI has been shown to be more effective than RAS blockade alone in (1) reducing blood pressure,[10] (2) improving morbidity and mortality in heart failure,[11-13] and (3) improving surrogate cardiovascular outcomes in diabetic patients.[14, 15] In diabetic, hypertensive rats, we have previously shown that ARNI lowers proteinuria and the development of focal segmental glomerulosclerosis, common features of diabetic kidney disease, more than ARB alone. This effect may even be independent of blood pressure.[16] We therefore hypothesize that dual inhibition may also provide better protection against DR, compared with ARB alone. We tested this hypothesis in transgenic (mRen2)27 (Ren2) rats made diabetic with streptozotocin (STZ). This is a well-known model of diabetic retinopathy.[17-20] We made use of the ARB irbesartan and the NEP inhibitor thiorphan.[16, 21]

Methods

Animal studies

Heterozygous Ren2 rats (age 10 weeks, weight 300-500 g) were obtained by crossing homozygous Ren2 and Sprague-Dawley (SD) rats. Studies were performed under the regulation and permission of the Erasmus MC Animal Care Committee. Diabetes mellitus was induced by administering streptozotocin (55 mg/kg i.p., Merck Millipore, Amsterdam, The Netherlands), and diabetic animals were studied for 5 or 12 weeks. In the 5-week group, heart rate and BP were measured by radiotelemetry transmitters, implanted 2 weeks before induction of diabetes.[22] Rats were checked daily for non-fasting blood glucose and β-ketone levels until day 3 after STZ injection, and thereafter once-weekly or every other week (5- and 12-week groups, respectively) (Precision Xceed, Abbott, Zwolle, The Netherlands). Only rats with glucose >15 mmol/L were considered diabetic; they received 2-4 U insulin per day (Levemir®, Novo Nordisk, Denmark). Rats in both groups were treated during the final 3 weeks of the study (i.e., during weeks 2-5 or 9-12) with vehicle (saline containing 0.2% DMSO), the ARB irbesartan (Sanofi-Aventis, Chilly-Mazarin, France; 15 mg/kg.day), or the ARNI irbesartan+thiorphan (Sigma-Aldrich, Zwijndrecht, The Netherlands; 0.1 mg/kg.day diluted in DMSO) making use of osmotic minipumps (2ML4 ALZET, Cupertino, USA). After three weeks of treatment, animals were sacrificed by i.p. pentobarbital injection (200-240 mg/kg). Renal, cardiac and vascular data on these animals have been reported previously.[16] Eyes were harvested, and fixated in 4% paraformaldehyde (PFA) for histological analysis, or snap frozen in liquid nitrogen for gene expression analysis.

Immunofluorescence

4% PFA fixed eyes were processed for paraffin embedding. Paraffin sections (4 µm thick) were cut and mounted on superfrost slides. Sections were deparaffinized in xylene and decreasing concentration of alcohol. This was followed by an antigen retrieval step where the slides were boiled for 20 minutes in sodium citrate buffer, pH 6.0. The sections were then incubated in blocking solution (5% BSA + 0.3 % Triton X100 in PBS) for 1 hour. This was followed by incubation with different primary antibodies: rabbit anti-glia fibrillary acidic protein (GFAP) (1:1000, Sigma-Aldrich, St. Louis, MO), rabbit anti-Iba-1 (1:200,

DAKO, Carpinteria, CA), rabbit anti-active caspase-3 (1:200, Millipore, Billerica, MA) diluted in the same blocking solution (overnight at 4°C). The sections were then incubated with the appropriate secondary antibodies conjugated to Alexa 488 or 594 (Molecular Probes/ Invitrogen, Eugene, OR) for 1 hour at RT. Sections were washed in PBS containing the nuclear counterstain DAPI (4',6 diamidino-2- phenylindole), and mounted in Dako mounting media (DAKO, Carpinteria, CA). For TUNEL staining the In Situ Cell Death Detection Kit, TMR red (Roche Applied Science, Indianapolis, IN) was used on paraffin sections in accordance to the manufacturer's instructions. The images were captured on a Keyence confocal microscope (KEYENCE Corporation, Itasca, IL) or on a spinning disc confocal microscope (Ultra VIEW Vox, PerkinElmer, Waltham, MA) using a 20x, 40x and/or 60x objective lens and were prepared for presentation using Adobe Photoshop (Adobe Systems Inc., San Jose, CA).

Trypsin digest preparations of retinal vasculature

Trypsin digest preparations of retinal vasculature were made as described previously.[23] Briefly, the retinas were dissected from the 4% PFA fixed eye cups, washed in water for an hour, and digested in 3% trypsin for 2-3 hours at RT. The tissue was then transferred into water and the network of vessels was freed from adherent retinal tissue by gentle shaking and manipulation under a dissection microscope. The vessels were then mounted on clean slides and allowed to dry and stained with PAS-H&E (periodic acid Schiff-hematoxylin and eosin; Gill No.3; Sigma-Aldrich, St. Louis, MO). After the tissue was stained and washed in water, it was dehydrated and mounted (Permount mounting media; Fisher Scientific, Pittsburgh, PA). The prepared retinal vessels were photographed by a Zeis microscope equipped with a high-resolution digital camera (AxioCam, MRC5, Zeis Axionvert 200) using both 20X and 40X objective lenses, 6-8 representative non-overlapping fields from each quadrant of the retina were imaged. Acellular capillaries were counted from images for each retina and expressed as the number of acellular vessels per square millimeter.

Western blot analysis

To quantify the level of GFAP expression in the different experimental groups, retinal tissues were collected in cold RIPA buffer (Sigma-Aldrich, St. Louis, MO) supplemented with protease inhibitors and were homogenized by sonication. Homogenized tissues were centrifuged at 12000 x g for 15 minutes at 4°C and the supernatant was collected. The protein concentration was detected by Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA). 20 µg of protein samples were loaded and separated on 10% gels for 1 hour at 120 V in Tris-glycine buffer and electrophoretically transferred onto polyvinylidene difluoride membrane. Immunodetection was performed on blots blocked in fluorescence-blocking buffer for 1 hour (Rockland Immunochemicals, Inc., Gilbertsville, PA) and then incubated with primary (rabbit anti- GFAP; 1:2000; Sigma-Aldrich, St. Louis, MO), and secondary (goat anti-rabbit IR Dye 800; 1:5000; Rockland Immunochemicals Inc., Gilbertsville, PA) antibodies. β-Actin antibody (mouse anti-β-actin; 1:5000; Sigma-Aldrich, St. Louis, MO) immunoblotting was used as a loading control. Immunoblots were visualized and quantified by using a Li-Cor odyssey infrared imager after normalizing it to β-Actin (Odyssey; Li-Cor, Lincoln, NE).

Real-time PCR analysis

Total RNA was isolated from frozen rat retinal samples using Trizol Reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instruction. Reverse transcription was performed using Enhanced Avian HS RT-PCR kit (Sigma-Aldrich) following manufacturer's instructions. Real-time PCR was carried out on a real-time thermal cycler (iCycler, Bio-Rad Life Sciences,

Hercules, CA) using iQTM Syber Green Supermix (Bio-Rad Life Sciences, Hercules, CA). The threshold cycle number (Ct) for real-time PCR was set by the cycler software. Optimal primer concentration for PCR was determined separately for each primer pair. Each reaction was run in duplicate or in triplicate, and reaction tubes with target primers and those with actin primers were always included in the same PCR run. The expression levels of the different genes were established based on the Ct compared with control housekeeping gene β-actin in each sample (amount of target = 2−ΔΔCt) and presented as fold change. The rat specific primers used in this experiment are listed in *Table 1*.

Table 1. Real-time qPCR primers.

Gene	Accession Number	Forward primer 5'-3'	Reverse primer 5'-3'
ICAM-1	NM_012967	CCCCACCTACATACATTCCTAC	ACATTTTCTCCCAGGCATTC
TNFα	NM_012675	CCTTATCTACTCCCAGGTTCTC	TTTCTCCTGGTATGAAATGGC
VEGF	NM_031836	TGCACCCACGACAGAAGGGGA	TCACCGCCTTGGCTTGTCACAT
MCP-1	NM_031550	GCAGCAGGTGTCCCAAAGAAGCT	AGAAGTGCTTGAGGTGGTTGTGGAA

Results

Induction of diabetes mellitus and general physiologic characteristics

Ren2 rats developed hypertension but had a normal blood glucose level, identical to that in SD rats as reported previously.[16, 17] STZ-induced diabetes mellitus increased blood glucose levels (*Table 2*). Treatment with ARB or ARNI did not affect the glucose level nor the body weight, but significantly reduced mean arterial blood pressure (*Table 2*).

