The expanding world of RAAS

Kamilic, Jelena

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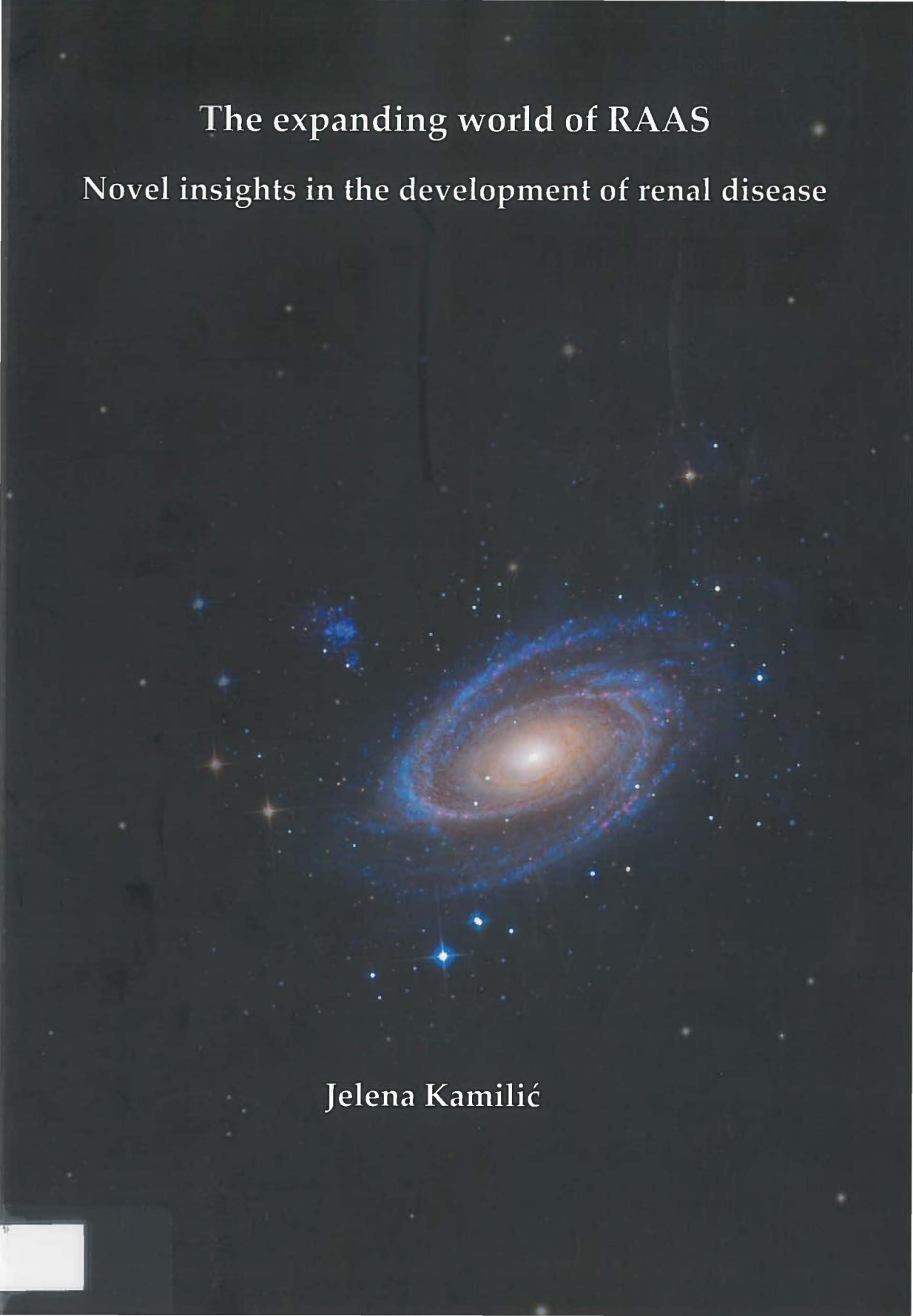
Novel insights in the development of renal disease



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Stellingen

bij het proefschrift

The expanding world of RAAS

Novel insights in the development of renal disease

1. Wistar rats should be genotyped for the *b/l Ace* polymorphism in studies of renal and cardiovascular diseases. *(dit proefschrift)*

2. The extent of renal damage after prolonged AngII insult depends on genetically predetermined high or low baseline ACE conditions. *(dit proefschrift)*

3. Local renal ACE inhibition is important in renoprotective therapy. *(dit proefschrift)*

4. ACE2 is not involved in the regulation of adult hypertension in rats. *(dit proefschrift)*

5. DNA methylation response on ischemia/reperfusion injury is genetically predetermined. *(dit proefschrift)*

6. All people are created equal, but some of us are more equal than the others. *(adapted G. Orwell)*

7. Its better to regret something you did, than something you did not do.

8. One of the most dangerous things in the world is an idea.

9. When you have eliminated the impossible, whatever remains however improbable, must be the truth. *(Sherlock Holmes)*

10. Character is tested when you are up against it. *(Dick Vermeil, Philadelphia Eagles, from Invincible)*

11. There is no such thing as perfect conditions.

Jelena Kamilić

4 april 2011

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"This above all: to thine self be true,
And it must follow, as the night the day,
Thou canst not then be false to any man."

W. Shakespeare

Contents

Chapter 1

General Introduction

Chronic kidney disease

Many patients diagnosed with chronic kidney disease (CKD) will eventually develop progressive renal function loss. These patients, now suffering from end stage renal disease (ESRD) require permanent renal replacement therapy. Reaching that point, these patients need dialysis until a compatible kidney becomes available for transplantation. To date, dialysis and kidney transplantation are the only long term therapy options for severe kidney failure. Health systems are facing a problem of high patient costs and the discrepancy between the need and the availability of renal transplants. With the annual number of ESRD patients steadily rising by 5-8% worldwide [1] CKD has become a severe global health challenge.

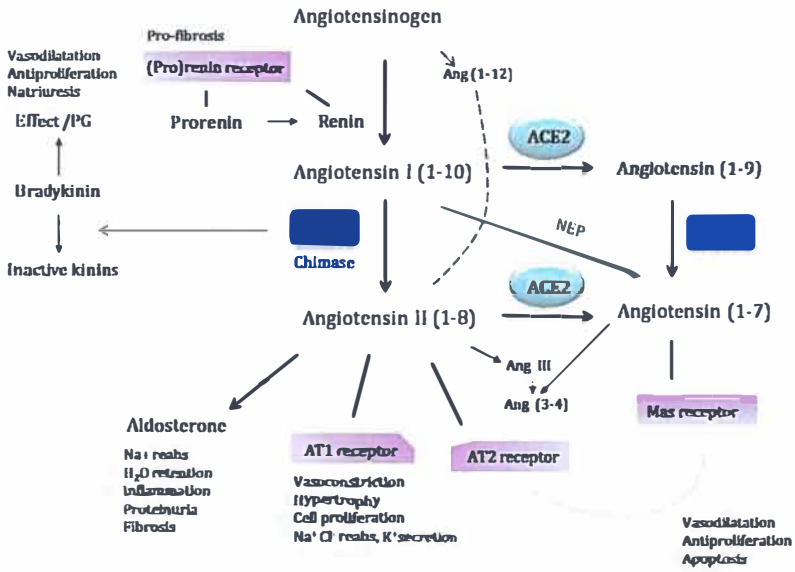
Recognized risk factors for CKD are proteinuria and hypertension [2-4], type 1 and type 2 diabetes [5], an atherogenic lipid profile, obesity, smoking [1] as well as individual genetic background [6]. Thus, both universal and selective prevention approaches are relevant in the battle against chronic renal disease. At the same time, early identification of individuals most likely to develop renal disease is crucial. The methods currently used for that purpose include urinary albumin/creatinine ratio, estimated GFR or calculation of serum creatinine and blood urea nitrogen (BUN) levels. The reliability and benefit of these methods in more global screening approaches has long been disputed. Only the latest meta-analysis of general population cohorts has confirmed albumin-to-creatinine ratio and estimated GFR as an independent risk factor for mortality in the general population [7]. This still remains to be implemented in the population screening guidelines. At this point, the diagnosis of CKD presents itself mostly at the stage when due to high blood pressure and proteinuria, ample loss of kidney function has already occurred. Considerable work is being done in trying to identify novel biomarkers of renal damage [8-10]. For example, kidney injury molecule 1 (KIM-1) is a promising biomarker which can be detected in urine early upon renal ischemia [11,12]. Also, identification of markers on the DNA level will become more common practice in the near future. Since the presence of different gene variants, mutations or heritable transcription regulation mechanisms could be implicated in the renal tissue response to various stimuli, they could possibly be used as a diagnostic and maybe even therapeutic tool. When a gene variant is proven to be involved in the onset and/or progression of renal disease, genotyping a patient presenting with proteinuria might help identify increased risk for renal failure, and guide in adjusting the therapy plan. Also, therapeutic gene modulation i.e. altering the expression of genes whose misbalance triggers a decline in renal function could lead to additional renoprotection. In order to improve and utilize the prospective diagnostic and therapeutic strategies it is necessary to better understand the mechanisms of renal disease progression and identify the factors involved in the susceptibility to renal damage. The ultimate challenge that would help hamper this ongoing health issue is to avoid the development of CKD or when once diagnosed, prevent its progression to the point when renal replacement therapy is the only remaining option.

The Renin-Angiotensin Aldosterone System (RAAS)

For decades, the RAAS seemed a simple, straightforward system. Inactive angiotensinogen produced in the liver is activated by renin released from the juxtaglomerular apparatus in response to a low renal perfusion pressure. Angiotensinogen is converted into angiotensin I (AngI) that is further, by actions of the central RAAS enzyme angiotensin converting enzyme (ACE), converted to angiotensin II (AngII). AngII is an active, vasoconstricting, profibrotic tissue signaling octapeptide with systemic and local tissue properties. Most of AngII biological actions are mediated through binding to the G-coupled Angiotensin II type 1 and Angiotensin II type 2 receptors (AT1 and AT2). Also, it stimulates proximal tubular Na^+ reapsorption, the release of aldosterone from the adrenal gland cortex and ADH secretion from pituitary gland. The picture was fine tuned with the negative feed-back mechanism present between AngII and renin.

Then, the effects of Ang(1-7), a heptapeptide thought to be an inactive byproduct/metabolite of AngII [13], and a new member of RAAS - the angiotensin converting enzyme 2 (ACE2), were identified [14,15]. The picture of RAAS has been expanding since. Next to the long existing ACE/AngII/AT1 axis, a new ACE2/Ang(1-7)/Mas receptor branch has now been added to the RAAS. An increasing body of data over the years has identified RAAS as a system with multiple mediators, multiple receptors and multifunctional enzymes [15-20]. As mentioned previously, AngII mediates its effect mainly through binding to the Angiotensin II type 1 receptor (AT1). AT1 belongs to a G protein-coupled receptor superfamily and acts primarily through phospholipase C and calcium channels. While most mammals have one gene encoding AT1, rodents express two related AT1a and AT1b receptor genes [21]. A cellular response to AT1 stimulation includes promotion of cell growth and proliferation, vasoconstriction, aldosterone and adrenal steroid secretion, ion transfer and sympathetic activation. AngII can also bind to AT2 receptor. Even though AT2 is more present during embryonic development, it still remains active in various tissue including adult kidney, heart and vasculature. Interestingly, a significant upregulation of the AT2 receptor was described after tissue injury. Activation of the AT2 receptor counteracts some of the AT1 effects, especially the growth responses [22]. Effect of Ang(1-7) on the other hand is delivered through the Mas receptor. Similarly as effects downstream of AT2 receptor, it antagonizes growth promoting signaling pathways. It is also shown to be protective against endothelial disfunction and leads to vasodilatation [23]. On the level of renin, a novel (pro)renin receptor, (P)RR, was recently discovered. Binding to (P)RR increases catalytic activity of renin, but it also can lead to AngII-independent pro-fibrotic effect which allows additional angle in renoprotective strategies [24].

Novel picture of the RAAS



The majority of the effects are influenced by the actions of the two catalyzing enzymes, ACE and its corresponding counterpart ACE2. The *ACE2* gene is located on the X chromosome in mouse, rat and human. It is a transmembrane protein, a Zn metaloproteinase/ carboxypeptidase that cleaves one amino acid from either AngI or AngII – leading to the production of Ang (1-9) and Ang (1-7) respectively. The tissue effect of Ang (1-9) has not yet been identified but the Ang (1-7) is an active metabolite with vasodilatory and anti-inflammatory properties [25]. ACE2 is abundantly present in the epithelial cells of many human tissues including kidney, heart, lung, small intestine and endothelial and smooth muscle cells [26]. Interestingly, ACE2 is also a receptor for SARS virus mainly in the lungs [27]. It is speculated that the balance between the two enzymes - ACE and ACE2 is in the center of RAAS regulation and that stimulating the ACE2 axis could be proven to be beneficial in renal and cardiovascular (CV) disease [28]. In line with this concept, renal tissue ACE2 levels have been studied in connection with (patho)physiological regulation of RAAS in health and disease. *ACE2* mRNA and protein levels were shown to be decreased in renal biopsies of patients with diabetic nephropathy [29] and in rats after subtotal nephrectomy [30], while ACE2 knock out mice developed more severe glomerular and tubulointerstitial damage after streptozotocin injection then the wild type mice [31], indicating the possible renoprotective properties of ACE2. In healthy rats, ACE and ACE2 were differentially regulated after modulation of RAAS activity by ACE inhibitor and low sodium intake [32]. In addition, the role of ACE2 has been studied in connection

with the regulation of hypertension. Over-activation of ACE/AngII/AT1 axis plays a crucial role in hypertension, the proof of principle being the effectiveness of ACE inhibitors in lowering the increased blood pressure. In the most simplified view, if the two axes are in balance, ACE2 would be expected to be decreased in hypertension. However, the evidence for the role of ACE2 in regulation of high blood pressure is still inconclusive.

I/D polymorphism of the *ACE* gene and global kidney methylation

If we take a step back from the complex (patho)physiological RAAS interactions and look at the disease at the nuclear level, again we are faced with the questions about the susceptibility to the development and progression of renal damage. Efforts to identify gene combinations by means of quantitative trait locus (QTL) analyses or targeted gene mutations have taught us of multifactorial genetic profiles in CKD patients [6].

One of the most studied heritable factors believed to influence the susceptibility and progression of CKD is the insertion/deletion (I/D) polymorphism in the *ACE* gene. In human, the *ACE* gene is located on chromosome 17 and consists of 25 exons that encode a 1306 amino acid long protein. The polymorphism in question is presented by an insertion (I) or a deletion (D) of a 287 bp sequence in intron 16 of the *ACE* gene [33]. This *ACE* I/D polymorphism accounts for almost half of the total phenotypic variance of plasma ACE levels and activity [33]. Homozygous DD carriers have the highest, homozygous II the lowest, and the heterozygous ID intermediate ACE levels in both plasma and tissue samples [34,35]. Since serum ACE levels are stable when sporadically measured in the same individual, but they differ greatly when measured in different individuals [36], the ACE I/D polymorphism is considered a major locus that determines plasma and tissue ACE. The function of this intronic polymorphism is still not fully understood.

With genetically predetermined ACE levels it is not hard to imagine that the I/D ACE polymorphism could have predictive properties and could determine different therapy approaches in respect to high or low ACE background. A meta-analyses confirmed a role of the D-allele as a possible renal and cardiovascular risk factor [37]. Although, some studies have shown the association of high ACE with risk of development and progression of diabetic and non diabetic chronic kidney disease [38-40], other studies have failed to establish such a link [41,42]. In regard to the therapy response, it was justly hypothesized that predetermined high ACE condition would react differently to ACE inhibition (ACEi) or Angiotensin receptor blocker (ARB) therapy. Here also, the data are contrasting [43-46]. Again, some studies showed better antiproteinuric response of DD genotype on ACE inhibition, others failed to find the correlation [47,48] or even showed better response in the patients with II genotype [49]. Also, a totally different approach has connected the D-allele to longevity [50,51] suggesting high ACE to be beneficial under healthy condition without previous renal or CV insult. Due to the heterogeneity of the studies performed, a small sample size or a short

follow up, the true practical advantage of knowing a patient's I/D *ACE* genotype still need to be demonstrated.

A similar intronic *Ace* polymorphism has been identified in rats between Brown Norway (b) and Lou (l) strains [52,53]. This b/l *Ace* polymorphism is associated with different levels of plasma and tissue ACE where the b allele is related to high and the I allele to low ACE levels. Furthermore, in an F2 cohort derived from a cross between Lou and Brown Norway, the degree of genetic determination of plasma ACE activity was estimated to be 94% hence validating the effect of this polymorphism [54,55]. However, the different genetic background between these two rat strains makes it very difficult to draw valid conclusions. Nevertheless, a good animal model would be a valuable tool in better understanding the RAAS in the conditions of high/low ACE levels. The characterization of the local renal RAAS at baseline conditions, and especially introducing various challenges, like RAAS stimulation, damage by ischemia or different therapy combinations in models of renal disease, would give better insight in the behavior of the system itself.

Next to allele differences and gene combinations, the phenotype can be influenced by regulatory mechanisms which are not bound to the underlying DNA sequence. These epigenetic mechanisms, such as DNA methylation and histone acetylation, have been shown to lead to changes in phenotype and gene expression [56]. Increased methylation of the CpG islands in the promotor region leads to gene silencing and inappropriate DNA methylation can have the same effect as if that gene had been mutated. Epigenetics has already been implicated in embryonic development, aging, cancer and various other disease pathways [57-60] and it is certainly a factor to be taken into account when determining the individual differential susceptibility to a renal insult.

Renoprotective therapy

The RAAS is the main regulator of body volume homeostatis and blood pressure. In case of volume depletion, the RAAS is activated and causes vasoconstriction and stimulation of Na^+ reapsorption, thereby serving as a mechanism of protection against excessive blood loss and hypoperfusion. On the other hand, it is hypothesized that the pathological over-activation of RAAS can lead to a cascade of events that have a role in progression but also initiation of CKD [61].

When it comes to renoprotection, blocking of the RAAS by means of ACE inhibition (ACEi) alone, or in combination with Angiotensin II type 1 receptor blockers (ARBs) is the first line of therapy in hypertension and proteinuric renal disease [62]. ACEi controls hypertension and decreases urinary protein excretion by reducing the detrimental hemodynamic and protein toxic effects on the renal parenchyma. Interestingly, the plasma levels of AngII are only temporarily reduced after the start of ACEi therapy, while the clinical effect remains even after AngII levels have been restored - the so called "angiotensin escape". When it comes to combination therapy with ARBs, a large long-term clinical trial in patients with CV disease showed no significant benefits of dual blockade over

monotherapy and was associated with worse renal outcome in patients with low renal risk while smaller trials have suggested a possible favourable effect of dual blockade in renal disease [63-66]. Whether a combination of ACEi and ARB is more beneficial then monotherapy [67,68] and in which indications, is still a matter of debate. However, latest data from a randomized controlled cross over trial (the DUAAL trial) show that volume depletion by means of low sodium diet potentiates the reduction of proteinuria and blood pressure in non diabetic patients with renal disease in both mono- and combination renoprotective therapy (BMJ in press). The combination with sodium restriction was also found to be effective in the group of patients previously shown to be resistant to RAAS blockade [69]. Since the current therapeutic approach only delays the progression of CKD, alternative therapy methods are continuously examined. Most of them are targeting other parts of the RAAS either by inhibiting the over-activated damaging components or by inducing possibly beneficial protective components. Renin inhibition is suggested to be one of them [70], stimulation of ACE2 activity and Ang(1-7) production another. As a negative feedback exists between AngII and renin, ACE inhibition leads to renin upregulation. One of the theories why ACEi and ARBs do not halt the renal function loss is because the RAAS is not being fully blocked. It was recently shown that renin binding to the (pro)renin receptor can activate profibrotic pathways and may be even involved in AngII formation on the cell surface [71]. At the moment, preclinical and clinical data are showing positive effects of direct renin inhibitors [72,73]. The results from another trial, combining renal inhibitor aliskrein and ACEi or ARB are awaited shortly (ALTITUDE study)[74]. Opposite of blocking the over activated RAAS is the stimulation of ACE2 or Ang(1-7) in the attempt to promote the possible protective RAAS axis. Most of the experimental studies so far are positive in showing reno- and cardioprotective properties of ACE2 or Ang(1-7) [75-77].

Widening the scope of investigation, the multi level approach and constant questioning the current knowledge of the interactions within the RAAS will help to develop new, better diagnostic and therapeutic tools for CKD.

The aim and scope of the thesis

The aim of this thesis is to deepen the present knowledge of factors involved in the initiation and progression of chronic renal disease and to investigate their potential diagnostic and therapeutic capacity. We have focused mostly on the interactions within the RAAS, but also looked at the role of epigenetics in renal (patho)physiology.

In vivo studies are the best way to investigate the behavior of a biological system under different conditions. For that, an easily reproducible animal model that replicates the human situation is a valuable tool. The outbred Wistar rat strain is constantly being used in studies regarding renal and CV pathology. With this in mind, we have studied the RAAS in the Wistar rats and have identified

and characterized the b/I polymorphism in the *Ace* gene which is described in **Chapter 2**. In **Chapter 3** we have used this model to study the reaction of this genetically predisposed high or low ACE RAAS condition on exogenous activation by means of chronic AngII infusion. Since the function of this intronic polymorphism is still largely speculative, and in the case of b/I *Ace* alleles it contains the CA repeats, in **Chapter 4** we have studied whether the length of the CA repeat in the intron 13 could account for the splicing efficiency of the *Ace* gene. In **Chapter 5** we have used an animal model of adriamycin induced nephrosis in trying to differentiate the renoprotective effects of ACE inhibition and Angiotensin receptor blockade in chronic renal disease. In an attempt to elucidate the role of ACE2 in blood pressure regulation, we have studied ACE2 expression and activity in the models of polygenic and monogenic hypertension and compared it to their respective normotensive controls, as presented in **Chapter 6**. Recently more attention is being given to the mechanisms that can regulate gene expression and can influence the phenotype but are not bound to the DNA sequence per se. One of these epigenetic mechanisms is DNA methylation. In **Chapter 7** we have studied the involvement of the global renal DNA methylation status in the severity of damage caused by ischemia/reperfusion injury in four different rat strains. Finally, in **Chapter 8** the results of the studies presented in this thesis are discussed and summarized as well as their practical implications and future perspectives.

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Chapter 2

Differential *Ace* expression among tissue in allele-specific Wistar rat lines

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Abstract

In humans, the insertion/deletion polymorphism in the angiotensin converting enzyme (*ACE*) gene accounts for half of the variance in plasma ACE activity. The deletion allele is associated with high plasma ACE activity, cardiovascular and renal disease. In rat a similar association is found between the *b* and *l* alleles of a microsatellite marker in the *ACE* gene. We identified the b/l variation in the Wistar outbred rat and bred two lines homozygous for the two alleles (WU-B and WU-L). ACE activity was measured in serum, heart, kidney and aorta homogenates. Immunohistochemistry and *Ace* mRNA expression were performed in heart, kidney and aortic tissue. Aortic rings were collected and stimulated with AngI, AngII and AngI with Lisinopril to measure ACE functional activity by vasoconstrictor response. Serum, heart, and kidney ACE activity and kidney mRNA expression were two-fold higher in WU-B. Kidney staining showed a clear difference in tubular ACE expression, with more staining in WU-B. While in aorta ACE activity and mRNA expression was two-fold higher in WU-L, functional conversion of AngI was higher in WU-B, indicating either a functional difference in AngI to AngII conversion between the two alleles due to different splicing or the presence of other factors involved in the conversion that are differentially expressed as the result of differences in the *Ace* alleles. The newly developed WU-B and WU-L lines show tissue-specific differences in ACE expression and activity. This provides an experimental tool to study the pathophysiological consequences of differences in *ACE* alleles in renal and cardiovascular disease.

Introduction

In human the insertion/deletion (I/D) polymorphism of the *ACE* gene, accounts for the half of the variance of circulating and tissue ACE levels, with the highest ACE levels in DD homozygotes, the lowest in II homozygotes, and intermediate values in heterozygotes [1]. The D allele of the *ACE* genotype has been associated with cardiovascular and renal disease [2-4]. However, the results are not uniform and analysis of both its pathophysiological significance and the underlying mechanisms is complicated by multiple interactions with other genetic and environmental factors [5,6]. The availability of an animal model to study the effects of different *ACE* alleles would therefore be useful.

Identification of a microsatellite marker in intron 13 of the rat *Ace* gene has allowed differentiation of *Ace* alleles among different rat strains [7,8], and their association with different levels of plasma and tissue ACE. The *Ace* locus determines 74% of the plasma ACE activity in Brown Norway (*b* allele) and Lou (*l* allele) rats after cross-breeding, where the *b* allele is related to high ACE activity and the *l* allele to low ACE activity [9,10].

Challah *et al*. demonstrated that ACE activity in cultured vascular cells and in neointima formation in the carotid artery after balloon injury were influenced by ACE levels [11]. Moreover, Ocaranza *et al*. showed there is an enhanced hypertensive response and more fibrosis in response to myocardial infarction in the Brown Norway rat with higher ACE levels [12,13]. When studying the effect of the *b* and *l* alleles and their difference in ACE expression on the phenotype, it is important to realize that the Brown Norway and Lou rat strains have completely different genetic backgrounds. Therefore, variations in other genes might have influenced the difference in susceptibility to damage.

In the outbred Wistar rat strain, a commonly used strain in renal and cardiovascular research, individual differences in renal ACE activity are independent predictors of the susceptibility to renal damage [14]. To provide a more suitable animal model to study the human *ACE* I/D genotype, we investigated the *Ace* genotype in the outbred Wistar rat and its relation to tissue and serum ACE activity (Hip-His-Leu cleavage), and bred two lines with respectively high and low genetically determined plasma ACE activity. Moreover, we investigated whether the difference in *Ace* alleles leads to a different conversion of AngI to AngII in the vascular wall. To this purpose, we investigated the contractile response to AngI in reference to the response to AngII in isolated aortic rings, as a functional assessment of vascular ACE activity.

Materials and Methods

Animals /Experimental protocol Wistar Unilever (HsdCpb:WU) rats were obtained from Harlan (Harlan Inc, Horst, The Netherlands) and genotyped for the microsatellite marker in the *Ace* allele as previously described by Hilbert et al. [15]. Animals were then selected to breed 27 homozygous

males for both the *b* allele and the *l* allele. Animals generated from this breeding scheme entered the study. All animals were housed in a climate-controlled space with a 14h light/10h dark cycle. Food and water were available *ad libitum*. After six weeks, 14 rats (seven of each line) were anaesthetized using Isoflurane/O2/N20 mixture. Thereafter, aortic tissue was collected and immediately placed in Krebs bicarbonate solution (compound from Merck, Darmstadt, Germany). At 12 weeks, the other 40 rats were placed in metabolic cages and 24h urine samples were collected. Afterwards rats were anaesthetized and the abdomen was opened through midline incision. A two-ml blood sample was obtained via aortic puncture to determine the serum ACE activity. Kidneys were saline perfused and harvested, followed by collection of heart and aortic tissue. Renal cortical tissue from the upper pole was processed to measure gene expression. Mid-coronal renal tissue slices were processed for immunohistochemistry. All animal experiments were approved by the University's Animal Care and Use Committee. The study was conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Genotyping

Genomic DNA was isolated from tail tips as previously described [16]. To determine the *Ace* genotypes, primers were used as described by Hilbert *et al* [17]. They amplify the microsatellite located at the 5' end of the intron between exons 13 and 14.

mRNA expression of Ace in the kidney

RNA was isolated using the Nucleospin RNA kit (Macherey-Nagel) according to the manufacturer's protocol. Samples were diluted and 2µg was used for cDNA synthesis using the Omniscript RT kit (Qiagen), with random hexamer primers. *Ace* mRNA levels were determined using a custom designed primer-probe set (Applied Biosystems) with the primers 5'-CACCGGCAAGGTCTGCTT-3', 5'-CTTGGCATAGTTTCGTGAGGAA-3', and the probe 6-FAM 5'CAACAAGACTGCCA CCTGCTGGTCC-3'TAMRA. The ABI Prism 7900 HT sequence detection system (Applied Biosystems) was used, which uses TaqMan chemistry for highly accurate quantitation of mRNA levels. *Ace* mRNA levels were expressed relative to those of the beta-2 microglobuline housekeeping gene (*B2M*), since geNorm VBA applet for Microsoft Excel (Vandensompele, 2002) determined *B2M* as the most stable housekeeping gene compared to the genes encoding glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and glucuronidase beta (*GUSB*). The *B2M* probe and primers Rn00560865_mi were provided as preoptimized control system (Applied Biosystems).

ACE activity

ACE activity was determined by its potential to cleave the substrate Hip-His-Leu in experimental conditions. In brief, renal cortex tissue was homogenized (and diluted 10 times for serum and 100

times for tissues), in a 50 mM K_2PO_4 buffer at pH 7.5. Subsequently, 50 µl of sample was diluted in 25 µl of demi water and the substrate (50 µl of 12.5 mM Hip-His-Leu (H4884, Sigma)) was added. This was incubated at 37°C for exactly 15 minutes. In this amount the substrate is present in excess and not rate limiting for the reaction. Adding 0.75 ml of 270 mM sodium hydroxide stopped the conversion of the substrate. Then 50 µl of 1% phtaldialdehyde, which adheres to the formed end product, bipeptid His-Leu, was added. The amount of tagged His-Leu was fluorimetrically determined at 355 nm excitation and 460 nm emission wavelengths. This yields a measure of the amount of His-Leu generated in the sample. In blank samples, sodium hydroxide was added to prevent conversion. The substrate was added after the incubation period. The coefficient of variation was 6% for these measurements of ACE activity using this method.

Immunohistochemistry

ACE immunohistochemistry was performed on frozen sections. Endogenous peroxidase was blocked with 0.075% H_2O_2 in phosphate buffered saline (PBS, pH 7.4) for 30 minutes. Primary antibodies (Chemicon International, CD143, MAB4051, clone 9B9) were incubated for 60 minutes at room temperature. Binding was detected using sequential incubation with peroxidase-labeled secondary and tertiary antibodies (Dakopatts, Glostrup, Denmark) for 30 min. All antibody dilutions were made in PBS supplemented with 1% BSA and to the secondary and tertiary antibodies 1% normal rat serum was also added. Peroxidase activity was developed by using 3,3'-diaminobenzidine tetrachloride for 10 min containing 0.03% H_2O_2. Counterstaining was performed using Mayer's haematoxylin.

When looking with a small magnification at the transverse section of the stained renal tissue, the ACE staining is presented as a band expanding from the cortico-medulary region laterally back towards the cortex. We have calculated the thickness of this band and presented it as a percentage of the length of the renal tissue from capsule to the end of the cortico-medulary section.

Contraction measurements in the isolated aorta

Immediately after removal, the aorta was placed in a Krebs bicarbonate solution equilibrated with 95% O_2 and 5% CO_2 [18]. After the blood vessel was cleaned of connective tissue, eight rings of 2-mm length were cut with a sharp razor blade, and care was taken not to touch the luminal surface. The rings were mounted between two stirrups in organ baths filled with 15 ml Krebs solution (37.5°C) and studied in parallel. One stirrup was anchored inside the organ bath, and the other connected to a displacement transducer to determine isotonic changes [19]. Rings were subjected to 14 mN and allowed to stabilize for 60 min, during which regular washing was performed. Rings were primed by evoking a contraction with 10 µ*M* phenylephrine (PE). Then a second response with 10 µ*M* PE was evoked, which served as a control contraction. These contractions were not

significantly different between the two rings studied in parallel or between different groups (data not shown). After washout and renewed stabilization, rings were incubated with 100 μ*M* N^G-mono-methyl-L-arginine (L-NMMA) for 45 min. Then, with L-NMMA still being present, parallel rings were stimulated with either AngI or AngII (0.1 n*M*-1 μ*M* bath concentrations). Finally, AngI induced response was studied in the presence of Lisinopril. These rings were pre-incubated with Lisinopril in addition to L-NMMA. All results are expressed as percentage change of the maximal AngII induced response for every pair of rings.

Statistical analyses

Data are expressed as median and inter-quartile range or mean and SEM for the response curves. They were analyzed using the statistical program SPSS 12.0.2 for Windows and Graph Pad, Prism, Version 4.03. Statistical differences were determined using Mann-Whitney U test. Significance was accepted at $P<0.05$.

To avoid non-specific (inter-assay) differences between rats, we evaluated the responses to AngI in comparison to concentration-response curves to AngII obtained with parallel rings of the same rat. To control for non-specific differences between rings from the same rat (intra-assay variance), contractile responses to AngI and AngII were first normalized by calculating the response as a percentage of the (reference) control contraction response to phenylephrine (%PE). In order to estimate the area between the concentration response curves of AngI and AngII, the area under the curve was determined separately. Subsequently, the difference between the areas under the curve was calculated. The same method was used to estimate the area between the concentration response curves to AngI in the presence of Lisinopril.

Results

Rat characteristics

Body weight and urinary protein excretion are shown in Table 1. Body weight was similar in WU-B and WU-L, both at week 6 (time point used for aortic rings) and week 12 (time point used for ACE

Table 1. Clinical characteristics of Wistar rats

	WU-B	WU-L
Body weight (gr.) week 6 (n=7)	122 (120-145)	92 (89-171)
Body weight (gr.) week 12 (n= 20)	375 (367-392)	354 (340-377)
Urinary protein (mg/24h) week 12 (n=20)	20 (17-26)	19 (15-21)

Median and quartile range is presented.

activity/mRNA/24h urine collection). Urinary protein excretion was low in both groups and not significantly different.

ACE activity and mRNA expression of Ace

ACE activity and *Ace* mRNA expression are presented in Figure 1. Expression is shown as the number of *Ace* mRNA molecules per molecule of *B2M* mRNA. ACE activity was approximately two times higher in the WU-B in serum, kidney and heart homogenates (all, $P<0.05$, panels A, B and D, left side). In the aorta homogenate, ACE activity was approximately two times higher in WU-L ($P<0.05$, panel C). Relative *Ace* mRNA expression was higher for WU-B in kidney tissue ($P<0.05$, panel B, right side), non-significantly higher in cardiac tissue ($P=0.06$, panel D, right side), and significantly lower in aorta ($P=0.02$, panel C, right side) compared to WU-L.

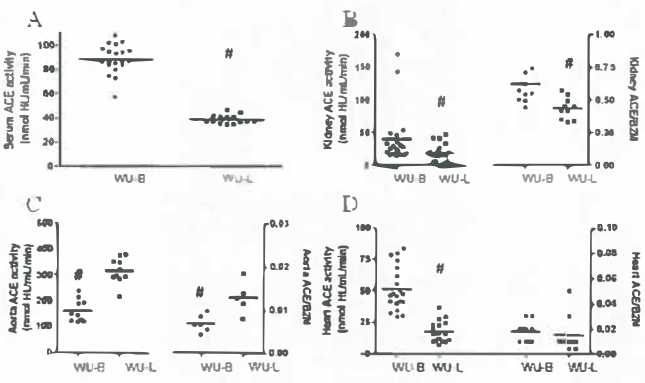

Figure 1. ACE activity and relative *Ace* expression in Wistar WU-B vs. WU-L. Expression is shown as the number of *Ace* mRNA molecules per molecule of *B2m* mRNA.The WU-B has higher ACE activity in serum, kidney and heart (panels A, B, D left side, # $P<0.05$). Relative *Ace* mRNA expression is higher in kidney tissue from WU-B (panel B, right side, # $P<0.05$) and borderline significantly higher in heart tissue from WU-B (panel D, right side, $P=0.06$). In the aorta, ACE activity and mRNA expression is higher in WU-L (panel C, # $P<0.05$).

ACE immunohistochemistry

In the kidney, expression of ACE protein was present in glomerular visceral and parietal epithelial cells, in the proximal and distal tubules and in the vascular endothelium of the large arteries, (Fig.2a and 2b, panels A-H). In WU-B there was a higher kidney ACE protein expression compared to WU-L, with the most remarkable difference in tubular ACE protein expression presented visually and calculated as a percentage of ACE staining/corticomedulary kidney region (Fig.3). No difference was observed in glomerular staining between the two genotypes. In heart tissue there was ACE protein expression in the vascular endothelial cells (Fig.2b, panels I and J), without differences between WU-B and WU-L. In WU-L aortic endothelial staining for ACE was positive. However, in aortic tissue from WU-B no endothelial ACE staining was observed (Fig.2b, panel K).

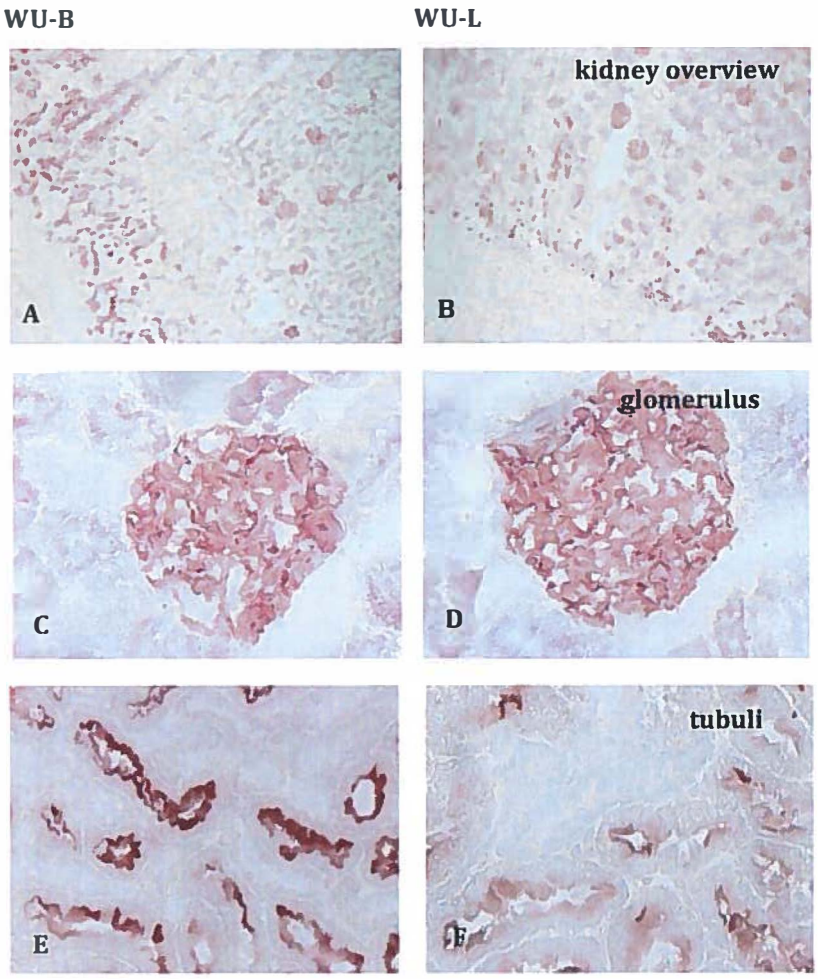

Figure 2a.

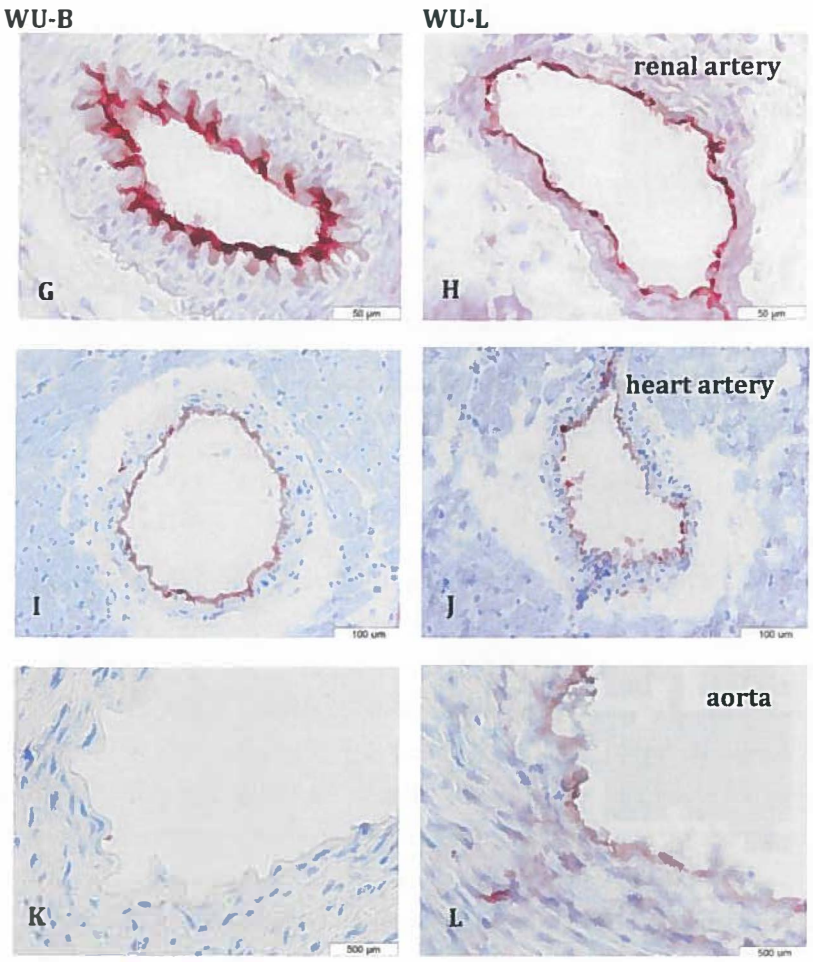

Figure 2b.

Figure 2. ACE staining in kidney (a) and blood vessels (b). Panels in 2a show kidney tissue of Wistar rat WU-B (kidney overview A, close up glomerulus C and proximal tubuli E) vs. WU-L (B, close up D and F) showing more staining of ACE in the tubules of the WU-B rat. Panels in 2b show kidney vessels of WU-B (G) and WU-L (H) with more staining in WU-B. Panels I and J show two coronary vessels with clear endothelial staining without differences between WU-B and WU-L. Lower panels (K and L) show aortic tissue, with no staining in WU-B (K) and endothelial staining in the WU-L (L) rat.

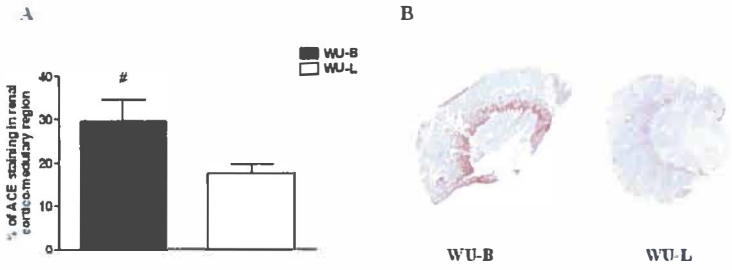

Figure 3. Percentage of ACE staining in renal cortico/medulary region. The ACE staining is presented as a band expanding from the cortico-medulary region laterally back towards the cortex (Fig. 3B). We calculated the thickness of this band and presented it as a percentage to the length of the renal tissue from capsule till the end of cortico-medulary section. The difference between WU-B and WU-L was statistically significant ($P<0.001$) with more staining present in WU-B lines (Fig.3A).

Vascular response

The contractile responses to equimolar concentrations of AngI and AngII in isolated parallelaortic rings are presented in Figure 4. Panel A and B show the responses to AngI, AngII and AngI + Lisinopril separately for WU-B and WU-L. It shows that AngI and AngII induced concentration-dependent vasoconstriction in both strains. It also shows that difference (area) between the concentration response curve to AngI and AngII was significantly higher in WU-L (panel C, $p<0.05$), indicating that there was more conversion of AngI in the WU-B. The effect of Lisinopril on the contractile response to AngI was studied to assess the contribution of ACE in the conversion of AngI to AngII. Lisinopril induced a significant shift to the right in the curves, demonstrating annihilation of the differences between the strains in conversion from AngI to AngII (panel D, P = ns).

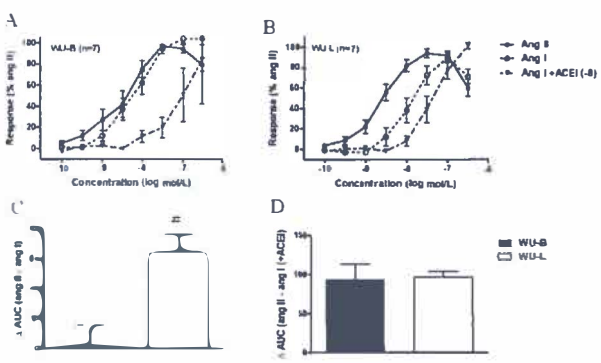

Figure 4. Concentration-response relationship for AngI and AngII in WU-B (panel A) vs. WU-L (panel B). Two parallel rings were stimulated with either AngI or II and studied for contractile responses. The results are expressed as a percentage of maximal response to AngII for every pair of rings (mean ± SEM). The area under the curves was determined for the individual pairs and compared; WU-L had a significant larger area, reflecting lower conversion from AngI to AngII (panel C, # $P<0.05$). The effect of

Lisinopril (ACEi) on the contractile response to AngI was studied, which showed a shift towards the right in the curves, with subsequent no differences between the conversion from AngI to AngII anymore (panel D).

Discussion

Genetically, deletion in intron 16 of the human *ACE* gene results in an increased ACE activity both on plasma and tissue level [20-22]. Numerous studies have been performed analyzing the possible association of the human *ACE* I/D genotype with cardiovascular and renal disease [23,24]. Whereas meta-analyses confirm a role for the D-allele as a renal and cardiovascular risk factor [25], the significance of genetically high/low ACE is still controversial [26]. A possible explanation for such inconsistency is the presence of multiple interactions with both genetic and environmental factors, i.e. sodium status, disease duration and sex.

Animal models would be helpful to unravel these complex interactions in well-controlled experimental conditions. Therefore, we investigated the previously described difference between the *b* and *l* allele (between the Brown Norway and Lou rats) [27], and identified these alleles in the outbred Wistar rats. We bred two lines homozygous for the two alleles (called WU-B and WU-L) and determined its phenotype related to ACE in serum, heart and kidney tissue. Both on serum and tissue level clear differences in ACE activity and mRNA expression were found between the two lines. The WU-B had higher levels of ACE activity in serum, heart and kidney. These differences were confirmed by immunohistochemistry with more ACE staining in tubules and kidney vessels of WU-B. This corresponds with the human plasma and tissue variation in relation to the *ACE* I/D genotype [28,29].

To identify the physiological mechanisms explaining the genetic modulation of ACE activity, we measured the ACE mRNA expression. Our data had similar tendency with higher ACE mRNA expression in kidney tissue but not significantly in heart tissue of the WU-B line (P=0.06). These measurements showed clearly that both the ACE activity and mRNA expression were related to the *b/l* alleles and suggested that the ACE activity is controlled at the transcriptional level. As mentioned before, Challah *et al.* described this polymorphism in inbred BN and LOU rats where *Ace* transcription rate was nearly two-fold higher in homozygote *b* rats compared to *l* rats. They also reported this difference in membrane ACE activity and mRNA expression in the lungs [30] while our investigation was more focused on renal and cardiovascular tissue but with the corresponding results. In the following studies between these two inbred strains higher left ventricular ACE activity was reported in connection with *b* homozygous *Ace* allele [31].

In order to provide a functional assessment of differences in ACE activity on tissue AngI conversion, we included measurements of contractile responses to AngI and AngII in isolated aortic rings. These results show a consistent higher conversion of AngI in the WU-B rat, which was reversible by the ACE inhibitor (ACEi) Lisinopril. Our experimental setup in isolated aortic rings was similar to

earlier studies that evaluated the efficacy of ACEi from the difference in the dose-response curves for AngI and AngII as a functional index of ACEi [32-34]. In comparison to the measurement of ACE activity in tissue homogenates, this setup has the advantage that the normal vascular architecture is preserved. This is physiologically relevant since the conversion of AngI seem to occur at the site of action [35]. However, this setup has the limitation that responses to exogenously administrated AngI and AngII may be similar [36], most likely due to high AngI converting capacity in rat aorta. Therefore, the clear difference in AngI responses, with higher responses in Wistar WU-B points towards a functionally relevant difference of the B/L allele. These differences are annihilated during pre incubation with Lisinopril.

In addition to the functional assessment of ACE activity in aortic rings, we measured ACE activity in aortic homogenates, as cleavage of the Hippuryl-His-Leu substrate, and performed both expression analysis and immunohistochemistry on aortic tissue. Interestingly, both ACE activity and mRNA expression was higher in WU-L. Also, the WU-B rat showed no staining of ACE in the aortic endothelium, which confirms the mRNA expression data. These allele-specific differences among the different tissues are not uncommon and imply that the ACE activity is not only controlled at the transcriptional level, but that the *cis*-acting loci regulating the transcription are under the control of a tissue specific mechanism.