ARNI reduced gliosis more strongly than ARB after 12 weeks of diabetes

Immunostaining for GFAP, an astrocyte-specific marker, was done in each group to evaluate the level of gliosis. In a normal healthy retina, GFAP expression is confined to the astrocytes at the inner limiting membrane. However, under pathological conditions like DR, GFAP expression is elevated and also observed in the Müller cell processes that extend across the retina from the inner limiting membrane to the outer limiting membrane. In a previous study in non-diabetic Ren2 rats, we observed that retinal GFAP expression is slightly

Table 2. Main characteristics of diabetic (DM) Ren2 rats that were treated for 3 weeks with vehicle, irbesartan (ARB) or irbesartan+thiorphan (ARNI). Treatment was started either 2 weeks ('5 weeks DM') or 9 weeks ('12 weeks DM') after the induction of DM. Results are means ± S.E.M. N=7-12 rats per group. *P<0.05 compared with baseline; # P<0.05 compared with vehicle.

	5 weeks DM			12 weeks DM		
Parameter	**Vehicle**	**ARB**	**ARNI**	**Vehicle**	**ARB**	**ARNI**
Body weight (g)	448 ± 13	452 ± 16	450 ± 11	448 ± 9	482 ± 20	430 ± 23
Blood glucose (mmol/L)	27 ± 0.6	27 ± 0.7	27 ± 0.5	27 ± 0.3	25 ± 1.9	26 ± 0.7
Baseline mean arterial pressure (mmHg)	157 ± 10	160 ± 10	154 ± 10	-	-	-
Change in mean arterial pressure (mmHg)	-9.5 ± 4.3	-45.0 ± 7.8*#	-47.7 ± 7.9*#	-	-	-

elevated versus age-matched SD controls in astrocytes.[17] In diabetic Ren2 rats, retinal GFAP expression was greatly elevated, and seen along the Müller cell processes (*Figure 1*). The longer the duration of diabetes, the higher the level of GFAP expression that was observed. ARB- and ARNI-treated groups showed similarly reduced retinal gliosis compared to the vehicle-treated group in the 5-week study. In the 12-week study, the ARNI-treated group showed a marked reduction in the level of GFAP expression when compared to the vehicle and the ARB-treated group (*Figures 1A and B*). Retinal sections from each group were also immuno-stained for Iba-1, which is a marker for activated microglial cells. In the 5-week study, ARB- and ARNI-treated groups displayed almost the same number of Iba-1-positive

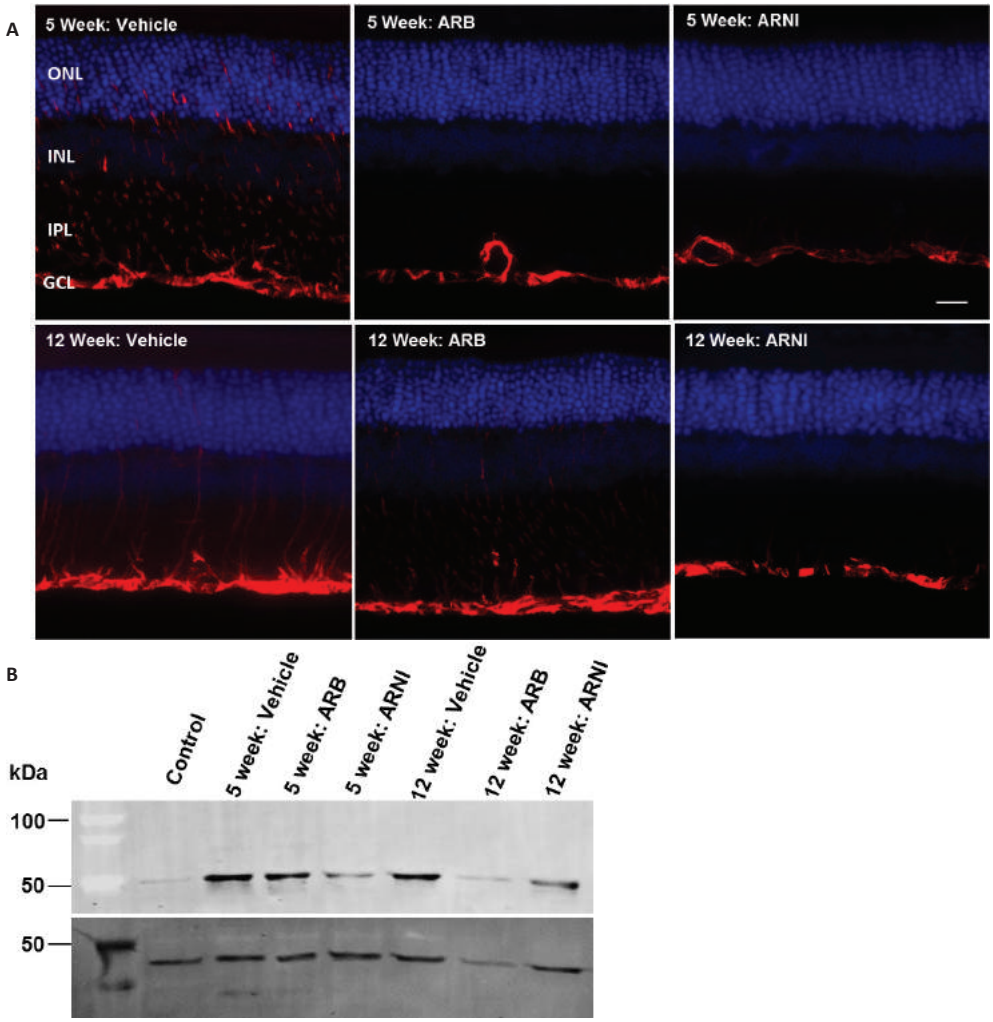

Figure 1. Immunofluorescence and western blot detection of GFAP expression in retina. (A) GFAP immunostaining in 5-week (top panels) and 12-week (bottom panels) diabetic rats, treated for 3 weeks with vehicle, irbesartan (ARB) or irbesartan+thiorphan (ARNI). Scale Bar = 20 µm. ONL: outer nuclear layer; INL: inner nuclear layer; IPL: inner plexiform layer; RGC: retinal ganglion cells. (B) GFAP western blot showing a reduction in GFAP expression in ARB- and ARNI-treated groups when compared to the vehicle group. Upper panel: GFAP and bottom panel: β-actin

cells as the vehicle-treated group. In the 12-week study, the total number of Iba-1-positive cells was significantly (P< 0.01) reduced in the ARNI-treated group when compared to the vehicle and ARB-treated groups (*Figures 2A and B*). ARB treatment alone also reduced the total number of Iba-1-positive cells versus vehicle.

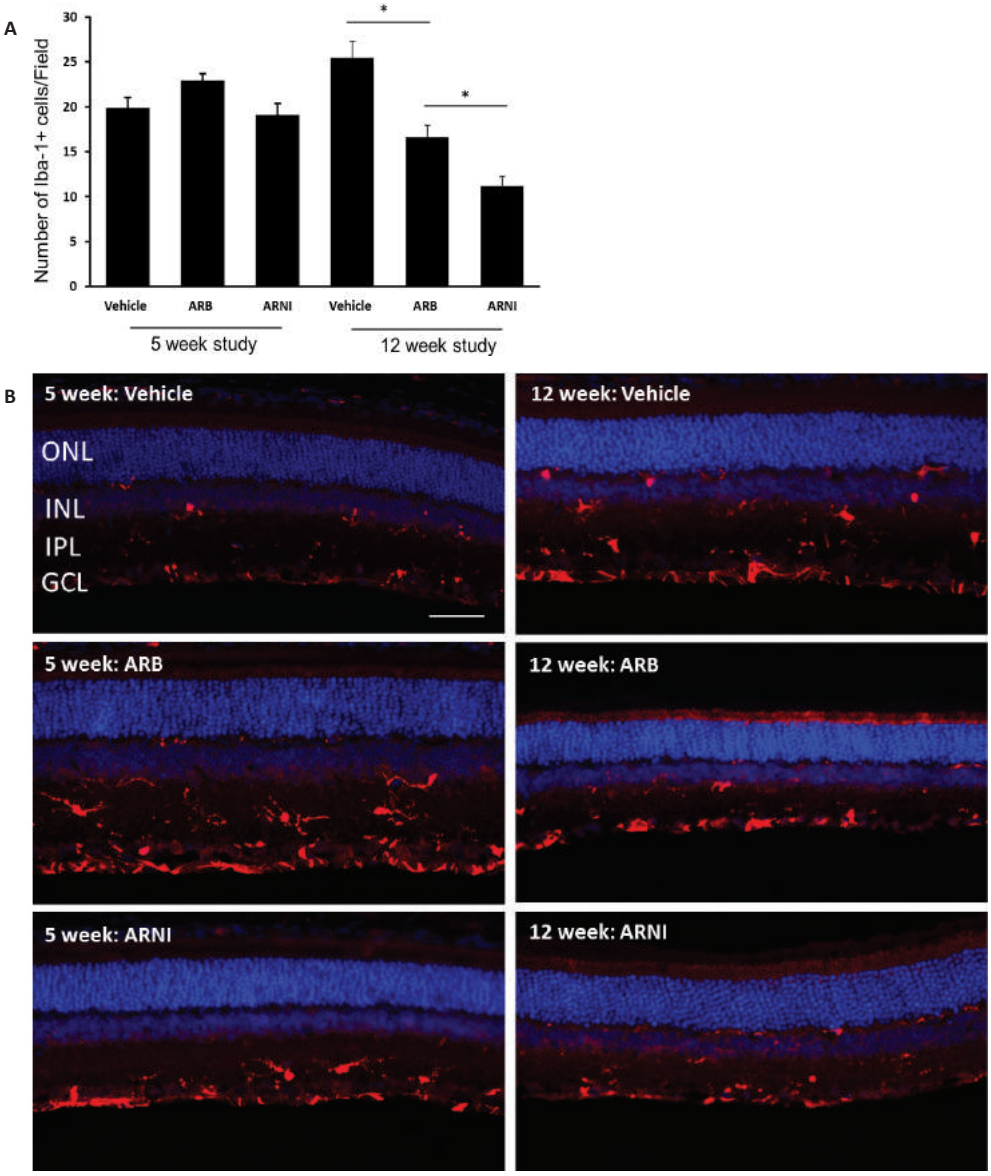

Figure 2. Immunofluorescence detection of activated microglial cells in retina. (A) Quantification of Iba-1 positive cells in 5-week ('5 week study') and 12-week ('12 week study') diabetic rats, treated for 3 weeks with vehicle, irbesartan (ARB) or irbesartan+thiorphan (ARNI). (B) Representative images of Iba-1 Immunostaining in rat retinal sections from different groups. Scale Bar = 20 µm. ONL: outer nuclear layer; INL: inner nuclear layer; IPL: inner plexiform layer; RGC: retinal ganglion cells. *P<0.05, N= 4 per group.

ARNI is more effective than ARB in reducing apoptotic cell death after 12 weeks of diabetes
Paraffin sections from each group were processed for TUNEL labeling to evaluate retinal degeneration. Both ARB- and ARNI-treated groups showed similarly reduced apoptotic cell death compared to the vehicle-treated group in the 5-week study. In the 12-week study, the ARNI-treated group showed a marked reduction (51%) in the number of apoptotic cell death when compared to the vehicle-treated group, while the ARB-treated group showed only a 25% reduction versus vehicle (*Figure 3A and B*).

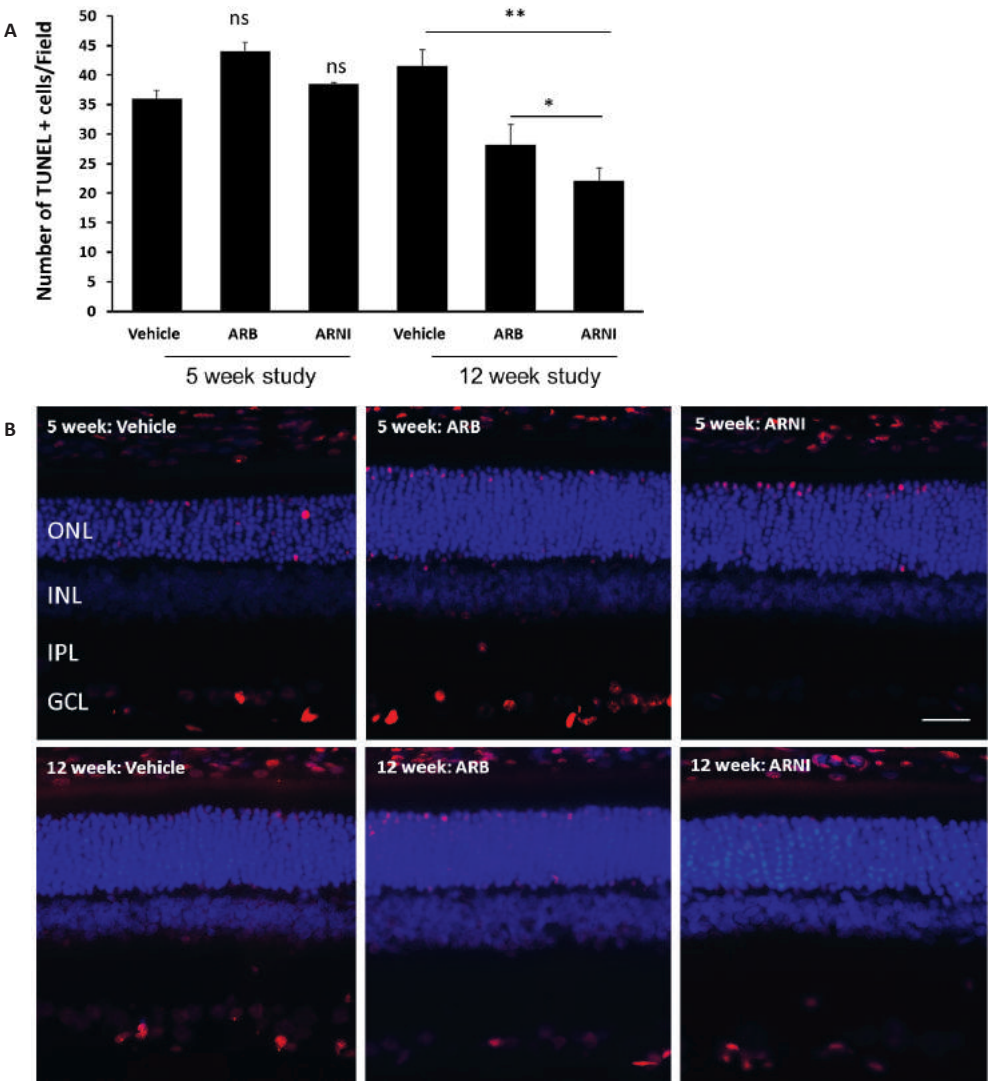

Figure 3. In situ cell death detection by TUNEL assay in retina. (A) Quantification of TUNEL positive cells in 5-week ('5 week study') and 12-week ('12 week study') diabetic rats, treated for 3 weeks with vehicle, irbesartan (ARB) or irbesartan+thiorphan (ARNI). (B) Representative images of TUNEL immunostaining in rat retinal sections from different groups. Scale Bar = 20 m. ONL: outer nuclear layer; INL: inner nuclear layer; IPL: inner plexiform layer; RGC: retinal ganglion cells. *P<0.05, **P<0.001, N= 4 per group.

ARNI reduced capillary loss more strongly than ARB after 12 weeks of diabetes
We have reported previously that the Ren2 rat retina shows an increased loss of capillaries when compared to age-matched SD rats.[17] STZ-induced diabetes in Ren2 rats further exacerbated this loss. ARB- and ARNI-treated groups showed a similar reduction in the number of acellular capillaries compared to vehicle-treated group in the 5-week study. In the 12-week study, ARNI treatment showed significantly more reduction in capillary loss (68% vs 43% reduction) than ARB treatment (*Figure 4*).

ARNI decreased expression levels of inflammatory markers more strongly than ARB after both 5 and 12 weeks of diabetes
Real-time RT-PCR was used to evaluate the expression level of inflammatory cytokines and angiogenic factors in the retina from each experimental group. In both the 5-week study and the 12-week study ARB and ARNI treatment significantly reduced the mRNA levels of tumor necrosis factor-α (TNF-α), intercellular adhesion molecule-1 (ICAM-1), vascular endothelial growth factor (VEGF) and monocyte chemoattractant protein-1 (MCP-1) when compared to the vehicle treated groups (*Figure 5*).