The expressional, biochemical and staining results of the aorta did not parallel the functional findings. This may have various explanations. First, with respect to the vasoconstrictor response to AngI, involvement of non-ACE dependent, like chymase AngII forming pathways cannot be excluded. However, it should be noted that non-ACE pathways are under debate in rats and that chymase can cause breakdown of AngII in rats [37]. The differences between AngI responsiveness were not present during pre incubation with Lisinopril. This raises the hypothesis of another isoform of ACE present in the aorta, which is inhibited by Lisinopril as well but does not cleave Hippuryl-His-Leu nor it cross-reacts with our immunohistochemistry antibody. The local vascular RAS activity in aortic tissue has been demonstrated before in rat by Igase et al. and distinguished from one in other vascular beds, namely carotid arteries [38]. Also, as noted above, in homogenates, in contrast to functional measurement in the aortic rings, the normal vascular architecture is destroyed, which may affect the conversion of the substrate in various ways that do not reflect the physiological condition. Since ACE activity assay measures the conversion of Hippuryl-His-leu to His-Leu, other substances than ACE, which cleave this product similarly, may have affected these measurements. Finally, the factor that would lead to discrepancies could be the difference in time points since aortic rings functional measurements were done in six weeks and plasma and tissue measurements in 12 weeks old rats. However, in our experience, AT1 receptor responsiveness in aortic rings gives best comparable results in younger, 6-7 week old animals (data not published). In order to illustrate functional differences in ACE activity on tissue AngI conversion between the two

alleles, we chose the experimental setup we believe would be sensitive and specific enough to do so. On the other hand, for the characterization of WU-B and WU-L lines our aim was to present the phenotype of an adult male animals aged 3 months (12 weeks) as this is the age that is mostly used by other investigators studying the renal and cardiovascular systems. Also, complete inversion of tissue ACE expression and activity in the matter of weeks is highly unlikely possible and a similar fact never been reported. Which of the above mechanisms might be involved in the discrepancies between functional ACE activity, biochemical ACE activity and immunohistochemistry data in aorta cannot be derived from our data at this point.

We did not perform blood pressure measurements. However, several studies in other rat strains with different *Ace* alleles: healthy congenic, transgenic and Brown Norway vs. Lou rats have shown no difference in blood pressure levels [39-41]. A study by Kreutz at al. of the *Ace* locus in rat showed that the *Ace* gene clearly influences plasma ACE levels but no genetic linkage was found between the *Ace* gene and blood pressure [42]. Similarly, majority of association studies in humans using the *ACE* I/D genotype did not show any significant relationship with blood pressure [43-46]. Therefore, it is not likely that there would have been a difference between the WU-B and WU-L. Wistar rats do not develop proteinuria without intervention. Phenotypic individual differences in renal ACE activity predict the severity of adriamycine-induced renal damage [47]. Furthermore, a positive correlation was found between renal ACE activity and proteinuria, interstitial fibrosis and focal glomerulosclerosis after adriamycin induced renal injury in Wistar rats [48]. In response to disease *ACE* is upregulated [49,50]. These results support the assumption that genetic differences in (tissue) ACE activity predispose to a less favorable course of renal damage and influences renal outcome. It would be very interesting in this respect to investigate whether this up regulation is influenced by the difference in *ACE* alleles.

Previous studies related pathophysiological differences between the Brown Norway and Lou strains to variation in *Ace b/l* expression and ACE activity. A serious limitation to the approach of comparing these two different inbred strains is the different genetic background where other genes could influence the phenotype. Within the inbred strains the loci are homozygous but between them they differ for many loci in addition to the *Ace* alleles. Therefore, a comparison of the effects of the *Ace* alleles between these two strains is not possible as one cannot rule out the effect of different alleles between the two strains for other genes influencing the phenotype. The best solution would be to study the effect of the *Ace* gene in a congenic model. However, producing congenic strains is technically difficult and time consuming. Hence, the Wistar rat seems a good alternative for a congenic strain, as we only selected for the ACE genotype, while keeping the rest of the genetic background random. Our lines are created from a Wistar colony with limited genetic variation. By keeping the colonies large enough and with specific breeding schemes to prevent inbreeding, both lines will maintain this random background and similar heterogeneity with similar

allele frequencies. By using large enough groups per line in experiments, allele frequencies will be equal in each group. This cancels the effect of other genes on the ACE phenotype in the two different lines making the results more applicable.

In conclusion, the newly developed WU-B and WU-L rat lines, bred and characterized in this study, provide a good model for studying the differences between the *Ace* B and L alleles. It is comparable to the human *ACE* I/D genotype with higher plasma and tissue ACE levels in WU-B in heart and kidney. Also, functional conversion of Angl is enhanced in WU-B, which is reversible upon ACEi. It is however, difficult to explain the differences between the functional measurement and ACE activity, gene expression, and immunohistochemistry data in the aortic wall. These data emphasize the importance of further studies trying to explain how the *ACE* gene polymorphism interact with cardiovascular pathologies and define more exactly its molecular basis in animal studies as well as in humans.

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Chapter 3

Rat *Ace* allele variation determines susceptibility to AngII induced renal damage

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Abstract

A polymorphism in the rat *Ace* gene was found to be associated to differential tissue ACE expression and activity, and to the susceptibility to renal damage. The same polymorphism was recently found within the outbred Wistar rat strain, with the b allele accounting for higher renal ACE compared to the l allele. This provided a model for studying the RAAS response behind the innate high or low ACE conditions. With this background in mind, we have investigated the reaction of different alleles on chronic Angiotensin II (AngII) infusion.

Wistar rats were genotyped and selected to breed male homozygotes for the *b* (WU-B) or *l* allele (WU-L) (n=12). At the age of nine weeks, rats were randomly divided into two groups. For each allele, one group (n=6) received AngII infusion via an osmotic minipump (435 ng/kg/min). The other group (n=6) served as a control. After three weeks of treatment, rats were anesthetized and kidneys were collected for processing. At baseline and before termination, blood and 24h urine was collected. Blood pressure was measured at baseline and at two weeks of treatment.

As expected, WU-B had higher ACE activity at baseline then WU-L. Interestingly, baseline renal ACE2 expression and activity was exactly opposite and higher in the WU-L. AngII infusion induced the same increase in blood pressure in both genotypes, but caused tubulo-interstitial renal damage with increased α-SMA and monocyte/macrophage influx only in WU-B. Rats with intrinsically low ACE (WU-L) did not develop renal damage. Proteinuria was not increased in either of the groups.

In conclusion, AngII infusion causes proteinuria independent renal damage in rats with genetically predetermined high ACE while rats with low ACE seemed to be protected against the detrimental effect of AngII. Differences in renal ACE2, mirroring those in ACE, might be involved.

Introduction

The *ACE* gene encodes for the Angiotensin-converting enzyme (ACE), a principal catalytic protease in the renin-angiotensin aldosterone system (RAAS). In humans, the functional ACE I/D polymorphism accounts for half of the variance in plasma and renal ACE [1]. A similar polymorphism resulting in differential tissue *Ace* expression has been identified in the rat [2]. This *Ace* polymorphism was recently reported also within the outbred Wistar rat strain. Wistar rats homozygous for the *b* allele (WU-B) had higher renal *Ace* expression when compared to Wistars homozygous to the *l* allele (WU-L), providing a model for studying the RAAS under high or low ACE conditions and against the background of similar genetic heterogeneity and allele frequencies [3].

Increased renal ACE, in both rodents and human, has been thoroughly studied in connection with the progression of renal and cardiovascular disease [4-9]. In rats, high renal ACE has been associated with the increased susceptibility to hypertensive end organ renal damage [10]. In the model of chronic renal transplant failure, rats with high levels of intrarenal ACE mRNA and enzyme activity tended to develop more easily intraglomerular hypertension and more renal damage [11]. Furthermore, after adriamycin induced nephrosis, the differences in baseline renal ACE activity predicted the severity of damage [12], while during nephrosis development, a positive correlation existed between renal ACE activity and proteinuria and glomerular and interstitial injury [13]. Additionally, genetically predetermined high ACE has been suggested as a genetic risk marker in renal disease [14,15]. The data on the whole are still conflicting and the exact role of predetermined high ACE in susceptibility to renal damage is still inconclusive. However, the response of the RAAS to various stimuli is probably affected by this ACE polymorphism.

Concurrently, with the expanding complexity of RAAS, ACE2 has emerged as a potential functional counterpart of ACE. Current premise is that the two enzymes stand in balance [16-19]. It was shown that ACE2 is able to catalyze AngII with high efficiency [20] hence preventing its accumulation [21]. Furthermore, increasing data provide evidence of reno- and cardio protective properties of the ACE2 axis [22-24]. Activation of ACE2 axis was reported to lead to the decrease of oxidative stress and cell damage [25], stimulates vasodilatation [26] and it was recently shown that supplementation of ACE2 attenuated diabetic kidney injury in mice [27].

The availability of a rat model that, with different kidney tissue ACE levels hypothetically has different RAAS symmetry, might give us an insight into the dynamics underlying the *b/l Ace* polymorphism. Enhanced conversion of AngI has been demonstrated in experimental setups in non-diabetic and diabetic humans [28,29], but considering the negative feedback within the RAAS, it is uncertain whether such acute experiments bear relevance to the susceptibility to chronic renal damage. An alternative hypothesis deserves consideration. Studying RAAS it was shown that disease development could be followed by combined increase of renal ACE and decrease of ACE2 [30,31] suggesting that genetically higher ACE may be associated with altered dynamics within the

RAAS other then AngI conversion. If such effects downstream of ACE would be involved in the association to genetically higher ACE and susceptibility to renal damage, an increased susceptibility of the kidney to AngII induced damage would be anticipated.

To test this hypothesis, we infused WU-B and WU-L rats with AngII for a period of three weeks. In our model we evaluated glomerular and interstitial damage as well as *Ace* and *Ace2* expression and activity response to an external AngII stimulation. Furthermore, we analyzed systemic and renal functional parameters (blood pressure, creatinine clearance and proteinuria).

Materials and Methods

Experimental protocol

Healthy Wistar Unilever (HsdCpb:WU) rats were obtained from Harlan (Harlan Inc, Horst, The Netherlands) and genotyped for the microsatellite marker in intron 13 of the the *Ace* gene that identifies the presence of the *b* or *l Ace* allele. In short, genomic DNA was isolated from tail tips as previously described [32]. Primers that amplify the microsatellite located at the 5' end of the intron between exons 13 and 14 were used to determine the *Ace* genotypes, as previously described by Hilbert et al. [33]. Animals were then selected to breed 12 males homozygous for each of the *b* and *l* alleles. All animals were housed in a climate controlled space with a 12h light/12h dark cycle. Food and water were available *ad libitum*.

At the age of nine weeks, rats of the same genotype were randomly divided into two groups. The first group received AngII infusion via an osmotic minipump (435 ng/kg/min). The second group served as a control. After three weeks of treatment, rats were anesthetized using an Isoflurane/O_2/N_20 mixture, the minipump was removed and kidneys were collected. Renal tissue from the upper and lower poles was snap-frozen in liquid nitrogen to be used for immunohistochemistry, and to measure gene expression and ACE activity. The middle section was fixed in formalin and embedded in paraffin for immunohistochemistry. At baseline and before termination, blood was taken via orbital puncture and rats were subsequently placed in metabolic cages for 24h urine collection. Body weight was measured weekly. Blood pressure was measured with a tail cuff method (CODA™, Kent Scientific Corporation, Torrington, Connecticut USA) at baseline and two weeks into the treatment. After five pre-measurements, the mean of best three measurements was determined for every animal.

All animal experiments were approved by the local Committee for Animal Experiments of the University Medical Center Groningen.

ACE and ACE2 mRNA expression and activity in the kidney

For RNA expression, parts of kidney poles were first homogenized in the presence of lysis buffer and β-mercaptoethanol. Kidney lysate was then used for mRNA isolation with the Nucleospin RNA

kit (Macherey-Nagel), according to the manufacturer's protocol. RNA concentration was measured with a Nanodrop spectrophotometer. For cDNA synthesis, 1000ng of template mRNA in 12µl total dilution was used according to the QuantiTect Reverse Transcription (Qiagen) protocol for cDNA isolation. *Ace* and *Ace2* mRNA levels were determined with RT-PCR on the ABI Prism 7900 HT sequence detection system (Applied Biosystems). Master mix (Eurogentec, Liege, Belgium), *Ace* primers and probe or *Ace2* primers, and 20ng of cDNA to a total volume of 10µl, were used in a reaction in triplet. For the *Ace* gene, a custom designed primer-probe set was used, with the primers 5'-CACCGGCAAGGTCTGCTT-3', 5'-CTTGGCATAGTTTCGTGAGGAA-3', and the probe 6-FAM 5'CAACAAGACTGCCA CCTGCTGGTCC-3'TAMRA (Biolegio, Nijmegen, The Netherlands). For the *Ace2* gene, a gene-specific assay was used (Rn01416295_m1; Applied Biosystems). *Ace* and *Ace2* mRNA levels were expressed relative to those of the hypoxanthine phosphoribosyltransferase (*Hprt*) housekeeping gene, which was determined to be more stable then β2 microglobulin housekeeping gene (*B2m*). Data were analyzed with SDS 2.1 (SABiosciences).

For ACE and ACE2 activity, kidneys were homogenized in the RIPA lysis buffer (pH=8) with addition of 4% (v/v) EDTA-free protease inhibitor cocktail (Roche Diagnostics Nederland BV) and 0.2% (v/v) benzonase nuclease (Novagen, Merck KGaA, Darmstadt, Germany). Every sample was incubated in Assay Buffer (Tris-HCl, pH 7.4), with and without an inhibitor (ACE inhibitor: Captopril; ACE2 inhibitor: MLN-4670), for 30 min at 37°C. The fluorescent substrate was added to the reaction (ACE: Abz-FRK(Dnp)-P; ACE2: Mca-APK(Dnp) (Biomol International LP)). Abz or Mca fluorescence is quenched by the DnP group, until ACE or ACE2 cleaves and releases it. The fluorescence was measured using a Wallac Victor 1420 Multilabel counter (ACE: Excitation: 320nm, Emission: 420nm; ACE2: Excitation: 340nm, Emission: 405nm) at 10 minute intervals for 400 min. Every sample was measured in triplicate in the presence and absence of the appropriate inhibitor, under conditions in which the initial reaction rate was linear, to determine ACE- and ACE2-specific activity. Activity was expressed in units of pmol product/min/µg protein.

Immunohistochemistry

Deparaffinised sections (4 µm) were stained by periodic acid-Schiff (PAS) on a DAKO automatic slide stainer (DAKO Group, Denmark) to evaluate renal morphology. To assess renal damage and cellular infiltration, a myofibroblast transformation marker α-smooth muscle actin (α-SMA) and a cellular marker specific for activated monocytes and macrophages (ED1) were detected. Mouse monoclonal antibodies were used (α-SMA: clone 1A4, Sigma, St. Louis, Mo, USA, dilution 1:10.000; ED1/CD68: Serotec Ltd., Oxford, UK, dilution 1:750). α-SMA staining was performed by an automated staining system (Dako Autostainer Universal Staining System, Glostrup, Denmark). Deparaffinised sections were incubated overnight in 0.1 M Tris-HCl buffer (pH 9.0) at 80°C for

antigen retrieval. Endogenous peroxidase was blocked with 0.3% H_2O_2 in PBS (pH 7.4) for 30 minutes followed by a 60 minute incubation of primary antibodies at room temperature. Antibody binding was detected by sequential incubation of peroxidase-labelled secondary and tertiary antibodies (Dakopatts, Glostrup, Denmark, dilution 1:100) for 30 minutes at room temperature. Peroxidase activity was developed by 3,3'-diaminobenzidine tetrahydrochloride (DAB) with 0.03% H_2O_2 for 10 minutes. All antibody dilutions were made in 1% bovine serum albumin (BSA) in PBS with addition of 1% normal rat serum (NRS) to secondary and tertiary antibodies. Counterstaining was performed using Mayer's haematoxylin.

Quantification of renal morphology

The degree of tubulo-interstitial fibrosis (TIF) was determined in PAS stained sections in a blinded fashion. TIF was scored positive when tubular atrophy and fibrosis of the interstitium were present simultaneously. Semi-quantitatively, damage scores of 0-5 were assigned to 20 cortical fields per slide: a score 0 indicated no interstitial fibrosis, score 1 indicated 0-10% involvement, score 2 10-25%, score 3 25-50%, score 4 50-70% and score 5 5-100% involvement of cortical field. The final damage score per slide was calculated by multiplying the degree of damage by the number of positive fields/score ratio and adding these scores.

Interstitial ED1 positive cells were counted in 40 interstitial fields per slide excluding glomeruli and large arteries.

The percentage of α-SMA positive staining was quantified by computerized morphometry. Glomerular α-SMA was determined on 50 glomeruli, interstitial α-SMA on 30 interstitial fields per kidney, excluding glomeruli and blood vessels. The surface area found positive was divided by the total area of the glomerulus/interstitial fields measured, providing a percentage of α-SMA-positive tissue. The average percentage of all glomeruli/interstitial field was then calculated for every group.

Proteinuria and creatinine measurements

Urinary protein excretion was measured in 24h urine with the Behring Nephelometer Analyzer II (BNII, Dade Behring Marburg GmbH, Marburg, Germany) by using a 20% trichloroacetic acid (TCA) solution as reagent and tuberculin protein prepared by means of the ultrafiltration method (TPU) as a control.

Urinary and plasma creatinine were determined colorimetrically using a multianalyzer (Modular Analytics System, modules: ISE900, P800 en E170; Roche Diagnostics GmbH, Mannheim, Germany). Creatinine clearance (Cr Cl) was calculated per 100g of body weight for every animal.

Statistical analyses

Data were expressed as median and inter-quartile range unless stated otherwise, and distribution was assumed to be non-parametric. Data were analyzed using the statistical program Graph Pad, Prism, Version 5.00. Statistical differences were determined using the Mann-Whitney U test. Statistical significance was set at $p<0.05$.

Results

Body and kidney weight

Body weight is presented in Table 1. In the course of the experiment all rats significantly gained weight except the WU-B AngII infused group. Baseline weight in the WU-L group (before AngII infusion), was somewhat higher then in the same WU-B group. Kidney weight at termination (3 weeks) did not differ between groups.

		WU-B		WU-L	
		Control (n=6)	AngII (n=6)	Control (n=6)	AngII (n=6)
Body weight (gr)	Baseline	303 (283-323)	303 (271-332)	318 (301-337)	345 (340-357) *
	Termination	369 (342-400) #	345 (288-372)	355(347-378) #	369 (360-380) #
Kidney weight (gr)		1,55 (1,35-1,7)	1,50 (1,3-1,53)	1,35 (1,28-1,45)	1,35 (1,28-1,5)

Table 1. Body weight at baseline and termination, and kidney weight at termination, in Control and Angiotensin II infusion (AngII) groups. In the three week course of the experiment all rats significantly gained weight except the WU-B group receiving AngII infusion. No difference was found in kidney weight between groups at termination. Data are expressed as median (interquartile range). # $p<0.05$ week 3 vs. baseline; *$p<0.05$ WU-B vs. WU-L, same timepoint.

Renal ACE and ACE2 mRNA expression and activity

Characterization of the model: As expected, the activity of renal ACE was higher in the WU-B than in the WU-L group. In the current study the difference in *Ace* mRNA expression level was smaller then showed previously in this strain [34], but nevertheless the effect of the *Ace* allele difference was confirmed with the presence of two different ACE phenotypes. Interestingly, the two phenotypes differed in baseline renal ACE2 levels. Both mRNA expression and activity of ACE2 was consistently significantly higher in the WU-L group compared to the WU-B group (Fig. 1).

AngII infusion: AngII lead to an increase of *Ace* mRNA in WU-L but not in WU-B when compared to their respective controls (Fig. 1). Also, the difference between genotypes in *Ace* expression was statistically significant ($p<0.05$). On the other hand, in both genotypes excess of AngII led to an increase of ACE activity. However, the ACE activity tendency between genotypes remained the same, with higher activity in WU-B compared to WU-L ($p<0.05$). As for *Ace2*, WU-L reacted by increasing mRNA, which was significantly higher then in WU-B ($p<0.05$). Conversely, ACE2 activity was increased only in WU-B rats after AngII infusion where ACE2 activity levels reached the levels detected in WU-L rats, which showed no difference between treated and untreated groups (Fig. 1).

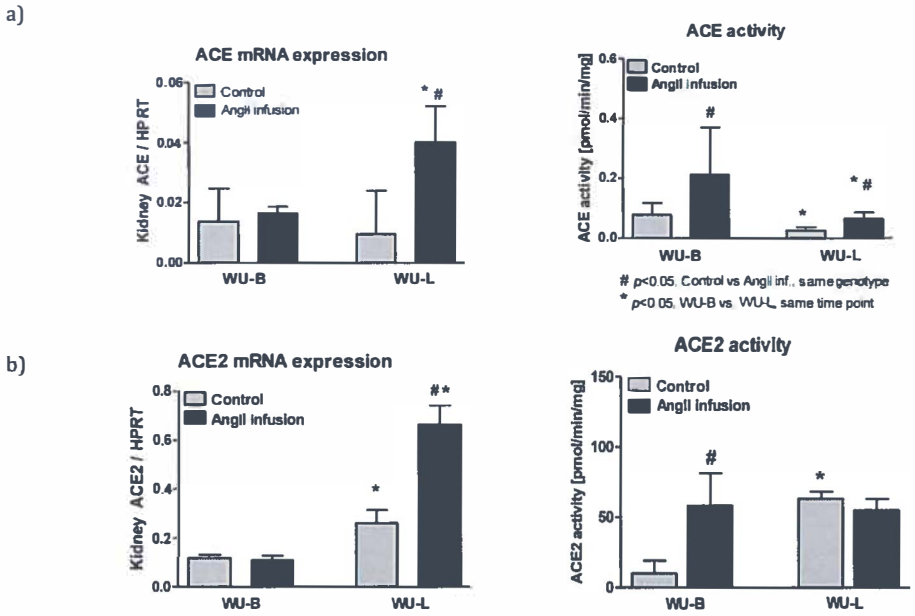


Figure 1. Renal ACE (a) and ACE2 (b) mRNA expression and activity in Control and AngII infusion groups in WU-B and WU-L genotype. In non treated groups ACE activity was significantly higher in WU-B then in WU-L. Direct opposite image was found for ACE2, where mRNA and ACE2 activity were higher in the WU-L. Three weeks of AngII infusion caused upregulation of both *Ace* and *Ace2* mRNA in WU-L, an increase in ACE and ACE2 activity in WU-B and ACE activity in WU-L. Data are presented as medium and interquartile range. # $p<0.05$, Control vs. AngII infusion, same genotype; * $p<0.05$, WU-B vs. WU-L, same timepoint.

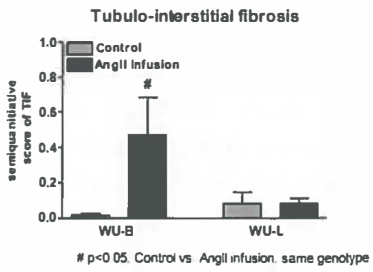
Renal histology parameters

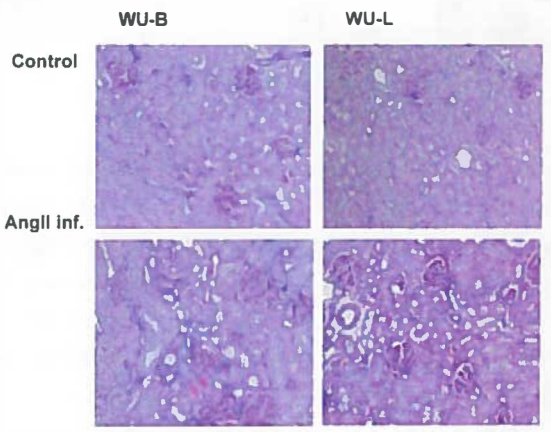
Characterization of the model: At the age of nine weeks normal renal structural damage scores were present in both genotypes. Interstitial fibrosis score was negligible and incidence of pro-fibrotic marker α-SMA was below 5% in both genotypes. The percentage of ED1 positive cells was similar between genotypes (8.15% (7.32-9.55) in WU-B and 8.23% (6.9-10.06) (Fig. 2)

AngII infusion: Three weeks of AngII infusion caused glomerular and interstitial renal damage only in WU-B rats ($p<0.05$)(Fig. 2). Tubulo-interstitial fibrosis (TIF) was significantly increased as was glomerular and interstitial pro-fibrotic marker α-smooth muscle actin (α-SMA) when compared to their respective controls. The number of cells expressing monocyte/macrophage marker ED1 was also significantly increased only in WU-B ($p<0.05$).

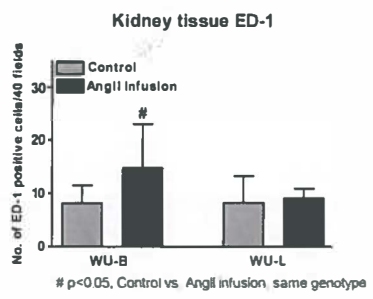
The consistent increase of these damage markers in the high ACE group indicates a pro-fibrotic and pro-inflammatory response of this genotype to high AngII On the other hand, in the WU-L group, AngII infusion did not result in renal damage. TIF, α-SMA and monocyte/macrophage influx were not increased in WU-L after AngII infusion, but remained at the control levels.