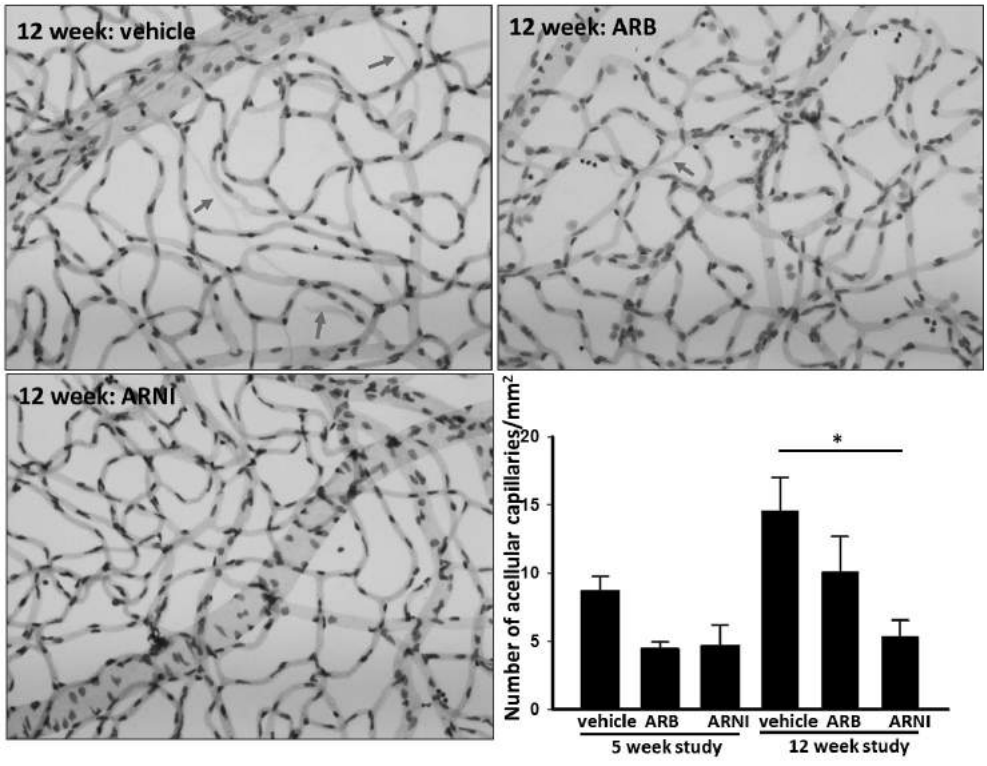

Figure 4. Histological detection of acellular capillaries in retina. (A-C) Representative images of trypsin-digested retinal vascular preparations from 12-week diabetic rats, treated for 3 weeks with vehicle, irbesartan (ARB) or irbesartan+thiorphan (ARNI). (D) Quantification of acellular capillaries in 5-week ('5 week study') and 12-week ('12 week study') diabetic rats, treated for 3 weeks with vehicle, irbesartan (ARB) or irbesartan+thiorphan (ARNI). *P<0.01, N= 4 per group.

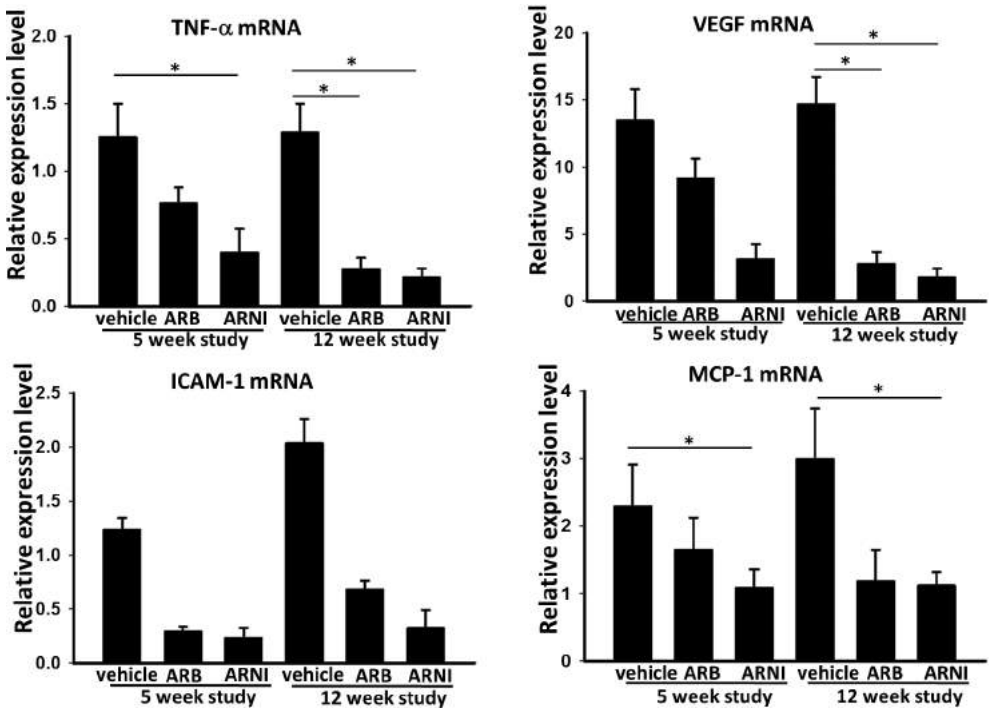

Figure 5. Real-time RT-PCR detection of inflammatory cytokines in retina. mRNA expression of TNF-α, VEGF, ICAM-1 and MCP-1, in fold change normalized to β-actin, in rat retinas of 5-week ('5 week study') and 12-week ('12 week study') diabetic rats, treated for 3 weeks with vehicle, irbesartan (ARB) or irbesartan+thiorphan (ARNI). *P<0.01, N= 4 per group.

Discussion

The present study firstly showed that STZ-induced diabetes in Ren2 rats resulted in increased capillary loss, inflammatory cytokine expression, gliosis and neuronal apoptotic cell death in the retina, which are all indicators of DR. This is consistent with our earlier work in this model.[17] Secondly, these symptoms exacerbated after a longer duration of diabetes; the increase in capillary loss, inflammatory cytokine expression, gliosis and apoptotic cell death were all significantly worsened after 12 weeks of diabetes compared to 5 weeks of diabetes. This could have important clinical significance in terms of developing potential therapeutics, as DR, a progressive retinal neuro-vascular disorder, is strongly associated with prolonged duration of diabetes in patients.[1] Thirdly, this study provides strong evidence that, in the 12-week study, ARNI is much more effective than ARB alone in ameliorating the retinal neurovascular dysfunctions observed in diabetic Ren2 rats, while the two treatment were equally effective at 5 weeks. Thus, the use of a NEP inhibitor in addition to an ARB seems to provide additional benefits effects on the retinal neurovascular system, making ARNI a promising therapeutic approach in the treatment of DR.

A large body of evidence suggests that a deficiency in the natriuretic peptide system in addition to a hyperactive RAS is associated with various renal and cardiovascular diseases like heart failure, hypertension and metabolic syndromes.[24-26] Natriuretic peptides are a family of hormones that help maintain sodium and fluid balance. This system comprises of three structurally similar peptides: atrial natriuretic peptide (ANP), B-type natriuretic

peptide (BNP) and C-type natriuretic peptide (CNP).[27] All three peptides have cardio-renal protective properties. Natriuretic peptides, in addition to having potent natriuretic, diuretic and vasodilator properties, also lower sympathetic drive, and have anti-proliferative and anti-hypertrophic effects.[28] These actions are mediated by the binding of ANP and BNP to the type A receptor, which is coupled to guanylyl cyclase. CNP is found mostly in the central nervous system, kidneys and vascular endothelial cells; it has anti-thrombotic and anti-fibrotic effects and binds to the type B receptor. The activation of both these receptors increases the expression of the second messenger cGMP which mediates most of the natriuretic peptide mechanisms of action. The natriuretic peptides are cleared from circulation via receptor-mediated internalization (involving the natriuretic peptide clearance receptor) and/or degradation by extracellular proteases like NEP.[29, 30] Natriuretic peptides and their receptors are expressed and functional in the rodent and human eyes in lens epithelial cells, vitreous, neural retina and retinal pigment epithelium (RPE).[31-33] Although their role in the retina is not yet clear, induction of diabetes in rats causes downregulation of retinal ANP expression. The activity level of NEP in the vitreous of patients with proliferative DR is increased compared to control patients.[33] Moreover, it has been shown that both ANP and CNP can suppress endothelial and RPE leakage, prevent choroidal neovascularization,[34, 35] and reverse advanced glycation end-product-induced retinal blood-barrier dysfunction in RPE.[32] All these processes precede the development of DR. Thus, increasing the level of natriuretic peptides in the retina could be beneficial in the treatment of DR.

Increasing the levels of natriuretic peptide by NEP inhibitors has been investigated as a therapeutic approach for treatment of hypertension and heart failure.[36, 37] However, the use of NEP inhibitors individually was not very beneficial, due to its broad effects on a variety of substrates other than natriuretic peptides.[28] Indeed, NEP is critical for the processing and catabolism of vasoactive peptides and peptides involved in diuresis, natriuresis and blood pressure regulation, like angiotensin II, bradykinin, substance P, adrenomedullin, glucagon, vasoactive intestinal peptide, and endothelin-1.[27] Therefore, in recent studies NEP inhibitors were used in combination with other cardiovascular agents such as ACEi and ARBs to at least counteract their potentiating effects on angiotensin II.[28, 38, 39] The combination of a NEP inhibitor and an ACEi was not useful because of a high incidence of angioedema, due to increased bradykinin levels [40, 41] related to the fact that NEP and ACE are the main enzymatic pathways for the breakdown of bradykinin. An alternative combination is that of an ARB and a NEP inhibitor (ARNI). ARBs do not disrupt bradykinin metabolism, and patients on ARNI did not seem to develop angioedema.[38, 39] This combination could be additionally beneficial because blockade of the angiotensin II type 1 receptor by ARB facilitates the binding of angiotensin II to the angiotensin II type 2 receptor that elicits several favorable actions. Clinical studies using ARNI for the treatment of heart failure and hypertension have shown much stronger favorable results when compared to ARB, without any extra associated negative side effects.[10, 12, 39, 42]

Possible chronic adverse effects of NEP inhibitors on the eye have not specifically been assessed yet. NEP has a possible neuroprotective effect as it brings about degradation of the amyloid β peptide.[33, 43] Elevated levels of amyloid β peptide in the brain is associated with cognitive impairment, and extracellular deposition leads to the development of Alzheimer's disease. Elevated levels of amyloid β peptide in the eye have been found in different progressive retinal neurodegenerative disorders such as age-related macular degeneration.[44-46] Hence, further studies need to be carried out to better understand the long-term effects of NEP inhibitors on the eye before they can be applied for the treatment of DR. Nevertheless, the beneficial effect that ARNI has over ARB on retinal neurovascular

symptoms in diabetic Ren2 rats suggest that this treatment holds promise as a novel potential therapeutic strategy for treatment of patients with DR.

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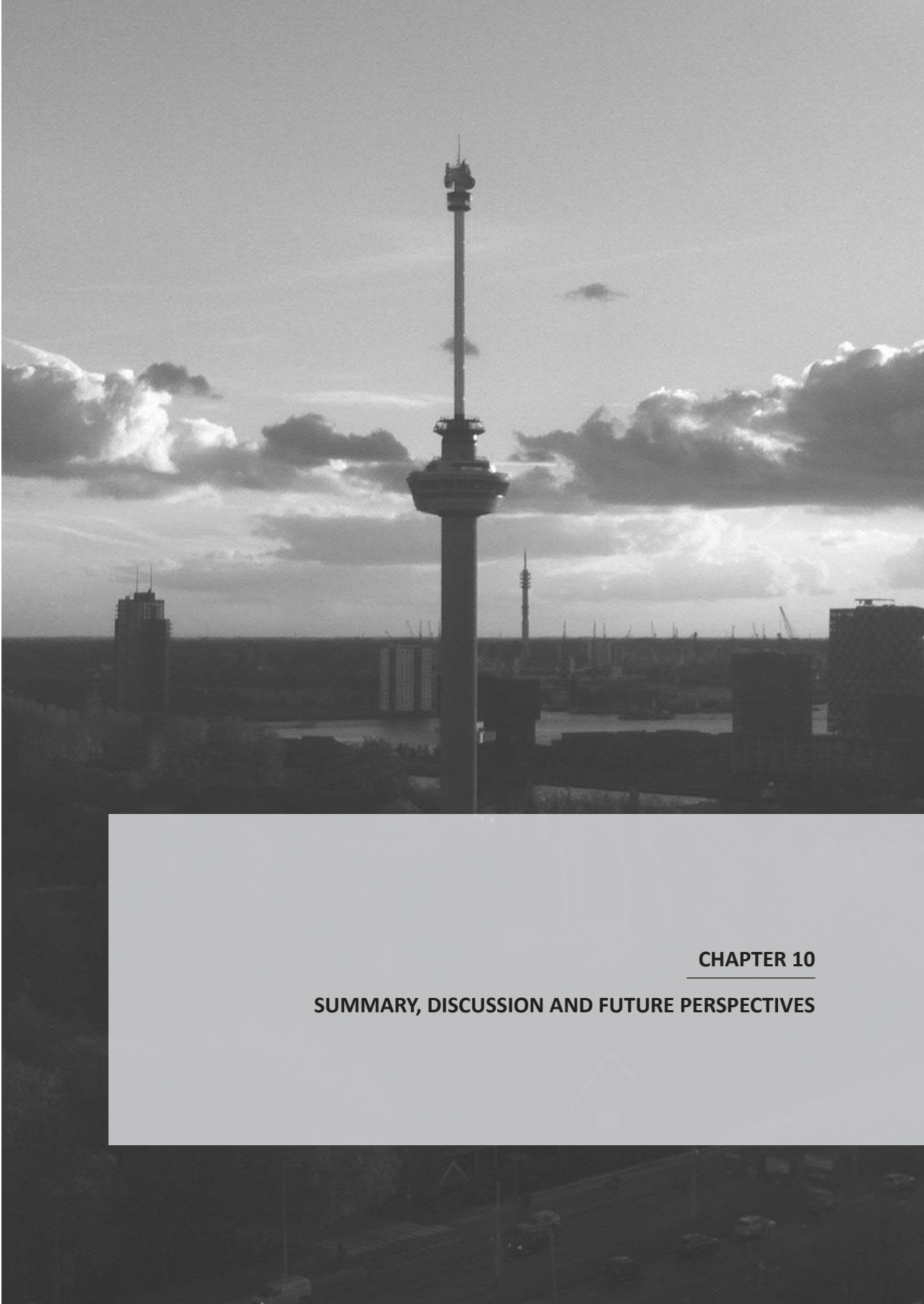

CHAPTER 10

SUMMARY, DISCUSSION AND FUTURE PERSPECTIVES

Summary

The intrarenal renin-angiotensin system (RAS)

In addition to the systemic (endocrine) RAS, it is generally believed that the kidney has its own (paracrine) RAS, since it is capable of local angiotensin II (Ang II) synthesis. Renal expression of other RAS components has been demonstrated in the proximal tubule (angiotensinogen and ACE), and the collecting duct (CD; renin). Another source of RAS components in the kidney may be endocytotic uptake from the ultrafiltrate. It has been suggested that RAS components in urine reflect the activity of the intrarenal RAS independently from the systemic RAS. This implies that urinary RAS components can be used as a marker for the activity of the intrarenal RAS, or even as renal disease markers. **Chapter 2** gives an overview of studies on urinary RAS components as markers of the intrarenal RAS. Renin, the rate limiting enzyme of the RAS, is not only expressed in the juxtaglomerular apparatus (JGA), but also in the CD. Renin expression in the CD is upregulated in diabetes.[1] Urinary renin is decreased in patients treated with a RAS blocker and increased in diabetics. However, it does not correlate with albuminuria, and therefore it seems unlikely that urinary renin reflects breakdown of the glomerular filtration barrier (GFB).[2] In **Chapter 4** we show that renin and prorenin are filtered by the glomerulus, and that filtration increases upon damage to the GFB. Even in rats with supraphysiologically elevated plasma prorenin levels, prorenin is still not detectable in urine. This is most likely because all filtered prorenin is reabsorbed from the tubular fluid by megalin. This does not appear to be the case for renin. When processing of reabsorbed proteins by megalin is disturbed, for instance in patients with Dent disease, urinary renin levels are higher. Prorenin then also becomes detectable in urine, confirming that in the normal situation, tubular reuptake of prorenin is highly efficient. Because urine of patients with Dent disease contained intact prorenin, prorenin-renin conversion in urine is unlikely. Such conversion should have occurred had urinary renin been derived from the CD, since synthesis at this site mainly concerns prorenin.[1] Based on these observations, fluctuations in renin excretion are more likely to be caused by fluctuations in tubular reuptake than release from the CD. Of interest, since angiotensin II may affect megalin expression via its type 1 receptor, it may also directly affect tubular reuptake.[3, 4] In the published literature, a variety of methods to measure urinary renin has been described. In **Chapter 3** we compare a new renin ELISA with the classical ways of measuring renin, either immunoreactively (making use of antibodies that recognize the active site of renin) or enzyme-kinetically (i.e., based on its capacity to generate angiotensin I). Our data suggest that the ELISA is less sensitive than the other two methods, and makes use of recombinant prorenin as a standard that has not been calibrated against the International Reference Preparation of human renin. This results in renin levels that are approximately 10 times higher than levels reported in studies using other assays. Hence, renin levels should be measured by established renin assays with internationally recognized standards.

In **Chapters 5 and 6** we focus on renal angiotensin-converting enzyme (ACE). ACE is present in the brush border of the kidney's proximal tubule.[5] Ang II generation at renal tissue sites depends on this renal ACE.[6, 7] Studies with low-renal ACE mice concluded that the (near) absence of renal ACE prevents the development of hypertension upon Ang II infusion or NOS inhibition.[8, 9] However, no attention was paid to possible regulatory changes in other pathways that are likely to occur after a decrease in ACE. For instance, an increase in renin could easily match a decrease in ACE, as is known from studies in humans during ACE inhibition.[10] Furthermore, during Ang II infusion the classical Ang II-metabolizing enzymes may become upregulated, or additional angiotensinases may come into play. It was also

overlooked that non-selective NOS inhibition may influence the effect of NO on renin release and its capacity to suppress the sympathetic nerve system, which interacts at various levels with the RAS. In a follow-up study, Giani et al. showed that a higher dose of NOS inhibition did raise blood pressure in low-ACE mice. [11] Clearly, therefore, NO formation is upregulated in these animals. Considering all the missing pieces of the puzzle, the evidence supporting a role for renal ACE in Ang II- or NOS inhibition-induced hypertension is still incomplete.