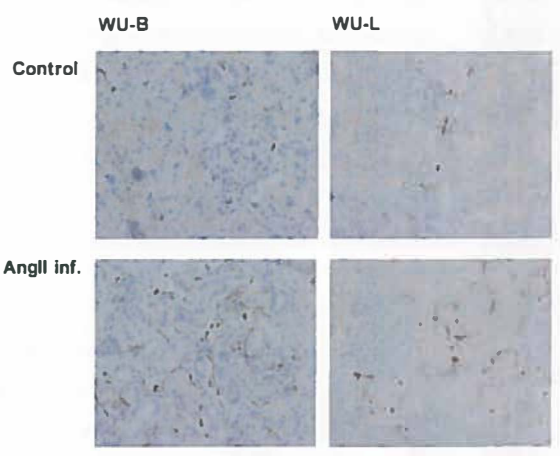
a)





b)





c)

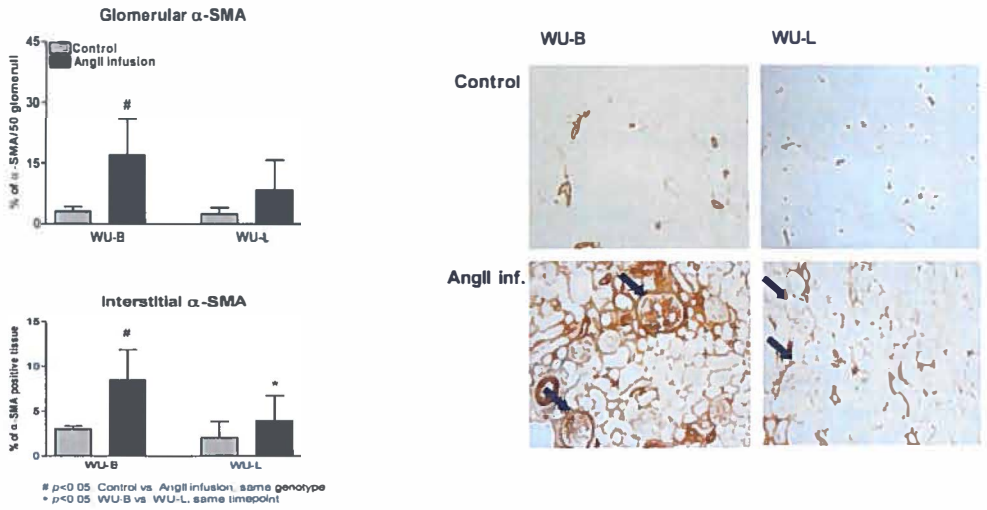


Figure 2. Renal damage markers in Control and AngII infusion groups in WU-B and WU-L genotype. (a) Tubulo-insterstital fibrosis (TIF) score and PAS staining of the kidney. **(b)** Kidney influx of monocyte/macrophage marker ED1 and staining in the kidney. **(c)** glomerular and interstitial α-SMA score and staining in the kidney. Arrows are indicating the difference in glomerular α-SMA between genotypes. Renal damage after AngII infusion was consistently present only in rats with WU-B genotype. The WU-L genotype seemed to be protected against AngII. # $p<0.05$, Control vs. AngII infusion, same genotype; *$p<0.05$, WU-B vs. WU-L, same timepoint.

Blood pressure and renal function parameters

Baseline blood pressure in all animals was 143 ± 15 mm Hg. AngII infusion increased significantly blood pressure in both genotypes ($p<0.05$) (Fig. 3). Although the WU-L control rats showed a slight increase in blood pressure compared to WU-B control rats, the effect of AngII infusion on blood pressure in both groups proved to be statistically significant.

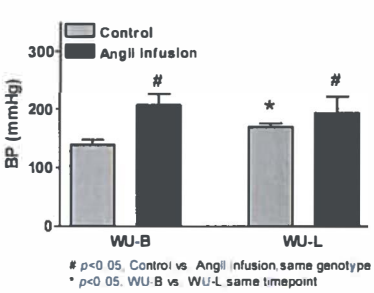


Figure 3. Blood pressure measurements at 2 weeks, in Control vs. AngII infusion groups in WU-B and WU-L genotypes. AngII infusion lead to a significant increase of blood pressure in both genotypes compared to Control, with no difference between genotypes after AngII infusion. Between the Control groups, WU-L had higher blood pressure then WU-B at the measured time point. # $p<0.05$, Control vs. AngII infusion, same genotype; *$p<0.05$, WU-B vs. WU-L, same timepoint.

Baseline proteinuria was not different between groups with an average of 27 ± 18 mg/24h. Three weeks of AngII infusion did not cause a significant increase in urinary protein excretion either in WU-B or in WU-L. As shown in a separate box, one rat in the WU-B group exhibited more sensitivity to AngII, while proteinuria levels of the five other rats in the WU-B group tended to cluster more closely. This accounted for the ascending proteinuria line for WU-B, but also for the lack of statistical significance when comparing to the other groups (Fig 4a).

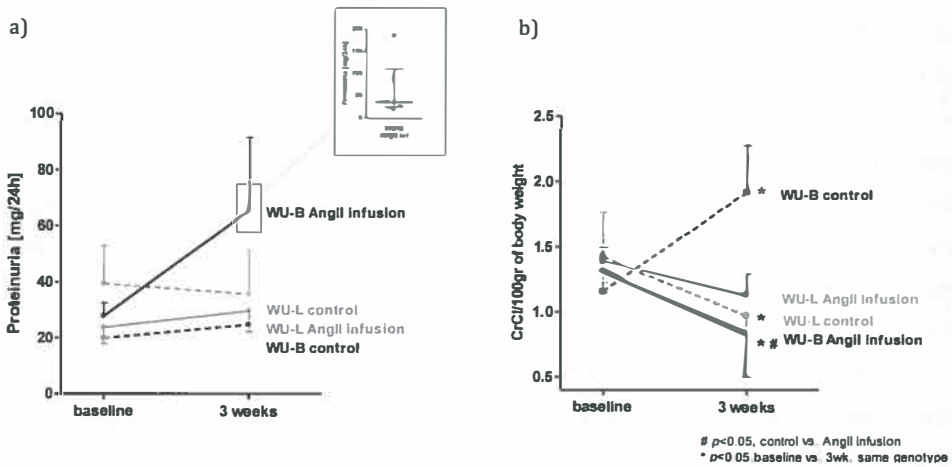


Figure 4. Proteinuria (a) and creatinine clearance (CrCl) (b) in Control and AngII infusion groups in WU-B and WU-L, measured at baseline and after 3 weeks. (a) AngII infusion did not cause an increase in 24h protein excretion in either of the genotypes. The ascending proteinuria line for WU-B was due to one rat with possible high AngII sensitivity (as shown in a separate box). The difference to other groups was not statistically significant. **(b)** At baseline, CrCl was not different between groups. At termination, CrCl has decreased in WU-B AngII infused group and has increased in WU-B Control group, making the difference between groups statistically significant. In WU-L Control group CrCl has also decreased, while in WU-L AngII infused group has not changed. Data are presented as median and interquartile range. *p<0.05, baseline vs. termination, same genotype; # p<0.05, Control vs. AngII infusion group, same timepoint.

Baseline Creatinine clearance (CrCl) was not different between groups with an average of 1.3 ± 0.3 ml/min/100g kidney tissue. Three weeks of AngII infusion led to a drop of CrCl only in the WU-B group (p<0.05 vs. baseline). At termination, in the WU-B control group CrCl was significantly higher than at baseline (p<0.05), while in the WU-L this was lower than at baseline (p<0.05) (Fig 4b).

Discussion

Three weeks of AngII infusion induces proteinuria-independent, pro-inflammatory and pro-fibrotic renal responce in rats with genetically determined high *Ace* expression. In our study, these WU-B

rats developed glomerular and tubulo-interstitial damage with increased monocyte/macrophage influx. Creatinine clearance and weight gain were reduced in comparison to controls indicating a decrease in renal function and welfare in WU-B. On the other hand, rats with intrinsically low *Ace* expression (WU-L) did not develop renal damage, hence seemed to be protected against increased AngII levels. As expected, AngII infusion led to a significant increase in blood pressure in both genotypes but interestingly without an increase in proteinuria. An excess of AngII is involved in the development of hypertensive nephropathy and nephrosclerosis [35] that is followed by an increase of proteinuria [36]. In other studies a significant increase in protein excretion was reported only after prolonged (6 weeks) AngII infusion [37]. Conversely, the absence of proteinuria in our study allowed us to investigate glomerular and tubulo-interstitial damage without the detrimental effects of high urinary protein levels.

The (patho)physiological effects of high or low ACE levels are still inconclusive. In humans, the high ACE activity determined by the D *ACE* allele has been extensively studied in connection with the development and progression of renal and cardiovascular disease. While many studies show a connection [38-42], others have failed to establish it in the risk and disease prediction [43,44]. The genetic variability as well as the multifactorial nature of cardio-renal diseases might account for these discrepancies. In rats, the significant effect of background ACE genotype on baseline renal hemodynamics was shown in a study by Lui *et al*. where renal plasma flow and GFR were lower in high ACE Ren2.F than in low ACE Ren2.L rats. They also identified the *Ace* gene as a modifier of hypertensive end organ damage [45]. On the other hand, a genetic linkage has been found between the *Ace* gene and plasma ACE activity, but not between the *Ace* gene and blood pressure per se [46], so it could be argued that possible detrimental effects of high ACE could be mediated by a more tissue-oriented pathway. Physiologically, ACE can regulate vascular reactivity and response to hypertension injury by altering the conversion of AngI and hence the concentration of AngII, which is known to have pro-inflammatory and pro-fibrotic properties [47-50]. However, negative feedback can be expected to offset these effects. Given that ACE holds one of the central roles in the RAAS, it can be hypothesized that with this basal high or low ACE levels goes also a baseline variation in RAAS system equilibrium. Consequently, the activation of a RAAS cascade in pathological situations [51] could lead to different response to damage as found in our study.

Further, the renal ACE and its homologue the angiotensin converting enzyme 2 (ACE2), are reciprocally expressed between the two genotypes. At baseline, higher ACE2 enzyme and activity seemed to have been combined with lower ACE activity in the kidney, and vice versa. It has been suggested that the two enzymes may be counter-balancing each other in vivo [17,52,53]. The ACE2 enzyme has a high substrate specificity for AngII by converting it to Ang(1-7) [54,55]. Endogenous ACE2 was suggested to have renoprotective properties in chronic kidney disease [56] and infusion of Ang(1-7) was recently shown to counteract AngII and reduces glomerulosclerosis in rat [57]. The

reciprocal nature of these two enzymes found between WU-B and WU-L, leads us to question whether the baseline predominance of one could lead to different susceptibility to AngII induced renal damage. Even though ACE2 activity was not increased in the WU-L after AngII infusion, the initial higher ACE2 baseline values might have been one of the beneficial factors in counteracting the renal effects of AngII.

We did not measure AngII or Ang (1-7) levels during the experiment, thus it is hard to speculate about the exact regulatory pathway in the presented different susceptibility to AngII infusion. In this regard, additional studies are necessary to clarify the possible mechanism.

In conclusion, rats with genetically predetermined high *Ace* expression are more susceptible to develop AngII induced renal damage. Behind this genetic *Ace* variability might exist different RAAS symmetry. The full understanding of this polarity is yet to be determined as well as the possible value in disease prediction, progression and therapy.

Acknowledgments

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Chapter 4

The intron 13 CA-repeat in the rat and mouse *Ace* gene determines splicing efficiency

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In preparation

Abstract

The gene encoding the angiotensin-converting enzyme (ACE) is similar in rat and mouse. It has been implicated as a gene underlying hypertension and kidney damage and, at least in rats, differences in alleles have been associated with differences in expression. In addition to the rat *b* and *l* alleles, we identified two additional alleles in rat and two different alleles in mouse that are determined by the length of a CA-repeat in intron 13. Because the length of the CA-repeat is associated with gene expression in both rat and mouse, we cloned the various alleles into a reporter vector designed for measuring splicing efficiency. We found that the length of the microsatellite determines splicing efficiency. Splicing either the *b* or *l* allele alters gene expression, but there is no direct correlation between length and efficiency, suggesting an effect of DNA structure.

Introduction

Angiotensin-converting enzyme (ACE) catalyzes the conversion of angiotensin I to angiotensin II and, as part of the renin-angiotensin aldosterone system (RAAS), plays a major role in blood pressure regulation. Both systemic RAAS and the activation of local tissue RAAS have been associated with hypertension, diabetes, and cardiovascular and renal damage[1-3]. In humans the insertion deletion (I/D) polymorphism in *ACE* accounts for approximately 50% of the variation in *ACE* expression[4], while the D allele is associated with cardiovascular and renal disease [5-7]. The full understanding of the effect of genetically predetermined high and low ACE in (patho)physiological conditions is still to be determined.

Identification of a CA-repeat in intron 13 of the rat *Ace* gene has allowed differentiation of *Ace* alleles among different rat strains [8,9], and their association with different levels of plasma and tissue ACE. The *Ace* locus determines 74% of the plasma ACE activity in Brown Norway (Ace^b) and Lou (Ace^l) rats after crossbreeding, with the *b* allele related to high ACE activity and the *l* allele to low ACE activity [10,11]. We bred two Wistar outbred lines (WU-L and WU-B) that were homozygous for either the *b* or *l* allele, and demonstrated that serum, heart, and kidney ACE activity and kidney mRNA expression were higher in WU-B and that kidney staining of tubular ACE was significantly elevated in WU-B [12].

The causative element responsible for the expression difference has not been identified, but one possibility is the CA-repeat itself. CA-repeats are a type of splicing enhancer that make up 0.25% of the human genome [13]. In the *eNOS* gene, a CA-repeat in intron 13 was shown to function as an intron splicing enhancer whose activity depended on the repeat length. hnRNP L, a protein that binds to pre-mRNAs at the CA-repeats, binds more efficiently to longer CA repeats, negotiating an increased efficiency of splicing in these regions [14]. The linear relationship between CA-repeat length and splicing efficiency found in the *eNOS* gene raises the question of whether the CA-repeat length also determines the splicing efficiency of *Ace* mRNA, possibly providing an explanation for the difference in *Ace* expression between the *b* and *l* alleles.

In this study, we determined allele sizes in rat and mouse inbred strains and their correlation with expression. We also tested whether the differences in expression could be due to splicing efficiency controlled by the CA-repeat.

Materials and Methods

Animals and housing

WU-B and WU-L rat lines were bred as previously described [15]. All animals were housed in a climate-controlled space with a 14h light/10h dark cycle. Food and water were available *ad libitum*. At 12 weeks the animals were sacrificed and kidneys were collected for RNA. F344/NHsd, WF/NHsd, BBDR/Wor, LEW/HanHsd, SHR/NHsd, SS/JrHsd, FHH/EUR, BH/Ztm, DA/OlaHsd,

AO/OlaHsd, PVG/OlaHsd, ACI/EUR, BN/RijHsd, and MWF/ZtmHsd rats are strains held at the animal facility in Groningen; tail tips were obtained for genotyping. Twelve-month-old kidneys from 18 mouse inbred strains (five animals per strain) were provided by the Jackson Laboratory's Shock Center study as previously described [16]. All animal experiments were approved by the University of Groningen's and The Jackson Laboratory's Animal Care and Use Committees.

Genotyping

Genomic DNA was isolated from tail tips as previously described [17]. To determine the *Ace* genotypes, primers were used as described by Hilbert et al.[18]; they amplify the microsatellite located at the 5' end of the intron between exons 13 and 14 inside the rat *Ace* gene. PCRs were performed, and products were purified with the QIAquick PCR purification kit (Qiagen) and sequenced in both directions on the MegaBACE 1000 DNA sequencing system (Molecular Dynamics & Amersham life science). Sequences were aligned using the sequence analysis program SeqMan 6.1 (Lasergene) and compared with published sequences of the Brown Norway rat and the C57BL/6J mouse obtained from Ensembl (v37).

Expression of Ace in the kidney

Kidney RNA was isolated using the Nucleospin RNA kit (Macherey-Nagel) according to the manufacturer's protocol. RNA was diluted and a 2 µg sample was used for cDNA synthesis using the Omniscript RT kit (Qiagen) with random hexamer primers. *Ace* mRNA levels were determined using a custom designed primer-probe set (Applied Biosystems) with the primers 5'-CACCGGCAAGGTCTGCTT-3', 5'-CTTGGCATAGTTTCGTGAGGAA-3', and the probe 6-FAM 5'CAACAAGACTGCCACCTGCTGGTCC-3'TAMRA spanning the intron between exons 3 and 4. The ABI Prism 7900 HT sequence detection system (Applied Biosystems) was used with TaqMan chemistry for highly accurate quantitation of mRNA levels. *Ace* mRNA levels were expressed relative to those of the beta-2 microglobuline housekeeping gene (*B2m*), since the geNorm VBA applet for Microsoft Excel [19] determined *B2m* as the most stable housekeeping gene compared to the genes encoding glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) and glucuronidase beta (*Gusb*). The *B2m* probe and primers Rn00560865_mi were provided as a preoptimized control system (Applied Biosystems).

Cloning of intron 13 in pBPLUGA

For the cloning of the exon13-intron13-exon14 fragment of the different alleles of the rat *Ace* gene, the following primers were developed: forward primer 5'-GTCGACTAGAGACTGATGAAGCC-3' and reverse primer 5'-GTGCTGCCTCCCAACGAGGATCC-3' containing a *Sal*I and *Bam*HI restriction site, respectively. PCR was performed under standard conditions on genomic DNA from ACI/SegHsd

(*Ace*^{a}), F344/NHsd (*Ace*^{f}), LEW/HanNhsd (*Ace*^{l}), and MWF/ZtmHsd (*Ace*^{b}). PCR products were purified and ligated into the pBPLUGA reporter plasmid [20] after both PCR product and plasmid were digested with *Sal*I and *Bam*HI, followed by transformation into JM109 cells. Constructs were verified by plasmid purification followed by standard sequencing.

Determination of splice efficiency

HEK293 cells seeded to 1.5x10^{6}/mL in 10% DMEM with fetal bovine serum were transfected using 3 µL of Gene Jammer transfection reagent (Stratagene) and 1 µg of plasmid. Following a 48-hour incubation, cells were washed with TEN (40 mM Tris.Cl, 1 mM EDTA, 150 mM NaCl) and centrifuged in 1.5 mL microcentrifuge tubes. Cell pellets were resuspended in 1X Tropix Lysis Solution (Applied Biosystems), vortexed to make homogeneous, and sent through a freeze-thaw protocol three times to activate the Lysis solution. The cell solution was centrifuged again, separating the transfected cell extract from the cell membrane pellet. Ten µL of each cell extract was transferred into a 96-well plate with 12.5 µL of Applied Biosystem's Dual light system buffer A. Immediately before the plate was read, 50 µL of Applied Biosystem's Dual light system buffer B (containing 10% Galacton Plus) was added to each well. Luciferase activity was measured with a VICTOR (Perkin Elmer). The plate was incubated at room temperature for 30 minutes. Following this incubation, 50 µL of Tropix Accelerator II was added and β-galactosidase activity was measured. To determine the splicing efficiency, the ratio between luciferase activity and β-galactosidase activity was calculated. All transfections and measurements were repeated six times for each construct in independent experiments.

Statistical analysis

Data are presented as mean ± SE. An ANOVA with Tukey's post-hoc test was used for statistical analysis between the groups. All statistical measurements were performed using JMP 7.

Results

Identification of different alleles for the intron 13 CA-repeat in Ace

Apart from the already known *b* allele ($[CA]_{26}$) and *l* allele ($[CA]_{23}$), we identified two novel alleles in rat intron 13. The first was a $[CA]_{24}$ repeat, which we called *a* because we found it in the ACI/EUR strain; the second was a $[CA]_{21}$ repeat, which we called *f* because it was first found in F344/NHsd (Table 1). We sequenced 18 inbred mouse strains for the homologous intron 13 and identified two different alleles ($[CA]_{19}$, and $[CA]_{20}$) with $[CA]_{19}$ being the most common (Table 2).

Table 1. Alleles of the intron 13 CA-repeat in rat

Allele	Repeat	Strains
f	$[CA]_{21}$	F344/NHsd, WF/NHsd, BBDR/Wor
l	$[CA]_{23}$	LEW/HanHsd, SHR/NHsd, SS/JrHsd, FHH/EUR, BH/Ztm, DA/OlaHsd, AO/OlaHsd, PVG/OlaHsd
a	$[CA]_{24}$	ACI/EUR
b	$[CA]_{26}$	BN/RijHsd, MWF/ZtmHsd

Table 2. Alleles of the intron 13 CA-repeat in mouse

Allele	Repeat	Strains
2	$[CA]_{19}$	129S1/SvImJ, BALB/cByJ, BTBR *T+ tf*/J, C3H/HeJ, CBA/J, FVB/NJ, KK/J, LP/J, NON/LtJ, RIIIS/J, SM/J, SWR/J
3	$[CA]_{20}$	C57BL/6J, C57BL/10J, C57BLKS/J, C57BR/cdJ, C57L/J, NZW/LacJ,

Expression differences between alleles

It has already been shown that Ace^{b} and Ace^{l} have different expression[21]. We confirmed this in our WU-B and WU-L lines, which were derived from outbred Wistar rats selected for the *Ace* alleles. WU-B, which has the *b* allele, has a significantly higher expression than WU-L, which has the *l* allele (Figure 1). We have previously shown that WU-B has significantly higher ACE activity in serum and kidney and a higher protein expression in kidney compared to WU-L [22]. We measured *Ace* expression in 18 mouse strains; the strains with a $[CA]_{19}$ repeat have a higher expression than strains with a $[CA]_{20}$ repeat (Figure 2A). When grouping all the animals by repeat length, this difference is significant (Figure 2B).

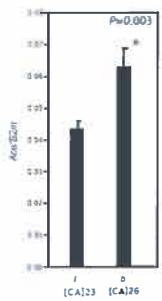

Figure 1. Renal *Ace* expression in WU-L and WU-B rats. Mean ± SE of *Ace* expression normalized to *B2m* is shown, where WU-L rats with a $[CA]_{23}$ repeat have a significantly (P=0.003) lower expression compared to WU-B rats that have a $[CA]_{26}$ repeat.

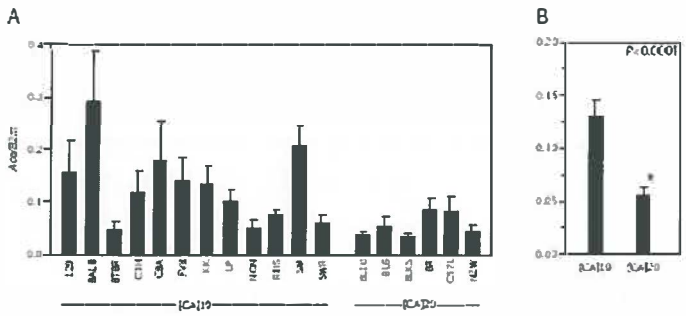

Figure 2. Renal *Ace* expression in mice. Mean ± SE of *Ace* expression normalized to *B2m* in 18 inbred strains with either a $[CA]_{19}$ or $[CA]_{20}$ repeat (A). Considerable variation exists among strains most likely because of trans-regulatory elements. However, but when comparing the mean expression by repeat length (B), shows that mice with a $[CA]_{20}$ repeat have a significantly ($P<0.0001$) lower expression compared to mice with a $[CA]_{19}$ repeat.

Differences in splicing efficiency

Plasmids for each of the four rat alleles were constructed, transfected into HEK293 cells, and after 48 hours, β-galactosidase and luciferase activities were measured in six independent transfections per construct. For $[CA]_{21}$, $[CA]_{23}$, and $[CA]_{24}$, we see a negative correlation with relative luciferase activity, indicating a decrease in splicing efficiency. For $[CA]_{26}$, however, splicing efficiency is greater than for the other constructs (Figure 3).

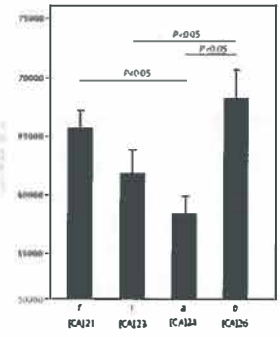

Figure 3. Splice efficiency of intron 13. The luciferase/β-galactosidase activity ratio in HEK293 cells after transfection is indicative of the splicing efficiency of the sequences.

Discussion

In humans and rats, various *Ace* alleles are known to be associated with differences in both gene expression and enzyme activity. To date, no functional polymorphism has been identified; however, a difference in splicing efficiency is one of several possible mechanisms.