Dual AT_1 receptor-neprilysin inhibitors

In **Chapter 7** we studied the combination of an angiotensin receptor blocker (ARB) with a neprilysin (NEP) inhibitor in hypertensive rats. This combination treatment is referred to as 'ARNI'. NEP is critical for the breakdown of a variety of substrates, both vasodilators and vasoconstrictors. Therefore, its net effect depends on the relative dominance of these substrates. When a NEP inhibitor is combined with a blocker of the RAS, the balance shifts to the positive, blood pressure-lowering side. In chapter 7 we confirm that ARNI has an additional blood pressure lowering effect compared to ARB alone. However, when the dose of the NEP inhibitor was increased, this effect was annihilated, likely due to an increase in endothelin-1. The endothelin-1 rise was accompanied by an increase in renal sodium-hydrogen exchanger 3 protein abundance, and an upregulation of constrictor vascular endothelin type B receptors. These results could explain why the high dose of the NEP inhibitor did not have the same blood pressure lowering effect as the low dose. In **Chapter 8** we studied the effects of ARNI in diabetic, hypertensive rats. We found that ARNI reduced heart weight, and improved proteinuria and the development of focal segmental glomerulosclerosis more strongly than ARB alone. Blood pressure in ARB- and ARNI-treated animals was equal, which suggests that the observed effects occurred independently of blood pressure. Similar protective effects were found on diabetic retinopathy in these rats in **Chapter 9**. After a short period of diabetes, ARNI treatment reduced markers of retinal inflammation more than ARB treatment, and in long-term diabetes ARNI reduced apoptotic cell death and capillary loss more than ARB treatment.

Discussion and future perspectives

The intrarenal renin-angiotensin system

Studying RAS components in blood and urine may help us to gain more insight in the pathophysiology of hypertension, the consequences of pharmacological interference in the RAS, and the regulation of sodium and water balance. However, we then need to be sure where the different RAS components originate and how they are processed. In pathophysiological states such as a high-salt diet, diabetes and hypertension, the CD is believed to generate (pro)renin, and, surprisingly, Ang II stimulates this (pro)renin synthesis.[1, 12, 13] Studies with CD-specific (pro)renin knockout mice showed that these mice have a normal blood pressure and sodium excretion under physiological conditions, but an attenuated hypertensive response to Ang II, and reduced membrane-associated protein levels of the epithelial sodium channel (ENaC).[14] This suggests that CD (pro)renin might be involved in the development or maintenance of hypertension during pathological states of high circulating Ang II levels. It has been suggested that this locally produced (pro)renin contributes to activation of ENaC, either indirectly via Ang II generation and subsequent Ang II type 1 (AT_1) receptor stimulation, or directly via stimulation of the so-called (pro) renin receptor (PRR). [14] The PRR is abundantly expressed in the CD.[15] Since renin synthesis and release in the CD mainly concerns prorenin, without evidence for its conversion into renin, the latter concept would imply a direct, functional role for prorenin. Moreover, recent studies suggest that Ang II induces the expression of the PRR, and that stimulation of this receptor (by prorenin?) is involved in the Ang II-induced stimulation of ENaC.[16, 17] Indeed, mice with a conditional deletion of the PRR in the CD exhibited a blunted blood pressure response to Ang II.[17] These data seem to imply that CD-derived prorenin via PRR signaling contributes to the fine-tuning of sodium and water excretion. However, CD PRR knockout mice displayed nephrogenic diabetes insipidus, due to altered arginine vasopressin responsiveness,[18] and elevated plasma renin activity, already at baseline. This might have caused their inability to raise blood pressure upon Ang II infusion. In a study by Trepiccione et al., applying a much higher dose of Ang II, CD PRR knockout mice responded identically to Ang II infusion as wild-type mice.[19] According to this study, PRR deletion caused impairment of vacuolar ATPase activity, which hampers autophagy, and thus impaired acid-base regulation by the kidney. It was suggested that the autophagic defect is causal for the dysregulation of ion transporter trafficking and turnover, independently of prorenin or the intrarenal RAS.[19] Clearly therefore, the function of prorenin and its potential site of action remain controversial. Our data do not support prorenin occurrence in urine. Our conclusion that urinary renin is not CD-derived, activated prorenin, but filtered plasma-derived renin is supported by very early work by Rappelli et al., who observed an increase in renin in urine in rats after reducing proximal tubule function with maleic acid and mercuric chloride[20] and in humans with proximal tubular dysfunction.[21] None of these studies found a correlation between urinary renin and plasma renin, or albuminuria. Apparently, tubular handling does not solely depend on plasma levels, and differs from tubular handling of albumin. This could be further explored by making use of drugs that inhibit tubular reabsorption by megalin (such as gentamicin), megalin knockout mice, or megalin antisense oligonucleotides (ASOs; single-stranded DNA molecules that can selectively inhibit mRNA translation).

Prorenin was not only detected in urine of patients with Dent disease, but also in urine of pregnant women, especially preeclamptic women.[22] Given that albuminuria is the main characteristic of preeclampsia, and that plasma prorenin levels rise during pregnancy,[23] the most obvious explanation of this observation is greatly increased glomerular filtration. Apparently, this cannot be matched by tubular reabsorption. This would fit with the finding

of decreased reabsorption of amino acids, β-microglobulin, uric acid, and glucose, during pregnancy.[24]

With regard to the role of renal ACE in hypertension and renal (patho)physiology, the current studies lack data on plasma and tissue levels of other RAS components. Information about renin levels is particularly essential when the indispensableness of renal ACE is assessed, since renin is the rate-limiting step of the RAS. In mice with a 70% reduction in plasma ACE, plasma renin levels were increased by almost 9-fold, causing a suppression of plasma angiotensinogen of 78% compared to control mice.[7] Selective, renal RAS suppression will increase the plasma concentration of renin, and similar (if not larger) renin increases are expected in the kidney.[25] This might explain why the renal Ang II content in ACE knockout mice is unaltered, most likely at the expense of renal angiotensinogen. Under such conditions, further rises in renal Ang II (e.g., by applying L-NAME) may be impossible, since tissue angiotensinogen is already greatly depleted. A pitfall in measuring the renal protein levels of angiotensinogen or renin after homogenizing the tissue, is the simultaneous detection of stored or reabsorbed renin or angiotensinogen.

Dual AT_1 receptor-neprilysin inhibitors

The ARNI LCZ696 has been shown to be superior to valsartan in the treatment of hypertension, and also to the ACE inhibitor enalapril in the treatment of heart failure.[26, 27] These promising results led to FDA approval in July 2015 and EMA approval in November 2015 for the use of LCZ696 (valsartan/sacubitril) in adult patients with symptomatic heart failure with reduced ejection fraction. Remarkably, LCZ696s effects on atrial wall stress were most pronounced in diabetic patients, evidenced by reduced levels of proBNP's N-terminal end, NT-proBNP.[28] NEP inhibitors have also been shown to improve vascular function in diabetes.[29] In diabetic, hypertensive rats, we have shown that ARNI improves proteinuria. Because blood pressure in ARB and ARNI treated animals was equal, the renoprotective effect of ARNI appears to be blood-pressure independent. Analysis of kidney histology showed that ARNI reduced focal segmental glomerulosclerosis more than ARB alone. Similar protective effects were found regarding diabetic retinopathy. A meta-analysis including all trials with LCZ696 that reported renal outcomes demonstrated that ARNI has favorable renal effects over RAS inhibition alone.[30] Furthermore, LCZ696 was found to be safe and well tolerated in hypertensive patients with renal dysfunction (eGFR 15-60 mL/min per 1.73m²).[31] These favorable effects of ARNI versus ARB on the microvascular complications of diabetes, support the investigation of ARNI in diabetic kidney disease patients in future clinical trials. The question remains how ARNI confers renoprotection. Hypertension and diabetes both induce changes in renal hemodynamics.[32] Diabetes may cause vasodilation of the afferent arteriole and vasoconstriction of the efferent arteriole. Consequently, hyperfiltration and increased glomerular capillary pressure arise, causing glomerular damage and proteinuria. ARNI may improve proteinuria and prevent further damage by regulation of renal vascular resistance. Furthermore, neprilysin may increase atrial natriuretic peptide (ANP) concentrations in the kidney. ANP directly regulates the vascular tone of both the afferent and the efferent arteriole. Moreover, ANP stimulates natriuresis. Salt-sensing by the macula densa provides additional signals for the regulation of glomerular filtration pressure.