The known *b* and *l* alleles of *Ace* in the rat are characterized by differences in a CA-repeat in intron 13 of the gene: the *b* allele has a 26-repeat while the *l* allele has a 23-repeat. In 2003 Hui et al reported that dinucleotide CA-repeats of variable length found in intron 13 of the human *eNOS* gene promote intron removal and that the effect is influenced by the number of CA repeats [23]. Splicing occurs within a macromolecular complex of ~150 proteins and a few small nuclear RNAs that assemble on the pre-mRNA and catalyze intron removal. It is currently believed that hnRNP L, a member of the hnRNP family, recognizes CA-repeats in a sequence-specific fashion and functions as a splicing enhancer.

We first sequenced intron 13 for 14 rat inbred strains and 18 mouse inbred strains to assess the number of different alleles present in the two species. In addition to the two previously identified alleles, we identified two novel alleles in the rat and, consistent with the *b* and *l* alleles, named them *a* (as it is present in ACI), and *f* (as F334 was the first strain in which we found the allele). We also identified two different alleles in the mouse. As many factors can influence gene expression, comparing *Ace* expression among strains does not reflect differences in the alleles of the gene alone. The only valid comparison in the rat is between the WU-L and WU-B lines, which were derived from outbred Wistar rats by selecting and breeding specifically for the *Ace* allele while keeping the remainder of the genome as random as possible. Renal expression shows a significantly higher expression in WU-B compared to WU-L, which confirms findings in a previous study in which *Ace* expression was compared between homozygous BB and homozygous LL rats from an F2 intercross population between the BN and Lou inbred strains [24]. We have previously shown that WU-B has significantly higher ACE activity in serum and kidney and a higher protein expression in kidney compared to WU-L [25], but that the basal blood pressure levels in both strains is the same (data not shown).

In mice we measured renal *Ace* expression in 12 strains with a $[CA]_{19}$ repeat and 6 strains with a $[CA]_{20}$ repeat. Most of the strains with the $[CA]_{19}$ repeat have a higher expression compared to the strains with the $[CA]_{20}$ repeat. When combining the data from all the strains, this is a significant difference ($P< 0.0001$).

To test whether the CA-repeat affects splice efficiency, we cloned all four alleles of the rat intron 13 with the flanking exons into the pBPLUGA reporter vector. The fragment is ligated between two reporters. The upstream reporter (β-galactosidase) is expressed regardless of splicing. Splicing removes the internal translation termination signals, placing the upstream reporter in frame with the downstream reporter (luciferase). The downstream reporter is expressed only after splicing. The ratio of luciferase activity to β-galactosidase activity depends on the proportion of transcripts that are spliced.

For *eNOS*, Hui et al showed that a longer CA-repeat results in an increase in splicing efficiency (testing a 19, 32, and 38 repeat) [26]. Our data (using different CA-repeats) show that a longer CA-

repeat results in both a decrease in efficiency (testing a 21, 23, and 24 repeat) and a significant increase in efficiency (testing a 26 repeat). One explanation might be that the secondary and tertiary structure of the DNA plays a role in the accessibility of the DNA sequence to the hnRNP L protein and that it is not a simple linear correlation but follows a more complex pattern depending on the conformation of the DNA. The currently available data for splicing and expression from the different published studies and ours suggest that with smaller repeat lengths there is a negative correlation, but after reaching a certain repeat length, the correlation turns positive. Whether this hypothesis is correct and is general or intron-specific must be tested using large series of CA lengths in different intron contexts.

In our specific case we show that the CA-repeat in intron 13 of the *Ace* gene determines splicing efficiency, and that the difference in splicing between the *b* and *l* alleles is reflected by the difference in both renal gene expression and protein activity. As these are the two alleles most commonly found in the rat inbred strains and outbred lines used for blood pressure related research, this finding can have important consequences for experimental design and interpretation of results. Especially when using outbred animals, knowing the *Ace* genotype will be essential. In a broader perspective, our work represents an important step in understanding the complex regulation of *Ace*.

Acknowledgments

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Chapter 5

The effects of ACE inhibition on renal ACE and on proteinuria contribute independently to renoprotection

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In preparation

Abstract

Data in man suggest that ACE inhibitors (ACEi) may possess specific renoprotective properties beyond proteinuria reduction. We hypothesized that inhibition of renal ACE activity might account for the added renoprotection. To test this, renal ACE activity was assessed in rats 6 weeks after adriamycin induced nephrosis. Subsequently, ACEi Lisinopril was given for a period of 6 weeks. Residual renal ACE was assessed by renal biopsy after stabilisation of antiproteinuric response (wk8). Healthy rats (N=8) and adriamycin rats treated either with AngII type 1 receptor antagonist (AT1RA, N=20), ACEi without pre-treatment biopsy (N=10) or vehicle (N=12) served as controls. ACEi reduced proteinuria by 78±3%, systolic blood pressure by 31±2%, and renal ACE activity by 58±8%. On univariate analysis, both residual proteinuria (R=0.90; P<0.01) and residual renal ACE (R=0.56; P<0.01) at week 8 predicted FGS at week 12. In AT1RA group only residual proteinuria at week 8 (R=0.68; P<0.01) predicted FGS at week 12. On multivariate analysis, residual proteinuria and residual renal ACE independently predicted FGS at week 12 (accounted for 63% of the variance). Analysed for % change from baseline of proteinuria and renal ACE, 54% of the variance in FGS was predicted by the model, with relative contribution of 28% for renal ACE. Creatinine clearance was independently predicted by both parameters as well. In summary, in addition to the effect on proteinuria, ACEi has a specific renoprotective effect by inhibition of renal ACE.

Introduction

Angiotensin converting enzyme inhibitors (ACEi) provide renoprotection in chronic proteinuric renal disease, under experimental conditions [1] as well as in human disease [2,3]. Since the renoprotective effect of ACEi exceeds the effect of mere blood pressure control, specific renal mechanisms must be involved. Proteinuria reduction is believed to be such a mechanism, decrease in local ACE and AngII content another. ACEi treatment restores the integrity of the glomerular barrier, reverses podocyte dysfunction and decreases intraglomerular capillary pressure [4,5], which might explain the distinct antiproteinuric effect. The residual proteinuria during therapy is a strong predictor of the renal outcome [6].

Various lines of research point towards an important role of the local renal renin-angiotensin system (RAS) activity in progressive renal damage [7,8]. In particular, tubuli of experimental animals with proteinuric disease show remarkable increase in angiotensinogen and ACE expression, and ACE activity [9,10]. Also AngII, the main product of ACE, was shown to be increased in renal injury and involved in upregulation of pro-fibrotic and pro-inflammatory cytokines [11,12]. Thus, interfering with the RAS at the level of local ACE could possible confer specific beneficial effects.

A meta-analysis of clinical renoprotection trials showed that for any given level of residual proteinuria during follow-up, the risk to reach a renal endpoint was lower in ACEi-treated patients, which suggests that ACEi exerts additional beneficial renal effects beyond proteinuria reduction [13], which might well be effects on local renal ACE. Recent human data, showing lack of superiority of dual RAAS blockade over monotherapy, have renewed interest in the specific merits, and mechanisms of action of ACEi as single RAAS blockade [14].

Thus, in the current study we tested the hypothesis that reduction of renal ACE activity as such may exert renoprotection on top of the effects on proteinuria.

To this purpose, we used the model of adriamycin-induced proteinuria in the rat. This model is characterized by a proteinuria-induced tubulointerstitial inflammation [15], mediated by NFκB in tubular cells [16], ultimately resulting in focal and segmental glomerulosclerosis [17]. It is associated with increased tubular ACE activity that may be involved in this cascade, so during ACEi treatment, the level of residual tubular ACE may be relevant to eventual renal outcome [18,19].

To test this, we investigated the renoprotective effect of ACEi and the respective roles of proteinuria reduction and renal tissue ACE inhibition with Lisinopril in the adriamycin-nephrosis rat model. Nephrotic rats treated with an AngII type 1 receptor antagonist (AT1RA) served as a positive control for reduction of proteinuria without pharmacological reduction of ACE activity.

Material and Methods

Adriamycin model

In our experimental setting, a single injection of adriamycin results in a non-hypertensive, proteinuric condition that usually stabilizes six weeks after the disease induction, with unremarkable abnormalities in renal morphology at that time, and subsequent progressive focal and segmental glomerulosclerosis (FGS) [20-22]. ACEi therapy started six weeks after disease induction consistently reduces proteinuria, and protects against progression of FGS. We used the adriamycin model because of its relevance to the clinical condition, where the renal disorder is usually well-established by the time that patients come to medical attention.

Experimental design

Eighty male Wistar rats (Hsd.Cpd.Wu; Harlan Inc., Horst, The Netherlands) were randomly divided in five groups according to the study protocol (Figure 1). After two weeks of acclimatization, nephrosis was induced in 72 rats by injecting 2 mg/kg adriamycin in the tail vein (body weight, 397 ± 4 gram), while 8 rats served as healthy controls. At week 6, the nephrotic rats were stratified according to proteinuria into three treatment groups, and a pre-treatment renal biopsy was performed (except in control animals as detailed below).

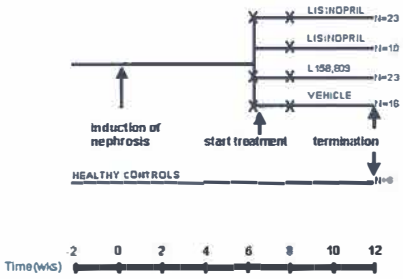


Figure 1. Schematic presentation of study design. X indicates renal biopsy.

Therapy was started after two days of recovery from the biopsy and continued until end of study at week 12. A second biopsy was performed at week 8, exactly twelve days after initiation of therapy. Group 1 (N=23) was treated with Lisinopril (75 mg/l drinking water [23]). A control group of N=10 nephrotic rats underwent all the same procedures (including week 8 biopsy), except for a biopsy in week 6, to serve as a control for possible effects of the pre-treatment biopsy on the course of disease. Group 2 (N=23) was treated with the AngII type 1 receptor antagonist (150 mg/l of L158,809 in the drinking water [24]). Group 3 (N=16) was treated with vehicle. The same procedures were followed as in the groups on active treatment, except in a subset of this group (N=6) that did not undergo renal biopsies.

Throughout the experiment, the rats had free access to drinking water and chow (low sodium diet, 0.05% NaCl, 20% protein, Hope Farms Inc., Woerden, The Netherlands) and were housed in a room maintained at 20°C and 60% humidity with a 12 hour light-dark cycle. All procedures were approved by the Committee for Animal Experiments of the University of Groningen.

Surgical procedures

All operations were performed by the same team of three workers and took place under anesthesia with isoflurane in N_2O/oxygen (1:2). Renal biopsies were performed via a dorsolateral incision; immediately after surgical removal of a small part of the underpole, gelfoam (Spongostan^R) was applied to accomplish hemostasis. Careful precaution was taken to obtain biopsies of similar size. One part of the renal tissue samples were snap-frozen in liquid nitrogen immediately after collection and stored at -80 °C, while a second part was fixed in 4% paraformaldehyde and later embedded in paraffin. Biopsies at weeks 6 and 8 were in all cases performed on the left and right kidney, respectively. During the terminal experiment in week 12, blood was collected by puncture of the abdominal aorta. In order to allow comparison with biopsy tissue, the kidneys were subsequently removed without being perfused. The overall mortality in nephrotic rats was 8/72 (highest in the vehicle group: n=4). The characteristics (body weight, blood pressure and proteinuria) of prematurely expired animals did not differ from the rest (data not shown) and mortality was in all cases biopsy-related. The prematurely expired animals were excluded from the statistical analyses.

Genotyping

Genomic DNA was isolated from kidney tissue as previously described [25]. Primers that amplify the microsatellite located at the 5' end of the intron between exons 13 and 14 were used for determining the *Ace* genotypes, as described by Hilbert et al [26].

Histological procedures

Paraformaldehyde fixed and paraffin embedded renal tissue samples were cut in four µm sections stained with periodic acid-Schiff (PAS) for determination of focal glomerulosclerosis (FGS). The degree of FGS was assessed semiquantitatively, by scoring the number of quadrants with mesangial matrix expansion and adhesion of the glomerular visceral epithelium to Bowman's capsule in 50 glomeruli/slide. First the score from 0 to 4 was determined per glomerulus, depending on the percentage of FGS present (25%, 50%, 75% or 100% respectively). The ultimate score was obtained by multiplying each score (0-4) by the percentage of glomeruli with the same score and then adding these figures, thus rendering a theoretical range of 0 to 400 [27]. Alpha-smooth muscle actin (α-SMA) was detected using a murine monoclonal antibody (clone 1A4, Sigma Chemical Co.,

St. Louis, MO, USA) [28] and measured using computer-assisted morphometry. Vessels and glomeruli were excluded from the measurements.

Measurements

Measurements of body weight and intake of food and water as well as the collection of 24-hour urine were performed weekly. Urinary protein concentration was measured by the Bioret method (Bioquant™, Merck, Darmstadt, Germany). Creatinine concentrations were determined by a multi-analyzer (SMAC, Technicon®). Systolic blood pressure was measured weekly by the tail-cuff method in trained conscious rats [29]. Enzymatic ACE-activity was established fluorimetrically by measuring the cleavage of Hip-His-Leu (Sigma), as described previously [30].

Data-analysis

Data are expressed as mean (SEM) unless stated otherwise. Baseline values for proteinuria are the mean of the values in week 5 and 6. Baseline blood pressures are the mean values of week 1 through week 6. The FGS score was log-transformed prior to testing in order to achieve a normal distribution. Within-group differences between pre-treatment and posttreatment were evaluated with paired, two-sided *T*-tests. Between-group comparisons were performed by a one-way analysis of variance (ANOVA) with a post-test according to Dunnett, using the group on vehicle treatment or the healthy controls as control group. Correlations were determined by the Spearman method. To test whether the antiproteinuric effect and renal tissue ACE inhibition contribute independently to the renoprotective effect of the ACE inhibitor, a step-wise linear regression analysis was performed, entering residual proteinuria and residual renal ACE activity (week 8) as independent variables and the FGS-score (or creatinine clearance, respectively) at the end of study as dependent variable. In order to take pre-treatment levels into account, the multivariate analysis was also performed by entering proteinuria and renal ACE activity as percentage change from baseline. To test the possible association of blood pressure regulation on residual proteinuria, residual renal ACE activity or the glomerular damage at the end of study correlation analysis was performed. All statistical calculations were performed using SPSS statistical software version 8.0 and Graph Pad, Prism, Version 5.00. Statistical significance was assumed at the 5% level.

Results

Proteinuria, blood pressure and histological renal damage

In healthy rats, proteinuria averaged 20 ± 3 mg/day. In the adriamycin groups, proteinuria developed rapidly to a level of 661 ± 29 mg/day (no differences between the groups) at week 6 and afterwards remained stable in untreated rats (Figure 2, left panel). Lisinopril effectively reduced proteinuria; the reduction in proteinuria (mean % change weeks 8 to 12) was similar in rats with biopsies at both week 6 and week 8 (-78 ± 3%) and those with only a week 8 biopsy (-78 ± 4 %),

indicating that a pre-treatment biopsy did not affect the response to ACEi (data not shown). AT1RA also reduced proteinuria (–59 ± 5 %) although slightly less effective than Lisinopril ($P<0.05$).

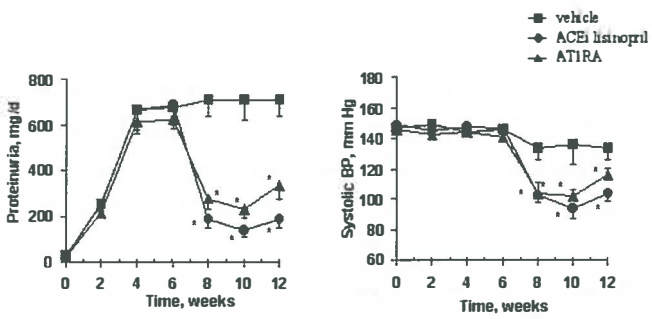


Figure 2. Proteinuria (left panel) and systolic blood pressure (right panel) over time among adriamycin nephrotic rats. Treatment with ACEi Lisinopril, type 1 receptor antagonist (AT1RA) L158,809 or vehicle was started at week 6. Each point represents the mean ± SEM of two consecutive weeks. *$P<0.05$ vs. Vehicle.

In healthy rats, the mean systolic blood pressure was 142 ± 2 mm Hg. Blood pressure remained unchanged after disease induction in all groups. The vehicle group remained normotensive throughout the study (Figure 2, right panel). Lisinopril effectively lowered blood pressure. The blood pressure response (mean % change weeks 8 to 12) was similar in the rats with two biopsies (-31 ± 2 %) and those with only a week 8 biopsy (-31 ± 6 %) (data not shown). Blood pressure was decreased in AT1RA group as well (–24 ± 4 %), although slightly less than with Lisinopril ($P<0.05$). When Wistar rats were genotyped for the *Ace* allele, homozygous ACE BB and heterozygous ACE BL genotypes were detected. The distribution of the two genotypes were similar in both ACEi and AT1RA groups, with 76,2% and 72,2% respectively having BB ACE genotype (Figure 3).

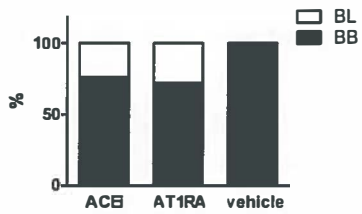


Figure 3. ACE genotype in ACEi, AT1RA and vehicle groups. There was a similar distribution in the ACEi and AT1RA groups, with more BB then BL ACE genotypes present.

At termination glomerular and tubulointerstitial damage were present in all nephrotic groups except in healthy controls (Table 1). FGS was significantly lower with active treatment than with vehicle. The creatinine clearance was slightly decreased in the nephrotic groups compared with the healthy controls.

Angiotensin converting enzyme: enzyme activity, mRNA expression and protein levels

Renal ACE activity was similar in all nephrotic groups before start of treatment (week 6). These values were not different from those obtained in healthy controls at sacrifice (Figure 4). At week 8, renal ACE activity had remained unchanged in rats treated with vehicle and AT1RA, whereas ACE activity was reduced significantly in the Lisinopril rats (-58 ± 8%; $p < 0,05$) without significant differences between animals with and without a prior biopsy. With Lisinopril, the reduction in renal ACE activity was maintained until termination. In the vehicle and the AT1RA rats, by contrast, a distinct increase in renal ACEactivity was present at termination. In the vehicle group the increase was similar for animals with (168 ± 49 nmol/g/min) and without control biopsies (163 ± 65 nmol/g/min, ns) (data not shown).

Table 1. Histological renal damage and creatinine clearance at the end of study

Treatment group	*N*	FGS	α-SMA	Creatinine clearance
Healthy control	8	6 (1, 10)	1.1 (0.6, 1.5)	0.48 (0.36, 0.60)
ACE inhibitor	22	45 (25, 66)[a,b]	7.0 (5.3, 8.7)[b]	0.33 (0.28, 0.39)[b]
AT1RA	20	53 (19, 87)[b]	8.0 (5.4, 10.6)[b]	0.36 (0.31, 0.40)[b]
Vehicle	12	103 (45, 161)[b]	10.2 (5.3, 15.1)[b]	0.34 (0.24, 0.60)[b]

Data are presented as mean (95% CI). FGS - focal glomerulosclerosis score; α-SMA- interstitial alpha smooth muscle actin; Creatinine Clearance (mL/min/100g); ACE inhibitor – Lisinopril; AT1RA – AngII type 1 receptor antagonist; [a] $P < 0.05$ vs. Vehicle; [b] $P < 0.05$ vs. Healthy controls.

The residual renal ACE activity was not correlated with the individual water intake (i.e. possible individual differences in drug dose) in either group.

At termination, plasma ACE was significantly reduced with Lisinopril, was increased in AT1RA group against healthy controls, while there was no difference in the vehicle group (data not shown). Renal expression of *Ace* mRNA (ratio mRNA *Ace/Gapdh*, in arbitrary units (AU)) at termination in the vehicle group showed a distinct elevation compared with healthy control. This rise occurred late in the course of disease, as after 6 weeks of nephrosis, *Ace* mRNA was only slightly but not significantly elevated (154 ± 15 vs. 115 ± 21 AU, data not shown).

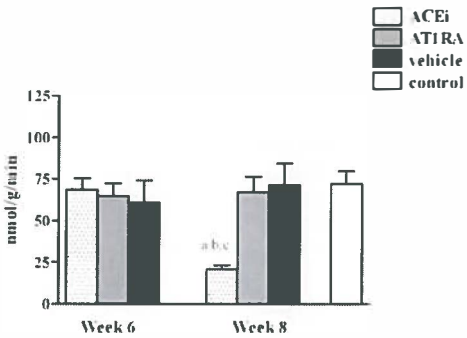


Figure 4. Renal ACE activity from homogenized tissue in untreated healthy control rats (white) and rats with established adriamycin nephrosis, sampled before (week 6) and after two weeks (week 8) of treatment. The respective groups were treated with ACE inhibitor Lisinopril (dotted), AngII type 1 receptor antagonist (AT1RA) L158,809 (grey) or vehicle (black). [a] $P< 0.05$ vs. week 6; [b] $P< 0.05$ vs. other group, same time point; [c] $P< .05$ vs. healthy control.

Subsequently, *Ace* mRNA remained unchanged at week 8 in the AT1RA and vehicle groups (153 ± 27 and 155 ± 55 AU respectively, data not shown), whereas Lisinopril treatment led to a transient rise in *Ace* mRNA at week 8 (208 ± 27 AU). In the Lisinopril group, *Ace* mRNA returned to pre-treatment level at termination (145 ± 10 AU).

Western blot analysis was performed on termination samples to test whether changes in mRNA were accompanied by corresponding changes in protein level. The prominent rise in *Ace* mRNA in vehicle rats was indeed accompanied by a rise in protein level – without further differences between the groups (data not shown).

Predictive value of proteinuria and renal ACE activity for long-term renoprotection by Lisinopril

- Residual proteinuria and residual ACE activity

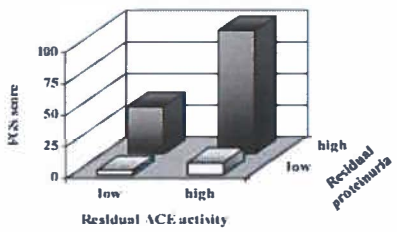
On the univariate analysis, as expected, residual proteinuria during Lisinopril predicted glomerular damage (Figure 6A, left panel) and creatinine clearance at termination (R= -0.61, $P<0.01$) (data not shown). Interestingly, residual renal ACE activity - assessed after 12 days of therapy - also predicted glomerular damage (Figure 6B, right panel) and creatinine clearance (R= -0.43, $P<0.05$) at termination.

These findings were supported by similar findings in the Lisinopril controls without a pre-treatment renal biopsy. As well in these animals, residual proteinuria (R=0.98, $P<0.01$) and residual renal ACE activity (R=0.7, $P<0.04$) both predicted glomerular damage. Residual renal ACE activity also predicted creatinine clearance at termination (R= -0.8, $P=0.01$), whereas no correlation between residual proteinuria and creatinine clearance found in this smaller group (R= -0.58, $P<0.1$).

Table 2. Independent contribution of proteinuria and renal ACE activity to the development of glomerulosclerosis

	Variance		*P*
	together	individual	
Residual proteinuria	63%	80%	<0.01
Residual renal ACE activity		20 %	<0.05
% change in proteinuria	54%	72 %	< 0.01
% change in ACE activity		28 %	<0.05

A possible independent role of residual proteinuria and residual renal ACE was addressed by multivariate analysis. Both residual proteinuria ($P<0.01$) and residual renal ACE activity at week 8 ($P<0.05$) were independent predictors of FGS at termination. Together, these two predictors accounted for approximately 63% of the variance in protection against FGS. Within the model, 80% of the variance in glomerulosclerosis was explained by residual proteinuria, and 20% by residual renal ACE activity (Table 2). The quantitative impact of both predictors is illustrated in Figure 5, providing the data on FGS in the Lisinopril treated rats. All group data were divided according to both low/high residual proteinuria and low/high residual ACE activity by a below or above group median break-up. The achieved blood pressure did not correlate with either residual proteinuria, residual renal ACE activity or the glomerular damage at the end of study.



	Low residual proteinuria	N	High residual proteinuria	N	*P*
Low residual ACE activity	4 (0 - 16)	6	38 (24 - 100)	5	0.003
High residual ACE activity	10 (0 - 78)	5	98 (58 - 160)	6	0.004
P	1.0		1.0		

Figure 5. Focal glomerulo-sclerosis score (FGS) after Lisinopril treatment in subgroups according to efficacy of renal inhibition of ACE and proteinuria reduction. Low /high residual ACE activity/ proteinuria is presented by below /above group median. Data are median (range). Kruskal-Wallis Test with post-test according to Bonferroni.

-Percentage reduction in proteinuria and renal ACE activity

Residual values of proteinuria and renal ACE, as analysed in the previous paragraph, may reflect pre-treatment differences, differences in pharmacological efficacy, or both. To distinguish the contribution of the pharmacological response as such, we analysed for the predictive values of the % change in proteinuria and renal ACE activity induced by Lisinopril.

The % reduction of proteinuria from week 8 to week 12, predicted the subsequent severity of FGS (R=0.8, $P<0.01$) as well as creatinine clearance at termination, although the latter correlation just fell short of statistical significance (R= -0.4, $P=0.07$). The % reduction in renal ACE activity also predicted FGS at termination (R=0.6, $P<0.01$) with a similar trend for creatinine clearance (R= -0.4, $P=0.058$). In the Lisinopril rats without pre-treatment biopsy, the correlation between the % change of proteinuria and glomerulosclerosis at termination was similar (R=0.83, $P<0.05$). In this group, creatinine clearance at termination was not predicted by the % change in proteinuria. On multivariate analysis, FGS at the end of study was independently predicted by the % change in proteinuria ($P< 0.01$) and the % change in renal ACE activity ($P<0.05$), together accounting for 54 % of the variance in protection against FGS. Within the model, 72% of the variance in FGS was explained by % change in proteinuria, and 28 % by % change in renal ACE activity (Table 2.)

The drop in blood pressure (% change from baseline) with Lisinopril treatment did not correlate with change in proteinuria, change in renal ACE activity or the glomerular damage at the end of study.

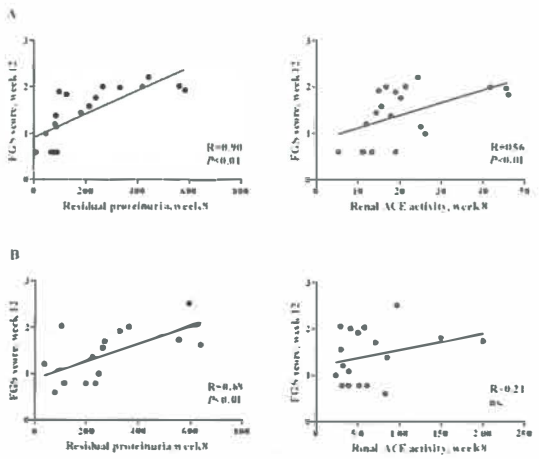


Figure 6. Scatterplot of rats treated with the ACE inhibitor Lisinopril (A) or type 1 receptor antagonist (AT1RA) L158,809 (B) with the focal glomerulosclerosis (FGS) score as dependent variable and residual proteinuria (left panel) or the residual renal ACE activity (right panel) as independent variables.

Predictive value of proteinuria and renal ACE activity for long-term renoprotection by AT1RA

In the AT1RA group residual proteinuria and the % change in proteinuria predicted FGS (R=0.68 and R=0.66, respectively, both: $P< 0.01$) and creatinine clearance (R= -0.64 and R= -0.67,

respectively, both: $P< 0.01$) at termination. Neither residual renal ACE activity, nor its % change predicted FGS or renal function at termination.

Discussion

The current study in the adriamycin model of proteinuria induced renal damage confirms the existing literature on renal disease with overt proteinuria, in showing that the residual proteinuria during ACE inhibitor treatment is consistently the strongest clinical parameter that predicts the long term renoprotective effect. The main new finding is, notwithstanding the predictive effect of residual proteinuria, residual renal ACE activity during treatment is an independent determinant of the long term renoprotective effect of ACE inhibitor therapy as well.

Both experimental and human studies suggested ACE to be involved in several pathways of renal damage, either directly or indirectly through profibrotic effects of AngII. In a recent study of adriamycin induced nephrosis in mice, similarly to our finding, only treatment with ACE inhibitor significantly decreased proinflammatory and profibrotic factors while no such effect was observed after AT1RA. Both therapies did, however reduce proteinuria and improved renal function [31]. Also in other rat models of diseased kidneys, induction of ACE expression coincided with infiltrating inflammatory cells and α-SMA positive cells in tubulointerstitium [32,33] while ACE inhibition limited tubular complement activation and interstitial inflammation [34]. This was consistent with human renal diseases, where ACE endothelial neoexpression co-localized with interstitial fibrosis [35]. These data support the pathogenetic relevance of renal ACE activity for development of FGS, which provides a pathophysiological basis for the independent predictive effects of residual renal ACE activity. Together, the data provide support for the notion that the renoprotective effects of ACE inhibitors could arguably stretch beyond attenuation of protein ultrafiltration and systemic and intraglomerular blood pressure reduction.

Better understanding of ACEi single drug therapy effect range is important also in the light of the current discussion regarding limited clinical outcome benefits and adverse tolerability after combined RAAS blockade [36-38]. A large long-term clinical trial in CVD patients recently showed that ACEi/ARB dual therapy has no significant benefits over monotherapy and was associated with worse renal outcome in patients with low renal risk. Although small trials have indicated a possible favourable effect of dual blockade on lowering blood pressure and proteinuria in CKD [39-42] the full benefit/side effect/cost ratio of the dual blockade in CKD is still unresolved [43,44]. In progressive renal disease an alternative to multidrug therapy approach could be individual monotherapy RAAS blockade optimization. Whether the best effect could be achieved by targeting renal ACE, or by interference with other RAAS components or other pathways [45] remains to be explored. Data showing better renoprotective efficacy with extremely high dose of ACEi [46] or the use of ACEi with higher tissue penetration and/or affinity than Lisinopril [47] suggest that specific

targeting of renal ACE might be fruitful. As for the current study and the predictive effect of residual renal ACE, it can be argued that the drug-effect on renal ACE might be a marker of the overall responsiveness to therapy. However, inhibition of renal ACE did not correlate with the percentage change of blood pressure or proteinuria. Second, renal ACE activity may be an indicator of other unidentified factors, that are the true mediators of renal damage [48-50] and a higher renal ACE may identify animals prone to develop renal damage - irrespective of its mechanism. It was shown in healthy rats that individual differences in renal ACE activity predict susceptibility to adriamycin induced nephrosis [51]. Third, low residual renal ACE activity may reflect more effective pharmacological blockade, with consequently better protection against FGS. From these considerations, it is clear that our study does not allow to conclude that the predictive effect of renal ACE for subsequence damage is due to a causal effect.

It would also be logical to assume that residual renal ACE activity in the Lisinopril rats reflects pharmacological efficacy of the ACEi regimen. However, this is not self-evident, as developed adriamycin nephrosis increases renal ACE expression and activity, as was also shown previously [52,53] pointing towards an association between intrarenal ACE and presence of advanced renal structural damage - an association that may also be relevant in human [54]. At week 8 in the present study, renal ACE activity in untreated nephrotic rats was still similar to the level in healthy control, hence adriamycin nephrosis may not yet have affected renal ACE activity, and consequently is not likely to affect residual renal ACE in the ACEi treated rats at that time.

As for the methodological factors that could have influenced renal ACE activity, first, we found no relation between individual water intake (i.e. drug dose) and ACE activity. Moreover, we previously demonstrated that doubling of the presently used dose does not further reduce ACE activity in kidney or plasma in this model [55]. Thus, the individual differences in residual ACE activity are not likely to reflect dose-response, as the present dose represents the top of the dose response. The sampling of renal tissue should also be considered: ACE activity was measured in homogenized renal tissue and, although the biopsies were taken according to a standardized protocol, some variation between the samples in the relative amount of cortical tissue cannot be excluded, resulting in differences in renal ACE activity. Finally, the effect of a pre-treatment biopsy must be considered as a potential source of bias. However, as shown by the data in the Lisinopril group without prior biopsy, the biopsy procedure did not affect subsequent proteinuria, FGS, or the predictors for FGS.

It should be noted that renal ACE activity can be altered not only in association with renal damage, but also in association with specific experimental conditions, such as sodium intake [56], and by genetic factors in experimental animals and human [57-59]. Therefore, additional studies in other models and other conditions would be needed to further delineate the role of reduced renal ACE activity on top of the proteinuria reduction.

Positive controls in our study were nephrotic rats that received AT1RA. As anticipated, in these rats AT1RA provided protection against FGS, and residual proteinuria predicted renal outcome. However, no independent contribution of renal ACE could be detected, suggesting that the predictive effect of renal ACE during ACEi is drug-specific, rather than a non-specific marker of susceptibility to FGS development.

In conclusion, this study demonstrates specific renoprotective effects of inhibition of renal ACE, by showing that the inhibitory effect on renal ACE, in addition to the effect on proteinuria, independently predicts the renal outcome in experimental proteinuric disease. Further studies - applying ACEi with high enzyme affinity and/or high tissue penetration, and/or very high dose of ACEi are needed to explore the therapeutic potential of this finding.

Acknowledgments

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Chapter 6

Renal ACE2 expression and activity is unaltered during established hypertension in adult SHRSP and TGR (mREN2)27

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Abstract

Differential renal expression of a homologue of the angiotensin-converting enzyme (ACE), i.e. ACE2, has been implicated as a genetic basis of polygenetic hypertension in the stroke-prone spontaneously hypertensive rat model. However, data on the role of ACE2 in hypertension are still inconclusive. Therefore, we analyzed kidney *Ace2* mRNA, ACE2 protein and ACE2 enzyme activity in the adult polygenetic stroke-prone spontaneously hypertensive rat (SHRSP) and the monogenetic TGR(mREN2)27 rat models, in comparison to their normotensive reference strains, i.e. Wistar Kyoto (WKY) and the Spraque Dawley (SD) rat respectively. Kidney *Ace2* mRNA was studied by quantitative real-time RT-PCR in cortex and medulla, while protein expression was scored semi-quantitatively in detail in different renal structures by immunohistochemistry. Furthermore, total renal tissue ACE2 activity was measured using a fluorimetric assay which was specified by the ACE2 inhibitor DX600. In SHRSP and homozygous TGR(mREN2)27 rats with established hypertension, kidney *Ace2* mRNA, protein and tissue ACE2 activity were not different from their respective WKY and SD reference strain, respectively. Additionally when we looked at renal localization, we found ACE2 protein to be predominantly present in glomeruli and endothelium with weak staining in distal and negative staining in proximal tubuli. Thus, our data challenges previous work that implicates ACE2 as a candidate gene for hypertension in SHRSP by reporting a significant reduction of ACE2 in kidneys of SHRSP. Taken together, renal ACE2 is not altered in the SHRSP and TGR(mREN2)27 genetic rat models with established hypertension.

Introduction

Angiotensin-converting enzyme (ACE), a key enzyme of the renin-angiotensin system (RAAS) plays a crucial role in renal (patho) physiology. It converts Angiotensin (Ang) I into Ang II, a potent vasoconstrictive, pro-inflammatory and pro-fibrotic peptide. The only known homologue of ACE, the angiotensin-converting enzyme 2 (ACE2) has been identified in humans and rodents [1]. ACE2 functions as a zinc metallopeptidase with carboxypeptidase activity. ACE2 hydrolyses Ang I to generate Ang(1-9), a peptide with unknown effects on the vascular bed. Additionally it catalyzes hydrolysis of Ang II to Ang (1-7), a potent vasodilator and antiproliferative peptide. The efficacy of ACE2 for the conversion of Ang I to Ang (1-9) in human is ~ 400-fold lower than for the generation of Ang (1-7) from Ang II [2,3]. ACE2 may act as a negative regulator of ACE and the RAAS by limiting the production or antagonizing the vasoconstrictive effect of Ang II and facilitating the formation of Ang (1-7).

It was initially hypothesised that disruption of the delicate balance between ACE and ACE2 would result in abnormal blood pressure control [4]. In that line of thinking increased ACE2 activity might protect against increases in blood pressure, and ACE2 deficiency might lead to hypertension. In early animal studies a potential role of ACE2 for blood pressure regulation and the pathogenesis of hypertension was supported by the fact that *ACE2* maps to the X chromosome and shows co-localization with blood pressure quantitative trait loci (QTL) [5] that have been identified in polygenetic rat models of hypertension including the stroke-prone spontaneously hypertensive rat (SHRSP)[6] and the salt-sensitive Sabra hypertensive (SBH/y) rat model of genetic hypertension [7]. In addition, it was reported that renal *Ace2* expression was decreased in these hypertensive strains when compared with the normotensive reference strains [5]. Moreover, stimulation of RAAS gene expression in SHR by all-trans retinoic acid (at-RA) for three weeks increased natively low kidney *Ace2* mRNA, which was associated with a reduction of blood pressure. However, other data were at variance with a causal role for ACE2 in the pathogenesis of hypertension. In the above study, ACE2 protein levels in SHR were increased and almost reached the levels of the control rats, with blood pressure elevated in comparison to the controls, thus putting into question a straightforward association between renal ACE2 and blood pressure [8]. In addition, in knockout mice lacking the *Ace2* gene blood pressure was normal or only modestly increased in comparison to their background strain, despite increased Ang II plasma and tissue levels [5,9]. These findings suggest that the role of ACE2 in blood pressure control is not a uniform phenomenon and is apparently context dependent. In human, data so far do not support a role for ACE2 in the genetics of hypertension, given the absence of association between single nucleotide polymorphisms in the *ACE2* locus and essential hypertension [10].

Thus, data on the role of ACE2 in blood pressure regulation and hypertension are still inconclusive. To address a possible role for renal ACE2 in established hypertension,

we analyzed kidney *Ace2* mRNA, ACE2 protein and ACE2 enzyme activity in two different genetic rat models of hypertension, namely the polygenetic stroke-prone spontaneously hypertensive rat (SHRSP) and the monogenetic TGR(mREN2)27 rat models in comparison to their normotensive reference strains, i.e. Wistar Kyoto (WKY) and the Spraque Dawley (SD) rat respectively.

Methods

Animals

This study was conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. All animal experiments were approved by the Animal Research Ethics Committee of the University Medical Centre Groningen. SHRSP, WKY, TGR(mREN2)27 and SD rats were obtained from our own colony in Berlin, respectively. SHRSP and WKY strains are directly derived from the original colonies from Heidelberg that were used in the co-segregation analysis in which the first blood pressure QTL on rat chromosome X was identified. Moreover, SHRSP and WKY animals with established hypertension were studied at a similar age of 14 weeks compared to the F_2 hybrids derived from SHRSP and WKY that were analyzed in the original linkage study [6].

Homozygous TGR(mREN2)27 animals develop severe hypertension at an early age and were therefore studied at 8 weeks of age as reported [11]. Rats were kept under conditions of regular 12h diurnal cycles using an automated light switching device and climate-controlled conditions at a room temperature of 22°C. The rats were fed a normal pelleted diet containing 0.2% NaCl and had free access to food and water. Systolic blood pressure and urinary albumin excretion were determined as previously reported [12].

Animals were sacrificed under 87 mg/kg body weight Ketamin HCl (Ketanest S, Pfizer, Karlsruhe, Germany) and 13 mg/kg body weight Xylazine (Rompun, Beyer, Leverkusen, Germany) anesthesia at 14 weeks of age. After induction of anesthesia, the abdomen was opened by a vertical incision. Blood was drawn from the abdominal aorta and through the same puncture saline was infused allowing both kidneys to be perfused before excision. This procedure in our hands precludes contamination of renal tissue by blood or serum components and by that eliminates possibility of serum ACE2 tissue contamination. The absence of blood is visually verified by the absence of erythrocytes in the lumen of renal blood vessels in Figure 2. Both kidneys were excised, the left kidney additionally dissected into cortex and medulla, and immediately frozen in liquid nitrogen and stored at –80°C.

Real-time RT-PCR for Ace2

To quantify mRNA expression of *Ace2* in kidney we employed the real-time quantitative RT ("TaqMan") PCR. Appropriate primers and fluorogenic probes were designed with the

PrimerExpress® software. The ABI PRISM® 7000 SDS instrument in conjunction with the ABI TaqMan Universal Master Mix (Applied Biosystems, Darmstadt, Germany) was used to perform the assays. The reaction volume was 25 µl with a final concentration of 900 nM for the primers and 200 nM for the probes. PCR conditions were used as recommended by the manufacturer. The primers were obtained from Proligo (Paris, France) with forward (F) and reverse (R) primer sequences (5'-3') F: GAGGAGAATGCCCAAAAGATGA and R: GAAATTTTGGGCGATCTTGGA. The fluorogenic probe was synthesized by TIB Molbiol (Berlin, Germany) (5'FAM- CTGCGGCCAAATGGTCTGCCTT - 3'TAMRA). Relative quantitation was done using the standard curve method. For each gene, a PCR fragment containing the sequence of the TaqMan-system was generated. Seven serial 1:10 dilutions of this fragment served as a standard curve that was assayed together with the corresponding unknown samples on each plate. Every sample was measured in triplicate. To normalize our expression data we used porphobilinogen deaminase (PBGD) as a housekeeping gene [11].

ACE2 immunohistochemistry

ACE2 immunohistochemistry was performed on frozen and paraffin kidney sections. Frozen slides were fixed in acetone for 10 minutes. Paraffin sections were incubated overnight at +80°C in TrisHCl buffer (pH 9). A polyclonal rabbit anti-ACE2 antiserum (a kind gift of the ACE2 antibody Millennium Pharmaceuticals Inc, Cambridge, MA)[13] diluted in in phosphate buffered saline (PBS) and supplemented with 1% bovine serum albumin, in concentration of 1:750 for frozen and 1:500 (WKY) or 1:750 (TGR(mREN2)27, SD and SHRSP) for paraffin sections was incubated for 1 hr at room temperature. Endogenous peroxidase was blocked for 30 minutes (0.075% H_2O_2 in PBS, pH 7.4), for paraffin sections before and for frozen sections after primary Ab incubation. Antibody binding was detected using sequential incubations with peroxidase-labeled goat anti-rabbit and peroxidase-labeled rabbit anti-goat antibodies (GARPO/RAGPO Dako, Glostrup, Denmark). Normal rat serum (1%) was added to the secondary antibodies to block aspecific binding. Peroxidase activity was developed by using 3-amino-9-ethylcarbazole (AEC) for 10 min containing 0.03% H_2O_2. Counterstaining was performed using Mayer's haematoxylin.

Three types of control tests were performed to determine the specificity of the antibody. First, control sections were incubated with anti-ACE2 antibody solutions, which were pre-incubated with the synthetic peptide to which the antibody was raised (peptide sequence: NTNITEENVQNMNNAGDKW aa51-69, Pepscan Systems BV, Lelystad, The Netherlands). Second, sections were incubated with unrelated rabbit polyclonal antibodies (anti-alpha1Inhibitor3 or anti-Nitrotyrosine) and third, sections were incubated with PBS in the absence of the primary antibodies. These control sections did not reveal any staining.

Analysis of structural changes

The structures positive for ACE2 were analyzed and scored semi quantitatively in a blinded fashion. The combined intensity and distribution of ACE2 immunostaining were determined on a scale of 0 to 2+ (0 absent; +/- very weak staining; + moderate staining; ++ strong staining) for different parts of the glomerulus (mesangium, endothelium, visceral epithelium, and parietal epithelium), tubuli (proximal and distal tubuli, collecting ducts), vascular structures in the cortex (vascular smooth muscle cells, vascular endothelium, peritubular capillaries) and vascular structures in the medulla (vasa recta).

ACE2 activity

ACE2 activity was measured according to the method by Vickers et al. [3]. Renal tissue was homogenized in assay buffer (50mM 2morpholinoethanesulfonic acid, 300mM NaCl, 10 µM $ZnCl_2$, 0.01% Brij-35, pH 6.5). Protein concentration was determined using Roti-Quant (Carl Roth GmbH and Co. KG, Karlsruhe, Germany) by the manufacturer's instruction. We used Mca-APK(Dnp) (Biosynthan GmbH, Berlin, Germany) dissolved in DMSO (50 µM, final concentration) as the ACE2 substrate. The assay was performed in assay buffer and was started by adding 10 µl of tissue homogenate. After 2 h incubation at ambient temperature (24°C), the reaction was suppressed by adding 100 µM *o*-phenanthrolin (final concentration). Parallel control tests were performed in the presence of 1 µM DX600 (R&D systems) (data not shown) [14]. After centrifugation (10 min, 10 000×g) the fluorescence was measured at 320 nm (excitation) and 405 nm (emission) with the Perkin-Elmer fluorescence reader Lambda 5 (Perkin-Elmer LAS GmbH, Rodgau, Germany). The molecular standardization was performed with Mca-AP (Biosynthan GmbH, Berlin, Germany) and calculated per mg protein. The functionality of the assay was proven by a standardized solution with defined, recombinant ACE2 activity (R&D Systems GmbH, Wiesbaden, Germany).

Statistics

Data are presented as mean ± standard deviation. Differences between the hypertensive strain and its normotensive control were analyzed with the Mann Whitney U test (SPSS 12.0). Significant differences were obtained when $P<0.05$, and all P-values were calculated from two-tailed tests of statistical significance.

Results

Rat characteristics

Table 1 summarizes the characteristics for the hypertensive and normotensive rat strains.

Table 1. Rat characteristics

	WKY (n=9)	SHRSP (n=8)	SD (n=6)	TGR(mREN2)27 (n=6)
Body weight (g)	289±23	260±18*	279±28	202±8*
SBP (mm Hg)	124±9	191±10*	125±16	197±38*
Urinary albumin excretion (mg/day)	0.55±0.23	0.71±0.45	0.30±0.21	20.1±13.41*

*P<0.05 compared to the normotensive reference strain. SBP = systolic blood pressure; WKY = Wistar-Kyoto; SHRSP = stroke-prone spontaneously hypertensive; SD = Spraque Dawley

Both SHRSP and TGR(mREN2)27 rats exhibit significantly higher blood pressure and lower body weights than their respective controls. Homozygous TGR(mREN2)27 rats demonstrate already a significant increase in urinary albumin excretion at 8 weeks of age while SHRSP show normal albumin excretion levels that are similar to the normotensive rat strains.

Ace2 mRNA

Ace2 mRNA levels were measured by quantitative RT-PCR in renal cortex and medulla. The results are presented in Figure 1. SHRSP rats have similar *Ace2* mRNA levels compared to WKY in both cortex and medulla. *Ace2* mRNA levels in TGR(mREN2)27 cortex were lower than in SD cortex, but this did not reach statistical significance. No differences in *Ace2* mRNA levels were found between TGR(mREN2)27 and SD medulla.