To answer the question if ARNI prevents renal damage by ANP-mediated regulation of renal vascular resistance it would be necessary to measure GFR and effective renal plasma flow. In addition to renal hemodynamics, ARNI may also affect infiltrating immune cells. The connection between diabetes, hypertension, and the immune system is increasingly

recognized. Hypertension and diabetes both induce an influx of activated immune cells in renal tissue.[33, 34] Local production of cytokines and reactive oxygen species induces renal damage, characterized by tubular atrophy and loss of nephrons. ANP can regulate macrophage function by inhibiting the production of tumor necrosis factor, nuclear factor-kappa B, and interleukin 12.[35] In addition, ANP promotes polarization of T-lymphocytes. ARNI may exert its anti-fibrotic effects by preventing the influx, activation and secretion of cytokines by immune cells. Ideally, infiltrating inflammatory cells in the kidney would be quantified using flow cytometry, and subsequently localized using immunohistochemistry. After this, the cytokines that are involved can be explored.

Studies as proposed above will improve our understanding of the mechanisms behind the positive effects of ARNI on diabetic complications in laboratory animals, and pave the way for the application of ARNI in human diabetes.

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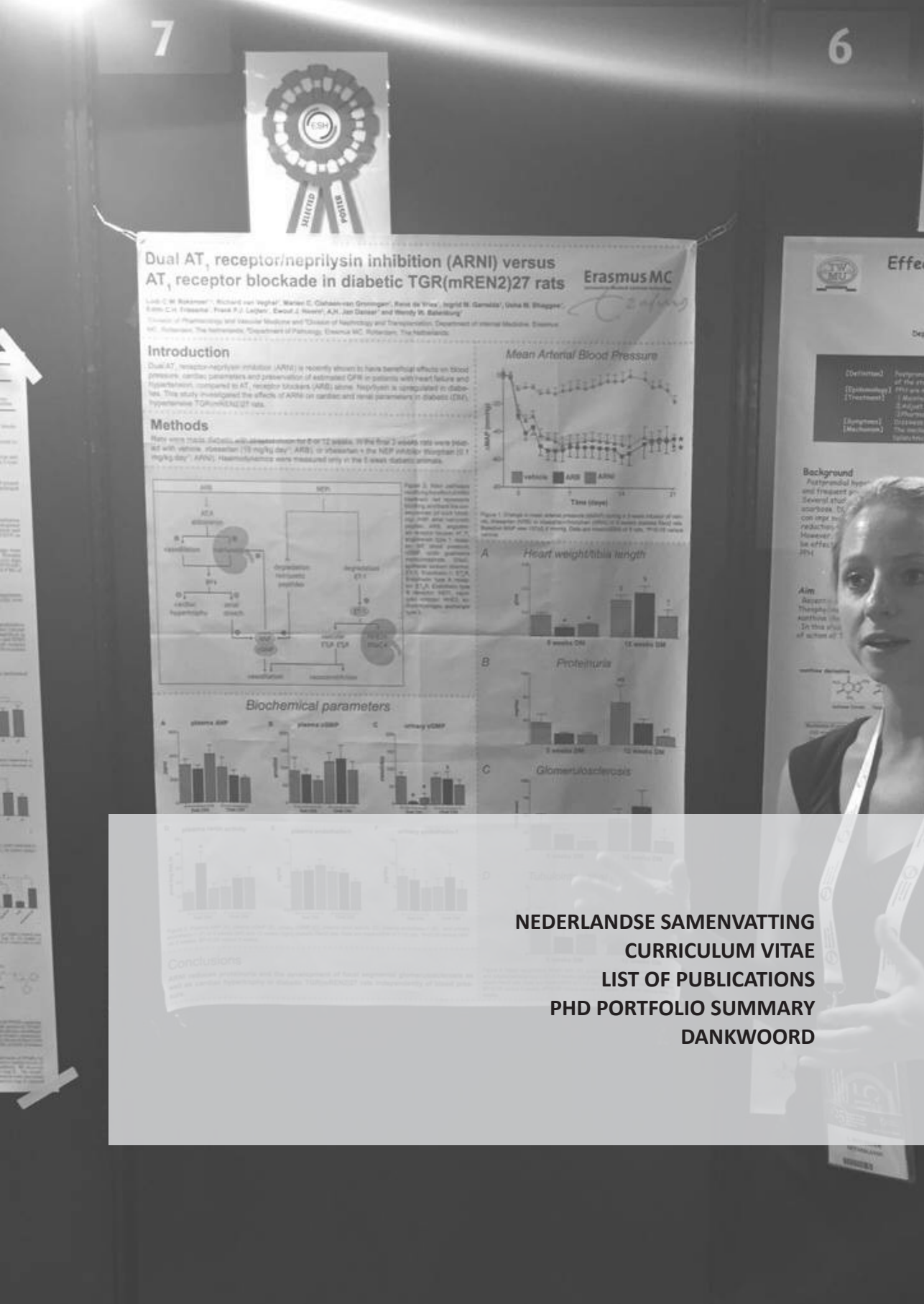

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Nederlandse samenvatting

Het intrarenale renine-angiotensine systeem (RAS)
Naast het systemische (endocriene) RAS, wordt aangenomen dat de nieren hun eigen (paracriene) RAS hebben, aangezien de nieren in staat zijn om lokaal angiotensine II te synthetiseren. Bovendien is er expressie van andere RAS componenten aangetoond in de proximale tubulus (angiotensinogeen en ACE) en de verzamelbuis (renine) van de nier. Een andere verklaring voor de aanwezigheid van RAS componenten in de nieren zou endocytotische opname uit de voorurine kunnen zijn. RAS componenten in de urine zouden de activiteit van het intrarenale RAS kunnen weerspiegelen, dat onafhankelijk is van het systemische RAS. RAS componenten in de urine zouden zodoende gebruikt kunnen worden als markers voor de activiteit van het intrarenale RAS, of zelfs als markers voor nierziekten. **Hoofdstuk 2** geeft een overzicht van studies die gedaan zijn naar RAS componenten in de urine als markers voor de activiteit van het intrarenale RAS. Renine, het snelheidsbeperkende enzym van het RAS, komt niet alleen tot expressie in het juxtaglomerulaire apparaat (JGA), maar ook in de verzamelbuis. Renine expressie in de verzamelbuis is verhoogd in diabetes.[1] Urine renine is verhoogd in diabeten en verlaagd in patiënten die behandeld worden met een RAS remmer. Urine renine correleert echter niet met albuminurie, wat het onwaarschijnlijk maakt dat urine renine een afspiegeling is van de afbraak van de glomerulaire filtratie barrière (GFB).[2] In **hoofdstuk 4** laten we zien dat renine en prorenine beiden gefilterd worden door de glomerulus en dat filtratie toeneemt wanneer er schade aan de GFB is. Zelfs bij ratten met suprafysiologisch verhoogde plasma prorenine spiegels, is prorenine niet meetbaar in de urine. De meest voor de hand liggende reden hiervoor is dat al het gefilterde prorenine door megaline heropgenomen wordt vanuit de voorurine. Dit is anders bij renine. Wanneer het opnameproces van megaline wordt verstoord, zoals bij de ziekte van Dent, dan is er meer renine in de urine. Prorenine wordt dan ook meetbaar in urine, hetgeen bevestigt dat in de normale situatie de tubulaire heropname van prorenine hoogst efficiënt verloopt. Aangezien de urine van patiënten met de ziekte van Dent prorenine bevat, is het onwaarschijnlijk dat er omzetting van prorenine naar renine in (voor)urine plaatsvindt. Een dergelijke omzetting zou wel moeten plaatsvinden als het renine in de urine afkomstig was geweest uit de verzamelbuis, aangezien daar alleen prorenine gemaakt wordt.[1] Het lijkt dus aannemelijk dat fluctuaties in urine renine veroorzaakt worden door fluctuaties in de tubulaire heropname en niet door veranderingen in afgifte vanuit de verzamelbuis. Angiotensine II kan zelfs, aangezien het de megaline expressie beïnvloedt via de angiotensine II type 1 receptor, een direct effect hebben op de tubulaire heropname.[3, 4]
In de gepubliceerde literatuur worden verschillende methoden beschreven om urine renine te meten. In **hoofdstuk 3** vergelijken we een nieuwe renine ELISA met de bestaande methoden om renine te meten, zijnde immunoreactief (gebruikmakend van antilichamen die het actieve deel van renine herkennen) of enzymkinetisch (gebaseerd op de capaciteit van renine om angiotensine I te genereren). Onze data suggereert dat de ELISA minder gevoelig is dan de andere twee methoden, en bovendien gebruik maakt van recombinant prorenine als standard, dat niet gekalibreerd is met het Internationale Referentiepreparaat van humaan renine. Dit resulteert in reninespiegels die ongeveer 10 keer hoger zijn dan de spiegels die gerapporteerd worden in studies die gebruik makend van andere assays. Reninespiegels zouden gemeten moeten worden met reeds gevestigde renine assays met internationaal erkende standaarden.