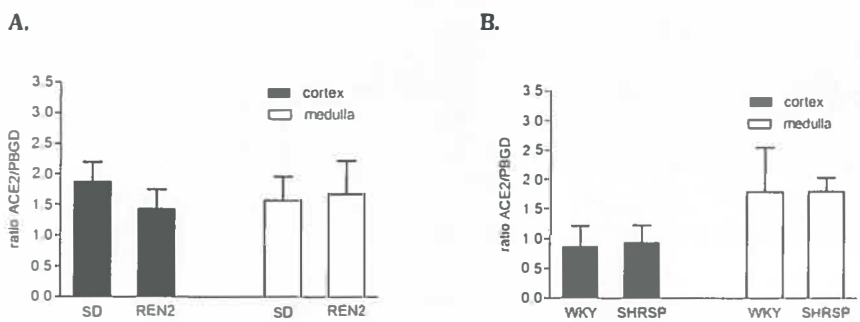
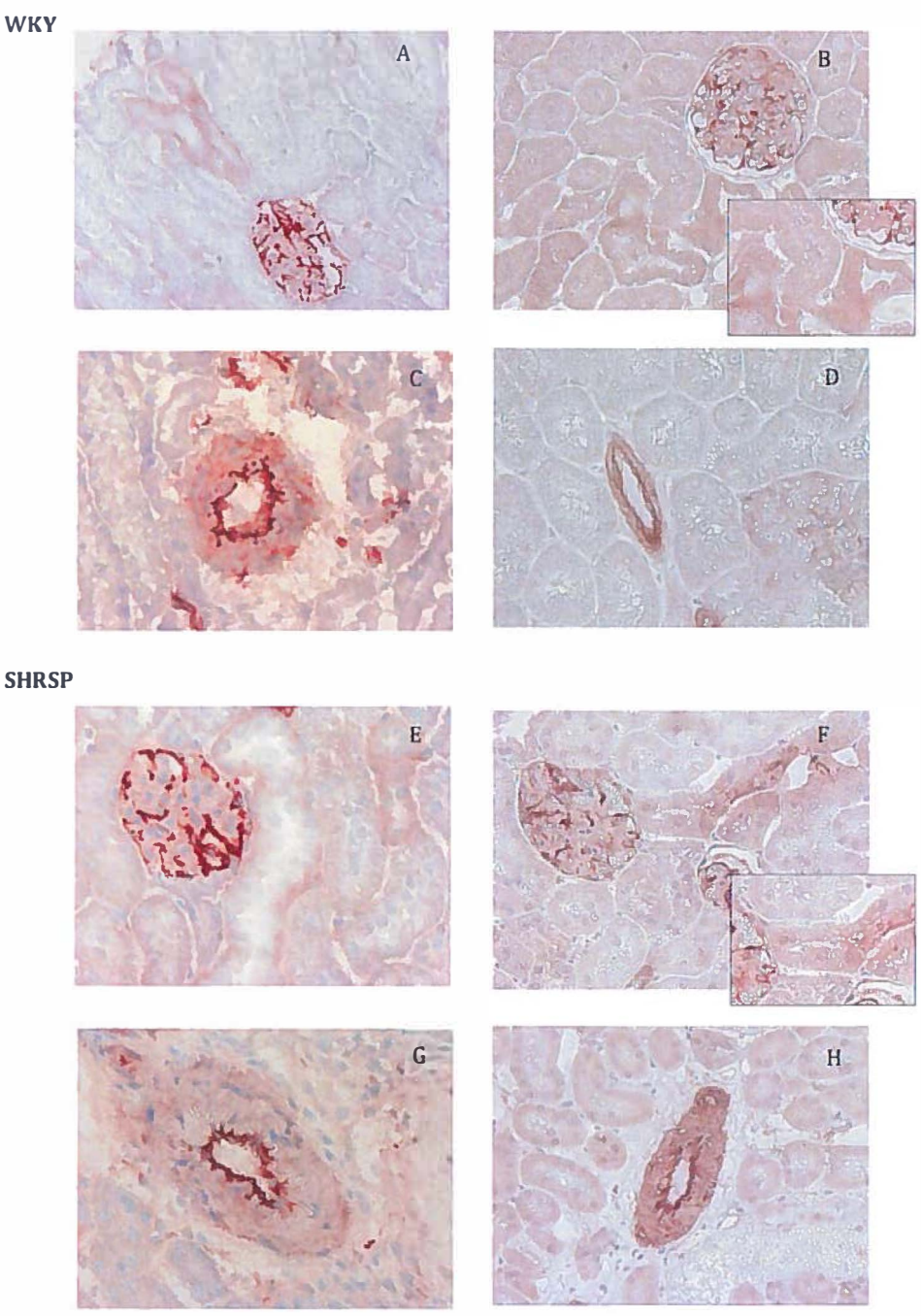


Figure 1. *Ace2* mRNA levels measured by quantitative RT-PCR in renal cortex and medulla. A. No differences were found in renal cortical and medullar ACE2 between stroke-prone spontaneously hypertensive rats (SHRSP) and Wistar-Kyoto rats (WKY). **B.** No differences were found in renal cortical and medullar ACE2 between TGR(mREN2)27 rats with renin-dependent hypertension and normotensive Spraque Dawley (SD) rats.

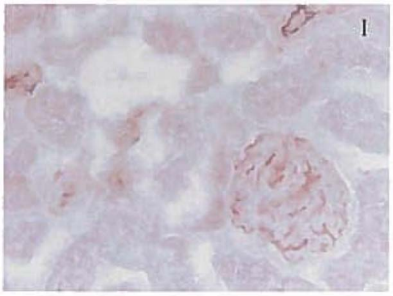
ACE2 immunohistochemistry

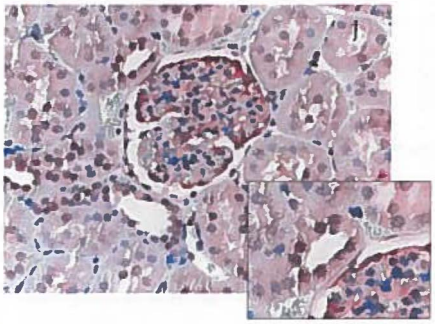
The immunohistochemical staining pattern of renal ACE2 was uniform in all rat strains, whether normotensive or hypertensive. Moreover, the pattern of ACE2 staining was consistent between

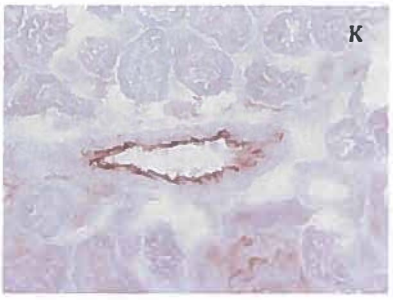


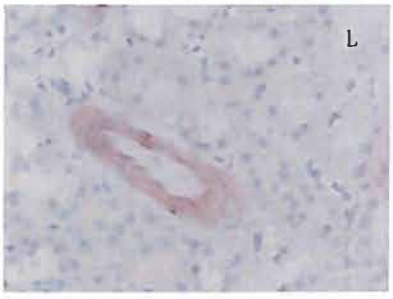
Frozen section **Paraffin section**

SD

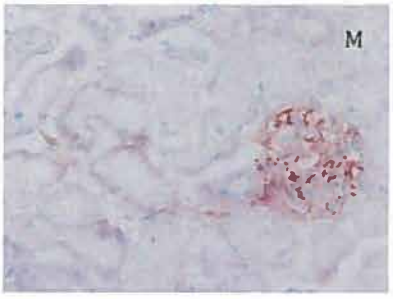


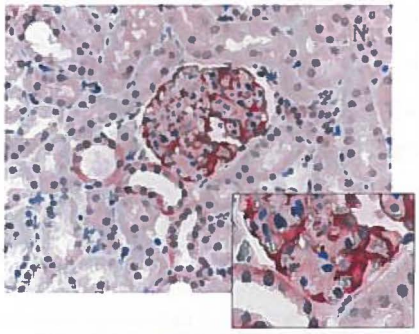


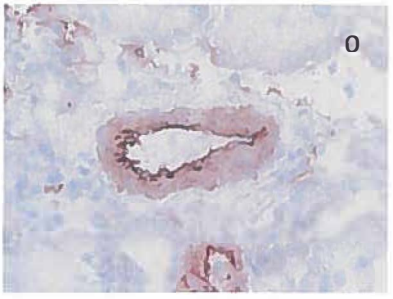


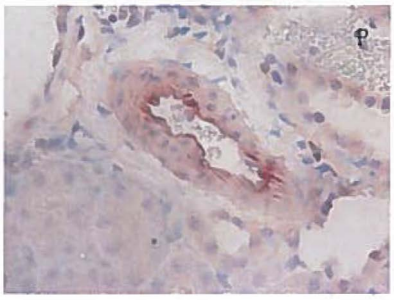


TGR(mREN2)2









CONTROLS

ACE2 peptide Anti NT PBS

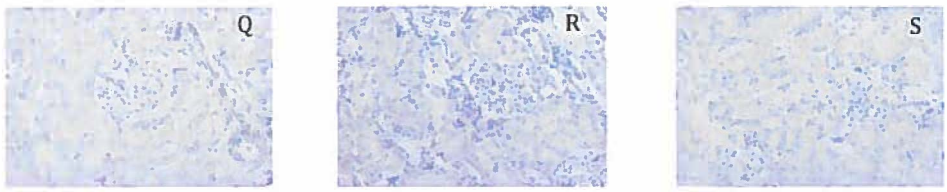


Figure 2. Immunohistochemical staining pattern of renal ACE2 of WKY (A-D), SHRSP (E-H), SD (I-L) and TGR(mREN2)27 (M-P) on frozen (left panel) and paraffin (right panel) sections and control sections (Q-S). Comparable renal ACE2 expression was found in all four rat strains. In glomeruli abundant ACE2 expression was present in visceral epithelial cells with moderate expression observed in parietal epithelial cells, whereas the glomerular mesangial and endothelial cells were consistently negative for ACE2. Overall weak ACE2 expression was present in the distal tubuli as seen on the high magnification fields. Consistent vascular ACE2 expression was observed in vascular smooth muscle cells and endothelial cells. Control sections were incubated with anti-ACE2 antibody solution in the presence of the synthetic ACE2 peptide **(Q)**; an unrelated rabbit polyclonal antibody anti-Nitrotyrosine (anti NT) **(R)** or with PBS in absence of the primary antibody **(S)**. These control sections did not reveal any staining.

frozen and paraffin sections. In the glomeruli, moderate ACE2 expression was observed in parietal epithelial cells and abundant ACE2 expression in visceral epithelial cells, whereas the glomerular mesangial and endothelial cells were consistently negative for ACE2 (Figure 2, Table 2). In vascular structures, ACE2 was abundantly expressed in vascular smooth muscle cells (VSMCs) and endothelial cells, with the exception of the endothelium of peritubular capillaries (Table 2). Remarkably, VSMCs of larger cortical radial (interlobular) arteries have less ACE2 expression than VSMCs of smaller preglomerular arterioles. This effect was most pronounced in SHRSP and WKY rats. Proximal tubuli did not reveal ACE2 expression while distal tubuli showed weak, predominantly intracellular ACE2 expression on the basal side of the cells. Collecting ducts were consistently negative for ACE2. The extent of ACE2 protein expression was scored semi-quantitatively for all the renal structures in cortex and medulla and the results are presented in Table 2. No differences in ACE2 expression were found between SHRSP and WKY rats and between TGR(mREN2)27 and SD rats (Figure 2).

Table 2. Renal ACE2 protein expression

	WKY (n= 9)	SHRSP (n=8)	SD (n=6)	TGR(mREN2)27 (n=6)
Glomeruli				
parietal epithelium	+	+	+	+
visceral epithelium	++	++	++	++
Endothelium	-	-	-	-
mesangial cells	-	-	-	-
Large arteries				
VSMCs	+	+	+/-	+/-
Endothelium	++	++	++	++
Preglomerular arterioles				
VSMCs	++	++	+/-	+/-
endothelium	++	++	++	++
Tubuli				
proximal	-	-	-	-
distal	+/-	+/-	+	+
Collecting ducts	-	-	-	-
Peritubular capilliaries	-	-	-	-
Vasa recta	++	++	++	++

Immunohistochemical staining pattern of renal ACE2 in hypertensive rat strains and their normotensive reference strain. The combined intensity and distribution of ACE2 immunostaining were determined on a scale of 0 to 2+ (- absent; +/- very weak staining; + moderate staining; ++ strong staining).
WKY = Wistar-Kyoto; SHRSP = stroke-prone spontaneously hypertensive; SD = Spraque Dawley; VSMC = vascular smooth muscle cells.

Tissue ACE2 activity

In accordance with the renal mRNA and protein expression data, no differences were found in tissue ACE2 activity between the hypertensive strains and their normotensive reference strain (Table 3). Notable, ACE2 activity was higher in SD and REN2 rats when compared to WKY and SHRSP rat strains.

Table 3. Renal ACE2 activity

	ACE2 activity mmol Mca-AP-OH/mg protein/h	
Rat strain	Mean	SD
WKY (n=9)	3.46	0.37
SHRSP (n=8)	3.38	0.32
SD (n=6)	10.97	2.63
TGR(mREN2)27 (n=6)	11.08	3.66

The amount of Mca-AP generated from Mca-APK(Dnp) by 1mg of protein from kidney tissues of rat within 1 h. WKY = Wistar-Kyoto ; SHRSP = stroke-prone spontaneously hypertensive; SD = Spraque Dawley.

Discussion

The major finding of our study is the absence of any differences in kidney ACE2 mRNA, ACE2 protein and ACE2 activity between the adult SHRSP, a model of polygenetic hypertension, and its normotensive reference strain, i.e. the WKY rat. Similarly, in a completely different model of monogenetic, renin-dependent hypertension, equal levels of kidney ACE2 mRNA, ACE2 protein and ACE2 activity were found in adult hypertensive homozygous TGR(mREN2)27 animals and their normotensive reference strain, a transgenic negative SD rat.

The hypothesis that linked ACE2 to blood pressure regulation was initially supported by a study that investigated renal ACE2 in genetic adult hypertension in rat models [5]. These findings would be theoretically in line with the hypothesis that in conditions with decreased ACE2 activity, blood pressure rises due to a prevailing AngII vasoconstrictor effect.

Recent studies have re-examined the role of renal ACE2 in blood pressure regulation with varying conclusions [10,15,16]. In experimental studies, first, in knockout mice lacking the *Ace2* gene, blood pressure is either normal or only mildly increased compared to control littermates [5,9,17]. In rats Pendergrass et al. found that hypertensive male mRen2.Lewis rats had lower cortical ACE2 activity than normotensive Lewis rats, but no difference in ACE2 activity was present between female hypertensive and normotensive rats in the same study. Paradoxically, male mRen2.Lewis had a higher blood pressure than females despite significantly higher renal ACE2 activity [18]. Thus, the association between lower renal ACE2 levels and higher blood pressure that was observed in the early studies, is apparently not a uniform phenomenon, and seems to be context dependent, as shown by the impact of gender in the Pendergrass study.

Human renal biopsy data illustrate the complexity of the possible associations between renal ACE2 and blood pressure. Wakahara et al showed that renal ACE2 is expressed synergistically with ACE, supporting pathophysiological relevance of the combination of the two, rather than for ACE2 alone. Moreover, they found that blood pressure was an independent confounding factor for renal ACE/ACE2 ratio, at expression as well as protein level, in patients with hypertension secondary due

to diverse renal conditions [19]. These data support the presence of an association between renal ACE/ACE2 balance and hypertension in renal patients, with a higher ACE/ACE2 ratio being associated with hypertension. These association data in renal patients, however, do not allow to dissect between hypertension as a cause or consequence of elevated blood pressure, or to conclude upon a role of ACE2 in essential hypertension, in the absence of renal disease.

Epidemiological data so far have not provided support for a role of ACE2 in hypertension. Neither in candidate gene studies [10] nor in two recent genome-wide association studies [20,21], the *ACE2* locus was associated with blood pressure or hypertension.

The intrarenal localization of ACE2 could potentially provide clues as to its functional role. However, data on the intrarenal localization of ACE2 are not consistent. Our current findings in rat renal tissue are somewhat at variance with earlier reports that provided varying results. In the current study we found predominant glomerular and endothelial ACE2 staining with weak staining in distal and negative staining in proximal tubuli. Others, however, have reported ACE2 positive proximal tubuli and/or ACE2 negative glomeruli in male SD rats using commercially available goat polyclonal anti-ACE2 antibodies [22] and in female SD rats with self-prepared mouse monoclonal and rabbit polyclonal anti-ACE2 antibodies [23,24]. These reports strongly emphasize the lack of ACE2 protein in glomeruli. On the other hand, with the above mentioned commercially available goat polyclonal anti-ACE2 antibody, ACE2 positive staining was subsequently reported to be present in tubular segments, but also in glomeruli and endothelial cells of male adult SD rats [25]. Similarly, in adult SHR and WKY rats, ACE2 mRNA and protein was present in proximal tubuli and large vessels with weak distal tubular and glomerular presence using the initial rabbit polyclonal anti-ACE2 [26]. Considering these between-laboratory discrepancies, in the current study we took great care to rigorously test validity and reproducibility of our immunohistochemistry data. To this purpose we tested different antibodies, and moreover, we reproduced the findings on paraffin as well as frozen sections. The similarity of our findings on renal ACE2 in paraffin and frozen sections, as well as the reproducibility of it for two different hypertensive and normotensive strains supports the robustness of our findings. So, whereas our findings are internally consistent, for the moment between-laboratory differences are still difficult to interpret and hamper a consistent overall conclusion on the intrarenal localization of ACE2.

It should be noted that there are species differences in the renal localization of ACE2. ACE2 expression pattern in human kidneys is similar to the one in mouse kidneys [1,22,27] with primary localization in the proximal tubular epithelium. However, inconsistencies can be found in mouse glomerular ACE2 presence as described even by the same group[28,29]. Besides, kinetic differences in Angl and AngII metabolism are shown to be exist between rat, human and sheep kidneys [25,30,31].

There are some limitations to our study. First, our data were obtained in adult animals with established hypertension and hence do not rule out a role for renal ACE2 in the early onset of hypertension. Second, due to technical issues, we only can provide data on ACE2 whereas the relevant pathogenetic entity might well be ACE/ACE2 ratio. These limitations taken in mind, our data do not support a role for renal ACE2 in established hypertension in genetically determined hypertension in rats. Yet, our data do not completely rule out a possible contribution to specific hypertensive conditions such as Sabra rat model, or an important (protective) role for tissue ACE2 at local organ-specific sites [28,32-35].

In conclusion, kidney ACE2 expression and activity is similar in polygenetic and monogenetic adult rat models of hypertension as compared to their normotensive reference strains. The precise role of renal ACE2 in hypertension remains to be established.

Acknowledgements

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Chapter 7

Differences in DNA methylation between rat strains after ischemia/reperfusion injury of the kidney

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In preparation

Abstract

DNA methylation is an epigenetic mechanism involved in regulation of gene expression, and as such, is subject to signals from extracellular environment leading to change in cellular phenotype. In the brain, DNA methylation status was shown to have an effect on damage caused by ischemia. In renal diseases, as in many other, ischemic status is an important indication of the outcome and can lead to acute or chronic renal failure. We investigated DNA methylation in renal ischemia/reperfusion (I/R) injury by comparing global DNA methylation and tissue damage in different rat inbred strains - Brown Norway (BN), spontaneously hypertensive (SHR), Dahl salt-sensitive (SS) and Munich Wistar Frömter (MWF) rat. Significant differences in DNA methylation levels between the four strains were found in ischemic kidneys. Methylation was increased in BN (P=0.002) and SS (P=0.025), while in SHR and MWF global methylation did not change after I/R injury. Inversely, renal tubulointerstitial damage after I/R injury was significantly higher in SHR (P<0.01), and in MWF (P=0.05), but not in BN and SS. Moreover, mRNA expression of pro-inflammatory cytokines *Il1b* and *Mcp1* was significantly increased only in the ischemic kidneys of SHR and MWF. Also, these two strains showed a two-fold higher expression of the acute kidney injury marker *Kim-1* when compared to the hypermethylated BN and SS. We did not find significant differences in DNA methyltransferase (DNMT) expression between the strains.

In summary, we found that DNA methylation status was associated with the level of tubulointestitial renal damage and secondarily, with the expression of damage markers after I/R injury. This supports the central idea that change in global methylation correlates with gene specific regulation and suggests that DNA methylation plays a role in the reaction of kidney tissue to ischemia/reperfusion.

Introduction

DNA methylation is an epigenetic mechanism, which is intimately related to gene expression, genomic imprinting, genome-wide stability, and replication. It plays a role in embryonic development and differentiation as well as in cancer and other diseases where aberrant methylation patterns have been reported [1-3]. DNA methylation is catalyzed by DNA methyltransferase enzymes (DNMTs), where a methyl group is added to the 5 position of cytosines in CpG dinucleotide sequences. Usually, a methylated gene promoter is inactivated, while a non-methylated one is active [4]. Tissue injury could contribute to changes in this specific epigenetic DNA modification ergo influencing cellular response, gene expression and possible general outcome [5,6].

DNA methylation activity was shown to be increased after ischemic brain injury [7]. Mice that had lower DNMT1 activity and consequently lower DNA methylation levels showed less damage after ischemic brain injury. Nonetheless, total absence of DNMT1 was not protective [8], indicating that alterations of methylation status is a fine-tuning mechanism that modifies cell reaction to ischemia.

Besides the brain, the heart and the kidneys are organs most vulnerable to insufficient blood supply. Renal ischemia/reperfusion (I/R) injury is one of the leading causes of acute renal failure and allograft dysfunction [9,10]. Early molecular changes like DNA damage and alterations in gene expression after an ischemic insult could be connected to the consequent degree of organ insufficiency. As a response to an ischemic insult a cascade of events starts, including synthesis and release of pro-inflammatory cytokines (IL-1, IL-6 and TNF-α), induction of chemokines, tubular epithelial cell activation, complement activation and leukocyte infiltration [11,12]. However, the same amount of injury does not always lead to the same outcome. In human renal pathology it ranges from minimum damage to severe fibrosis and renal failure. The primary regulation of the expression of the early signalling markers might play a role in this difference of tissue survival. Variation in DNA methylation in ischemic kidney injury is a potential modifier of this long-term outcome by influencing the heritable transcriptional permissiveness. Recognizing it as a possible parameter involved in regulation of kidney response to ischemia could be an additional step towards understanding the complex pathogenesis lying behind it. Moreover, it might bring us closer to identifying some of the factors prone to therapeutic interventions.

In the current study, we investigated rat strain differences in DNA methylation after renal ischemia/reperfusion and its associations with the tubulointerstitial damage, the expression of tissue damage markers and the DNA methylation enzymes.

Materials and Methods

Animals and surgical procedures

Six male Brown Norway BN/RijHsd (BN), spontaneously hypertensive SHR/NHsd (SHR), Dahl salt-sensitive SS/JrHsd (SS) and Munich Wistar Frömter MWF/ZtmHsd (MWF) rats were obtained from Harlan Nederland B.V. All animals were housed in a climate controlled space with a 14h light / 10h dark cycle. Food and water were available *ad libitum*. At nine weeks of age, animals were anesthetized with isoflurane (2% Forene; Abbot, Hoofddorp, The Netherlands), N_2O (50%), and O_2 (50%). Unilateral renal ischemia was induced previously described [13]. The right renal artery was clamped for 45 min to induce ischemia, followed by reperfusion. After 24 hours, the rats were sacrificed and blood and kidneys were collected. One part of kidney tissue was immediately placed in liquid nitrogen for DNA and RNA isolation, and another part was fixed in formalin and subsequently embedded in paraffin for immunohistochemistry. All animal experimentation was approved by the University's Animal Care and Use Committee.

Luminometric methylation assay (LUMA)

DNA was isolated from both the ischemic kidney and the untreated kidney as previously described [14]. Genome-wide methylation was determined using the LUMA method [15].The method is based on digestion of genomic DNA by the enzymes *Hpa*II and *Msp*I. The target for both enzymes is CCGG sequence within the genome, with the difference that if internal cytosine is methylated, *Hpa*II is unable to cut the substrate, while *Msp*I is insensitive to this modification. To eliminate sequences devoid of CpG dinucleotides, an internal *Eco*RI digestion control is added to the reaction. Briefly, 200 ng of genomic DNA was cleaved with *Hpa*II + *Eco*RI or *Msp*I + *Eco*RI in two separate 20 µl reactions containing 33 mM Tris-acetate, 10 mM Mg-acetate, 66 mM K-acetate pH 7.9, 0.1 mg/ml BSA and 5 units of each restriction enzyme. The reactions were set up in a 96-well format and incubated at 37°C for 4 h. Then 20 µl annealing buffer (20 mM Tris-acetate, 2 mM Mg-acetate pH 7.6) was added to the cleavage reactions, and samples were placed in a PSQ96 MA system (Biotage AB, Uppsala, Sweden). The instrument was programmed to add dNTPs in four consecutive steps including Step 1: dATP; Step 2: mixture of dGTP + dCTP; Step 3: dTTP; and Step 4: mixture of dGTP + dCTP. Peak heights were calculated using the PSQ MA software. The *Hpa*II/*Eco*RI and *Msp*I/*EcoRI* ratios were calculated as (dGTP + dCTP)/dATP for the respective reactions. The *Hpa*II/*Msp*I ratio, determined as (*Hpa*II/*EcoRI*)/ (*Msp*I/*EcoRI*), defined DNA methylation levels within the genome. If DNA were completely unmethylated, the *Hpa*II/*Msp*I ratio would be 1.0, and if DNA was 100% methylated the same ratio would approach 0.

Immunohistochemistry

Periodic Acid-Schiff (PAS) staining was performed using a DAKO automatic slide stainer (DAKO Group, Denmark) on deparafinized kidney tissue. Immunohistochemical localization and quantitation of ED-1, HO-1 and KIM-1, were carried out with mouse monoclonal antibody against macrophages anti-CD68 (ED-1), anti-heme oxygenase-1 (HO-1) antibody, and rabbit-anti KIM-1 peptide 9 antibody, obtained from AbD Serotec Ltd., U.K., Stressgen Bioreagents, Ann Arbor, USA, and V. Bailly, Biogen Idec. Boston, USA, respectively. Deparafinised sections (4µm) were incubated in 0.1 M Tris-HCl antigen retrieving buffer (pH 9.0) at 80°C overnight, followed by blocking of endogenous peroxidase for 30 min. (0.075% H_2O_2 in PBS) the next day. Primary antibodies were diluted in 1% BSA/PBS (1:750 for ED-1; 1:1000 for HO-1 and 1:400 for KIM-1) and sections were incubated for 60 minutes. Incubation with peroxidase-labeled secondary and tertiary antibodies (DakoCytomation) followed (diluted in 1% normal rat serum and 1% BSA/PBS). 3,3'-diaminobenzidine tetrachloride (DAB) reaction (10 min) was used to visualise peroxidase activity. Tissue HO-1 and KIM-1 was quantified by morphometric calculation using the Leica QWin program. Sections stained with ED-1 were counterstained with haematoxilin and manually counted.