In **hoofdstukken 5 en 6** richten we ons op angiotensine-converterend enzym (ACE) in de nier. ACE is in de nier aanwezig in de borstelzoom van de proximale tubulus.[5] Angiotensine II productie in de nier is afhankelijk van dit renale ACE.[6, 7] Naar aanleiding van studies met muizen die weinig ACE in de nier hebben, is geconcludeerd dat de (bijna volledige) afwezigheid van renaal ACE beschermt tegen de ontwikkeling van hypertensie na infusie van angiotensine II of NOS remmers.[8, 9] Er is hierbij echter geen aandacht besteed aan mogelijke veranderingen in andere regulatoire routes, waarvan het waarschijnlijk is dat zij geactiveerd worden bij een afname van ACE. Een afname van ACE kan gemakkelijk overreden worden door een toename van renine. Dit is een bekend fenomeen bij mensen die ACE remmers gebruiken.[10] Bovendien kan het zo zijn dat tijdens angiotensine II infusie de klassieke angiotensine II-metaboliserende enzymen worden geactiveerd, of andere angiotensinases van belang worden. Daarnaast beïnvloedt non-selectieve NOS remming mogelijk het effect dat NO heeft op renineproductie, en de capaciteit van NO om het sympathisch zenuwstelsel te onderdrukken, dat op verschillende niveaus op het RAS inwerkt. In een vervolgstudie zag men dat een hogere dosis NOS remming wel leidde tot een bloeddrukstijging, in hetzelfde type muizen met een verlaagd renaal ACE.[11] Hieruit blijkt dat de NO productie in deze dieren verhoogd is. We concluderen hieruit dat, gezien het grote aantal missende puzzelstukjes, het nog steeds onduidelijk is wat de rol van renaal ACE in angiotensine II- of NOS remming-geïnduceerde hypertensie is.

Gecombineerde AT_1 receptor-neprilysine remmers

In **hoofdstuk 7** bestuderen we de combinatie van een angiotensine II type 1 receptor blokker (ARB) met een neprilysine (NEP) remmer, in hypertensieve ratten. Deze combinatiebehandeling wordt 'ARNI' genoemd. NEP is belangrijk voor de afbraak van zowel bloedvatverwijdende als bloedvatvernauwende stoffen. Het netto effect van NEP (of NEP remming) is daarom afhankelijk van de relatieve aanwezigheid van deze stoffen. Wanneer een NEP remmer gecombineerd wordt met een blokker van het RAS, schuift de balans naar de positieve, bloedvatverwijdende (en dus bloeddrukverlagende) kant. In hoofdstuk 7 bevestigen we dat ARNI een groter bloeddrukverlagend effect heeft dan ARB alleen. We zagen echter ook dat wanneer de dosis van de NEP remmer verhoogd werd, dit extra effect verdween, waarschijnlijk als gevolg van een toename van endotheline-1. Dit ging gepaard met een toename van de natrium-waterstof wisselaar type 3 in de nier en een toename van de bloedvatvernauwende variant van de endotheline type B receptor. Deze verschillen zouden kunnen verklaren waarom de hoge dosis van de NEP remmer niet hetzelfde bloeddruk verlagende effect heeft als de lage dosis. In **hoofdstuk 8** bestudeerden we de effecten van ARNI in hypertensieve ratten met diabetes. Dieren die behandeld waren met ARNI hadden een lager hartgewicht, de proteïnurie verbeterde en ze ontwikkelden minder focale segmentele glomerulosclerose dan dieren die alleen ARB hadden gekregen. De bloeddrukken in beide groepen waren gelijk, wat suggereert dat de geobserveerde effecten onafhankelijk zijn van de bloeddruk. Gelijksoortige beschermende effecten werden gevonden op diabetische retinopathie in deze ratten, beschreven in **hoofdstuk 9**. Na een korte periode van diabetes waren er bij ARNI-behandelde dieren minder tekenen van retinale ontsteking dan bij ARB-behandelde dieren, en na een langere duur van de diabetes zagen we in de retina van dieren die met ARNI behandeld waren minder apoptotische celdood en minder verlies van haarvaten in de retina dan bij ARB-behandelde dieren.

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Curriculum vitae

Lodi Roksnoer werd geboren op 29 november 1985 in Mierlo. Na het behalen van haar middelbare school diploma aan het Gymnasium Juvenaat in Bergen op Zoom in 2004, startte zij met de studie Geneeskunde aan de Erasmus Universiteit Rotterdam. Tijdens haar studie was zij actief lid van studentenvereniging 'het Rotterdamsch Studenten Gezelschap', waar zij van 2007 tot 2008 een fulltime bestuursfunctie vervulde. Daarnaast werkte zij van 2005 tot 2009 op de afdeling Thoraxchirurgie in het Erasmus Medisch Centrum. In 2009 deed zij haar afstudeeronderzoek, getiteld 'de aminozuurbehoefte van à terme en preterme neonaten', aan het Fudan University Children's Hospital in Shanghai, China, in samenwerking met het Sophia Kinderziekenhuis in Rotterdam, onder supervisie van Prof. dr. J.B. van Goudoever. Na het behalen van haar artsexamen in januari 2012 was zij werkzaam als arts-assistent op de afdeling Interne Geneeskunde in het Maasstadziekenhuis in Rotterdam. In december 2012 startte zij als promovenda op de afdeling Inwendige Geneeskunde van het Erasmus Medisch Centrum, bij de sectie Farmacologie en Vasculaire Geneeskunde en de sectie Nefrologie. Onder supervisie van Prof. dr. A.H.J. Danser, Prof. dr. R. Zietse en Dr. E.J. Hoorn werd het promotieonderzoek uitgevoerd dat in dit proefschrift beschreven is. In januari 2017 zal zij starten met de opleiding tot internist.

List of publications

Roksnoer LCW, van Veghel R, Clahsen-van Groningen MC, de Vries R, Garrelds IM, Bhaggoe UM, van Gool JMG, Friesema ECH, Leijten FPJ, Hoorn EJ, Danser AHJ and Batenburg WW. Blood pressure-independent renoprotection in diabetic rats treated with AT_1 receptor-neprilysin inhibition versus AT_1 receptor blockade alone. Clin Sci. 2016 Jul;130(14):1209-1220

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PhD Portfolio Summary

Name PhD student:	Lodi Roksnoer
PhD period:	2012-2016
Erasmus MC department:	Internal Medicine Division of Pharmacology and Vascular Medicine Division of Nephrology
Promotor(s):	Prof. dr. A.H.J. Danser Prof. dr. R. Zietse
Supervisor:	Dr. E.J. Hoorn
Research school:	Cardiovascular Research School Erasmus University Rotterdam (COEUR)

PhD training	**Year**	**Workload (ECTS)**
General academic skills		
- Laboratory Animal Science	2013	3.0
- Biomedical English Writing and Communication	2014	3.0
- Biostatistical Methods I: Basic Principles (NIHES)	2014	5.7
In-depth courses		
- Cardiovascular pharmacology (COEUR)	2013	1.5
- Cardiovascular medicine (COEUR)	2013	1.5
- 5th Rotterdam Course in Electrolyte and Acid-Base disorders	2013	0.3
- Winter School Dutch Kidney Foundation	2014	1.2
- ERA-EDTA (WGIKD) course: “renal fluids and electrolytes: from genes to bedside”	2014	0.9
- Molecular biology in cardiovascular research (COEUR)	2014	1.5
- Summer School European Society of Hypertension (Sirmione, Italy)	2016	2.1
Symposia and conferences		
- European Society of Hypertension (Milan, Italy) **	2013	1.5
- Gordon Conference and Seminar on Angiotensin (Barga, Italy) *	2014	2.1
- International Society of Hypertension (Athens, Greece) *	2014	1.2
- Dutch Nephrology Days **	2015	0.9
- European Society of Hypertension (Milan, Italy) *	2015	1.2
- American Society of Nephrology Kidney Week (San Diego, CA, USA) *	2015	1.5
- Gordon Conference and Seminar on Angiotensin (Barga, Italy) **	2016	2.1
- FIGON Dutch Medicine Days **	2016	0.6
Didactic skills		
- Supervising practicum first year medical students	2013-15	0.6
Other		
- Erasmus MC Internal Medicine science days *	2013,2015	1.2
- PLAN (platform AIOS Nephrology) day **	2013-15	0.9
- COEUR PhD day	2013-15	0.9

- COEUR lectures: 8	2013-16	0.8
- COEUR seminars: 3	2013-16	1.2
- Department of Internal Medicine sectormeetings and work discussions **	2013-16	2.7
Total		**40.1**

** oral presentation(s), * poster presentation(s)

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