Measurement of tubulointestitial damage

Tubulointerstitial damage was determined on the PAS stained kidney slides. Every slide was first scanned with ScanScope digital scanner (Aperio Technologies, Inc.) and then analysed with Aperio ImageScope programme v10.1.3.2028. The area of damaged tubuli was calculated as a percentage of the whole kidney slide area. Under 10x magnification, tubuli exhibiting necrosis with flattening of the epithelial cells, loss of nuclei and luminal debris were considered to be damaged. Slide artefacts and the middle sections containing connecting tubuli were excluded from the calculation.

Quantitation of Kim-1, Il1b1, Mcp1, Dnmt1, Dnmt3a and Dnmt3b

RNA was isolated from kidney samples using the Nucleospin RNA kit (Macherey-Nagel) according to the manufacturer's protocol. Samples were diluted and 2µg was used for cDNA synthesis using the Omniscript RT kit (Qiagen) with random hexamere primers. mRNA levels for the six genes were determined in kidneys using custom designed primer sets (Table 1) except for *Kim-1* for which expression assay was ordered from Applied Biosystems (*Havcr1*, Rn00597703_m1). The ABI Prism 7900 HT sequence detection system (Applied Biosystems) was used with Sybr green chemistry (Abgene) for highly accurate quantitation of mRNA levels. Renal mRNA levels were expressed relative to those of the Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh)*, because with geNorm VBA applet for Microsoft Excel (Vandesompele, 2002) *Gapdh* was found to be the most stable housekeeping gene compared to the genes encoding beta-2 microglobuline (*B2m*) and glucuronidase beta (*Gusb*).

Statistical analysis

Results of the methylation analysis were expressed as *Hpa*II/*Msp*I ratio for all kidney tissue. mRNA expression of *Kim-1, Il1b and Mcp1* as well as the enzymes *Dnmt1, Dnmt3a, Dnmt3b* were expressed as number of molecules relative to that of *Gapdh*. Student's t-test was used for statistical analysis between independent samples. *P*-value was set at <0.05 indicating presence of statistical significance. Statistical measurements were performed using SPSS version 14.0 and Graph Pad Prism 5.00.

Table 1. Sequences of the primers used for Realtime PCR.

Gene	Sequence (5'-3')
Kim-1	Havcr1, Rn00597703_m1, Applied Biosystems
Il1b	CACCTCTCAAGCAGAGCACAG
	GGGTTCCATGGTGAAGTCAAC
Mcp1	CAGATCTCTCTTCCTCCACCACTAT
	CAGGCAGCAACTGTGAACAAC
Dnmt1	GCTAAGGACGATGATGAGACGC
	CTTTTTGGGTGACGGCAACTC
Dnmt3a	CAGCGTCACACAGAAGCATATCC
	GGTCCTCACTTTGCTGAACTTGG
Dnmt3b	GAATTTGAGCAGCCCAGGTTG
	TGAAGAAGAGCCTTCCTGTGCC

Results

DNA methylation

Global kidney DNA methylation patterns of the four rat strains are presented in Figure 1. Increased methylation (hypermethylation) after an ischemic insult was present in kidneys of BN and SS strains (*P*=0.002 and *P*=0.025, respectively) as compared to the non-treated, contralateral kidney. In the two other investigated strains (MWF and SHR) the amount of global DNA methylation in the ischemic kidney did not differ from the basal methylation in the control kidney (normo-methylated kidneys). Furthermore, when comparing methylation levels of ischemic kidneys alone, the two hypermethylated strains (BN and SS) showed 43.5% and 23.5% respectively more methylated sites then in ischemic normo-methylated ones.

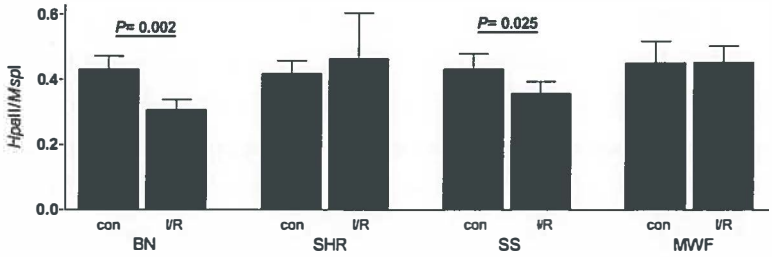

Figure 1. DNA methylation analyzed by LUMA in the control and ischemic (I/R) kidneys of the four inbred rat strains. The BN and SS strains showed increased levels of methylation in ischemic kidneys (P=0.002 and P=0.025, respectively) when compared to contralateral non-treated kidney. The kidneys of two other strains (SHR and MWF) exhibited no methylation difference regardless of injury. DNA methylation is defined as *Hpa*II/*Msp*I ratio, where *Hpa*II targets only nonmethylated while *Msp*I targets all CpG islands. As a result for a completely unmethylated DNA, the *Hpa*II/*Msp*I ratio would be 1.0, and if DNA is 100% methylated, the same ratio would approach 0.

Tubulointerstial damage

Ischemia/reperfusion injury leads to tubulointerstitial damage in the kidneys presented by tubular epithelial cell necrosis. In the SHR, where I/R did not lead to a change in global methylation status, the percentage of tubulointerstital damage was significantly higher then what we measured in the hypermethylated BN and SS kidneys (P<0.01).

The MWF, another normo-methylated strain, had significantly higher tubulointerstitial damage when comapared to the ischemic kidneys of BN and SS (P=0.05) (Figure 2). Interestingly, BN and SS were the two strains exhibiting increased global methylation as response to I/R. In all four strains, the contralateral, control kidney did not have tubulointerstitial damage (data not shown).

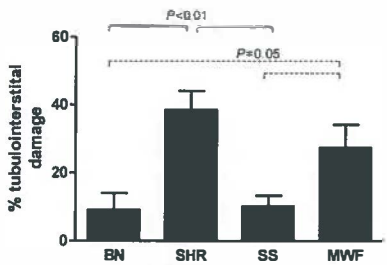

Figure 2. Tubular damage in ischemic kidneys of four inbred rat strains. Ischemia/ reperfusion injury induced tubular damage in all strains. However, the percentage of tubular damage was higher in SHR and MWF strains. Damage in BN and SS was significantly lower then in SHR (P<0.01) and MWF (P=0.05).

mRNA expression of Kim-1, Mcp1 and *Il1b*

mRNA expression after I/R of *Kim-1*, *Mcp1* and *Il1b* is presented in Fig. 3. Kidney injury molecule 1 (Kim-1) is an early marker for proximal tubular damage. It is barely detectable in healthy kidneys and strongly upregulated after renal damage. Although its function is still largely unknown, it is believed that it plays a role in damage/repair mechanisms and removal of apoptotic cells from the ultrafiltrate [16]. Upregulation of *Kim-1* expression was present in ischemic kidneys of all four rat strains. Also, the normo-methylated animals (MWF and SHR) showed a two-fold higher *Kim-1* expression compared to the hypermethylated BN and SS animals.

Cytokines IL1B and MCP1 are signalling molecules and significant factors of the inflammatory response pathway. The expression of *Il1b* and *Mcp1* mRNA was significantly increased in injured kidneys of normo-methylated MWF and SHR. In the two other strains, the increase in expression was not significant when compared to the non-treated kidney.

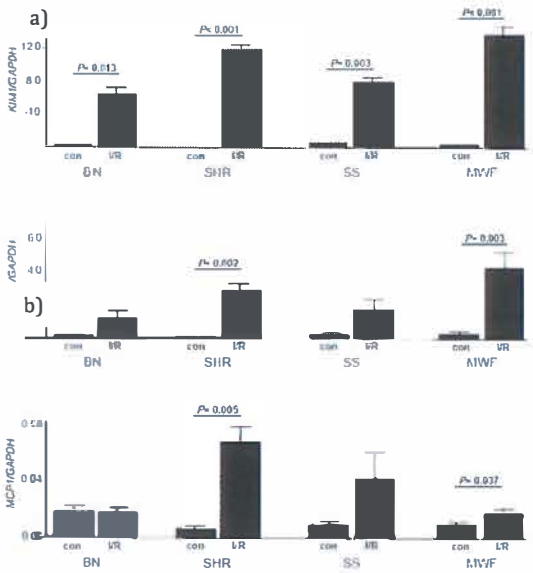

Figure 3. Renal mRNA expression of damage markers. a) Kidney injury molecule 1 (*Kim-1*) was increased in ischemic kidneys of all four strains. When looking in regard to kidney methylation levels, the normo-methylated strains (SHR and MWF) showed 2 fold higher expression of *Kim-1* when compared to expression in hypermethylated kidneys. b) Expression of *Il1b* and *Mcp1* was significantly increased in kidneys of two normo-methylated strains (SHR and MWF). In other words, levels of methylation and levels of expression markers exhibit reciprocal effects.

Immunohistochemistry of ED-1, HO-1 and KIM-1

The reaction of kidneys to oxidative injury is depicted by the presence of heme oxygenase 1 (HO-1). Oxidative stress and hypoxia are the factors that are activating the transcription of this enzyme [17]. There was a significant increase of HO-1 after I/R in all four strains. When looking at the relative amount of the HO-1 in the tissue of the injured kidneys, it follows the pattern of DNA methylation. In the two normo-methylated strains there was a greater induction of HO-1 than in the

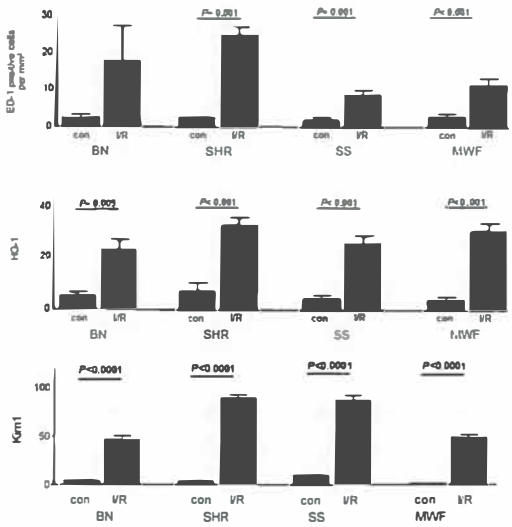

Figure 4. Quantification of ED-1, HO-1 and Kim-1 staining in the ischemic (I/R) and control kidneys of the four inbred strains. All damage markers were increased in injured kidneys. When looking at the relative amounts of HO-1 in kidney tissue, it follows the pattern of strain distribution of methylation, with more oxidative stress in normomethylated kidneys SHR and MWF).

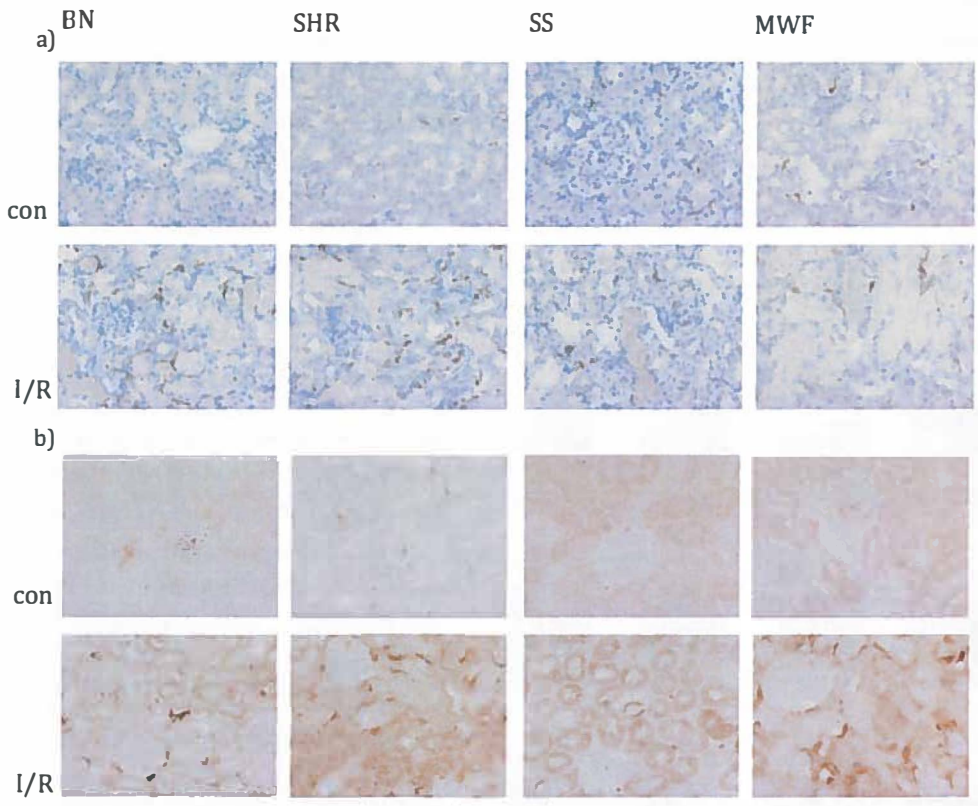

Figure 5. Immunohistochemical staining pattern of ED-1 (a) and HO-1 (b) in the ischemic and control kidneys of the four rat strains. Ischemia caused an increase of ED-1 positive cells and the development of oxidative stress in all four rat strains. Stronger HO-1 staining was clearly present in SHR and MWF, two strains with normo-methylated kidneys.

two hypermethylated strains (Fig. 4 and Fig. 5b). An early marker for tubular damage Kidney injury molecule 1 (Kim-1), was upregulated after I/R injury in all four rat strains (P<0.0001) (Figure 4). Also, there was an increase of macrophage influx upon injury measured by ED-1 in all four rat strains (BN: P=0.035; SHR: P=0.001; SS: P=0.001 and MWF: P=0.001) as presented in Figure 4 and Figure 5a.

mRNA expression of the DNA methyltransferases Dnmt1, Dnmt3a and Dnmt3b

mRNA expression of three investigated DNA methyltransferase (DNMT) genes in ischemic and contralateral kidneys are presented in Figure 6. DNMTs are enzymes that are catalysing the addition of methyl groups on promoter region of the genes. DNMT1 is primarily known as a maintenance enzyme with high affinity for hemimethylated DNA, while DNMT3A and DNMT3B are *de novo* enzymes.

There was no difference in *Dnmt1* mRNA presence in our samples (Figure 6). Expression of *Dnmt3a* was significantly higher in the ischemic kidney of MWF animals (P=0.037), and there was a significant increase of expression of *Dnmt3b* in three of the four strains (SHR: P=0.004; SS: P=0.007; MWF: P=0.003). The scheme of these differences does not reflect the differences in methylation status in our model, and as opposed to studies with brain ischemic injury [18,19] at this point could not be explained by them.

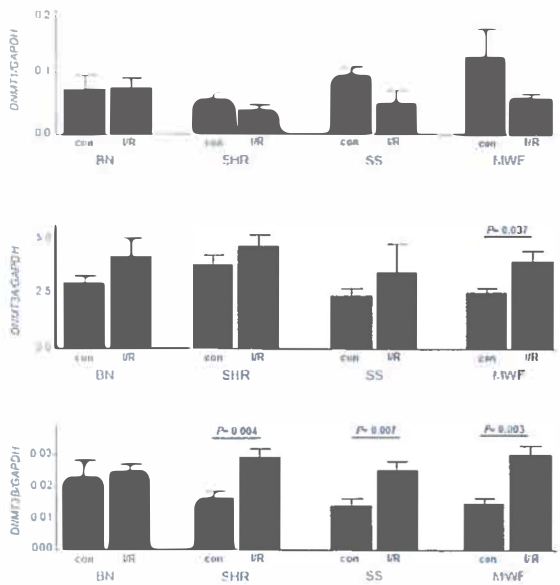

Figure 6. Renal mRNA expression of the DNA methyltransferase genes *Dnmt1*, *Dnmt3a*, and *Dnmt3b*. There was no significant difference of the *Dnmt1* the maintenance methyltransferase, in four strains between ischemic and contralateral non treated kidney. *Dnmt3a* was significant only in MWF and *Dnmt3b* in SHR, SS and MWF strains. Distribution of expression of analysed methyltransferases could not in our study explain differences in methylation patterns between strains.

Discussion

The major finding of this study is that renal ischemia/reperfusion generates an increase of global DNA methylation in the BN and SS rat strains, whereas this hypermethylation was not observed in MWF and SHR. Furthermore we found that the expression of renal tubulointerstitial damage was negatively associated with the kidney methylation status. The two strains that displayed hypermethylation developed less tubulointerstital injury and also showed suppression of pro-inflammatory *Il1b* and *Mcp1* genes, with a similar pattern in regard to *Kim-1* mRNA expression. This suggests that DNA methylation as a regulatory system that controls gene transcription could be involved in the overall renal response to an ischemic injury. The differences in methylation and tubulointestitial damage could not be explained by differences in expression of the DNA methyltransferases as shown in a model of ischemic brain injury [20].

Ischemia/reperfusion injury is mediated by oxygen derived free radicals, changes in cell metabolism, release of multiple chemokines, cytokines, and other molecules associated with autocrine and paracrine cell communication (cadherins, Bcl-2 proteins, growth factors etc) [21]. The order and specificity of these released molecules together with the severity of damage can determine the destiny of the cell itself. In the case of significant insult, cells might go into apoptosis or necrosis. The major difference is that in apoptosis the reaction is contained and followed by little or no inflammation. On the other hand, necrosis is associated with an inflammatory response of surrounded tissue with leukocyte infiltration, repair and/or scaring that is often followed by loss of function and interstitial fibrosis [22].

On a more molecular level, as a response to an ischemic insult a great number of genes become up or down regulated [23]. It can be argued that the modification of gene transcription plays a crucial role in cell reaction to ischemic injury, thus determining the level of tubulointerstital damage developed after such an insult. DNA methylation in promoter regions is involved in epigenetic mechanisms that suppress gene transcription [24]. Together with other epigenetic mechanisms, like histone deacetylation, methylation regulates gene expression under different physiological and pathological situations. In our study, in the MWF and SHR rats, where ischemia caused no difference in renal global DNA methylation levels, the tissue reaction was shifted towards more pro-inflammation, resulting in a more abundant response to damage. These data indicate that renal DNA methylation is involved in the response to an ischemic injury. Relative mRNA amounts of pro-inflammatory cytokines, *Il1b* and *Mcp1* corresponded to the pattern of measured tubulointerstital damage and secondarily to the renal DNA methylation status. IL1B and MCP1 are both mediators in the inflammatory response, involved in a variety of cellular activities including monocyte recruitment (MCP1), and cell proliferation and apoptosis (IL1B). Moreover, a protective role of inhibition of Ccl2/MCP1 pathway in the ischemic renal injury was shown during the inflammatory response [25].

The mRNA expression of *Kim-1*, an early marker of ischemic renal damage, was increased in all kidneys subjected to ischemia, while it was virtually non-detectable in the contralateral kidneys as previously shown [26]. Additionally, a two-fold increase in expression of *Kim-1* mRNA was present in the two normo-methylated strains. There has been a lot of debate about the role of KIM-1 in renal injury. It has been connected to an early reaction to ischemia, it is presented in proximal tubuli, shedded in urine and it co-appears with regenerative and dedifferentiated cells [27,28].

The upregulation of *Kim-1* in the hypermethylated rat strains may be explained in several ways. Methylation in the *Kim-1* upstream regions may prevent the binding of a repressor, as was shown in the IL-8 promoter in breast cancer [29]. Alternatively, increased methylation in the promoter of a repressor gene important for silencing the *Kim-1* may be involved. Finally, methylation in an intron region harboring an insulator binding site may prevents its binding and allow enhancer/*Kim-1* promoter interaction, a principle which has been described for the *IGF2* regulation [30]. However, sequence analysis of *Kim-1* and 10,000 bp of sequence on either side of the gene using the EMBOSS CpGPlot and CpGReport tools [31] did not result in any predicted CpG islands. Therefore, it is most likely that the differential expression of *Kim-1* is a downstream consequence of the methylation differences.

DNA methylation is catalyzed by highly conserved DNA methylation enzymes. It has been reported that mice that have reduced levels of the DNMT1 enzyme show significantly lower brain infarction area and higher cell viability after brain ischemic injury [32]. In our model the different methylation patterns of the four rat strains could not be explained by differences in expression of DNMTs. DNMT1 is known as a maintenance methyltransferase while DNMT3a and DNMT3b the two *de novo* DNMTs [33]. The activity of DNMTs is not the only player here however. Accessory factors are also needed to ensure appropriate gene regulation, like presence and variability of chromatin remodelling proteins that are enabling these enzymes to access DNA [34] or presence of proteins that show binding specificity to methylated DNA and in turn recruit histone modifying enzymes.

The data presented and differences discussed in this study represent only one point in time, taken 24h after reperfusion has occurred. The long-term effects of found differences in methylation levels upon I/R injury of the kidney cannot be addressed at this point and are a possible path for future studies, especially since epigenetic marks are responsible for long-term alterations and adaptations to a dynamic environment. In addition, the strain susceptibility to kidney damage does not correspond to the presented methylation pattern since SS and MWF are susceptible while SHR and BN are resistant strains to the development of renal disease as shown previously [35-37]. This makes another interesting path for investigating the progression of damage coupled with epigenetic modifications and genetic predisposition to kidney damage.

In conclusion, we have shown that in kidneys of BN and SS rat strains global DNA methylation levels are increased after I/R injury. The differences in DNA methylation could not be explained by expression of the three investigated DNA methyltransferases (*Dmnt1*, *Dnmt3a* and *Dnmt3b*).

Acknowledgements

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Chapter 8

Summary and Future perspectives

The characterisation of the B/L ACE polymorphism

In the first part of this thesis we have identified and characterized the *b/l Ace* polymorphism in the outbred Wistar rat strain. The line homozygous for the *b* allele was named WU-B and the one homozygous for the *l* allele was named WU-L. In the WU-B, serum ACE activity, renal *Ace* mRNA expression as well as renal and heart ACE activity were significantly higher than in the WU-L. This was an expected finding since the *b Ace* allele was previously connected to the higher ACE levels. ACE staining in the kidney followed the pattern of mRNA expression and activity with a clear difference between WU-B and WU-L. Interestingly, the ACE expression and activity levels in the aorta were lower in the rats carrying the B allele. This was confirmed by ACE protein staining of aortic tissue. But when we investigated the functional activity of the aortic rings, we measured lower conversion of AngI to AngII in the WU-L regardless of higher protein levels. This difference may be explained by the possible difference in functional activity of the ACE enzyme underlying the *b/l* polymorphism. What's more, after ACE inhibition with Lisinopril, the difference in conversion between the two genotypes did not occur. It can be argued however, that the amount of Lisinopril used was excessive and that the drug titration study could reveal the real difference in conversion. In all, the WU-B and WU-L lines described in Chapter 2 are a good model for studying the RAAS under genetically predetermined high or low renal and plasma ACE conditions. Especially since we also found that baseline ACE2 expression and activity were reciprocal to the one of ACE. Knowing that ACE and ACE2 are main enzymes in the two parallel axes within the RAAS, we found this a unique opportunity to investigate the behaviour of two systems with a different innate axis balance. Increase of AngII is argued to be the one of the key factors involved in the pathophysiology of CKD. The *b/l Ace* polymorphism is similar to the I/D ACE polymorphism in human, which is extensively studied in connection to the susceptibility and progression of renal damage, hence we have created an environment with increased AngII (by AngII infusion) in this model. We aimed to get an insight into how these two systems react in high AngII conditions. Interestingly, AngII infusion lead to proteinuria independent renal damage only in WU-B. This line had increased glomerular and tubulointerstitial damage and macrophage/monocyte influx. Conversely, there was no damage in WU-L after AngII infusion and these rats seemed to be protected against increased levels of AngII. One explanation could be that due to the initial higher levels of ACE2 in WU-L, more AngII was converted to Ang (1-7) and, acting through the Mas receptor, "sidetracked" from the downstream detrimental effects of high AngII. We unfortunately did not measure Ang (1-7) levels in our study, but latest report supports this hypothesis since stabilizing Ang (1-7) was shown to be extremely protective during inflammation [1]. It could also be due to the innate bigger ability of one of the systems to compensate within the (patho)physiological levels of the two axes. These data go in line with the numerous studies hypothesising the connection of high ACE with disease development. Previously found variations within the RAAS and the variability in response to renal insult, at least

in the Wistar rats, may now be explained by this polymorphism and the baseline phenotype behind it.

Both *I/D* and *b/l* ACE polymorphisms are characterised by an intronic difference. In human it stands for intronic insertion/deletion, in rat it is a difference in intronic CA-repeat. The functional background in both is still unknown. The difference in CA repeat was previously brought in the connection to splicing efficiency [2] and in chapter 4 we have hypothesises that CA repeat might play a role in differences seen between *b* and *l Ace* alleles. Indeed we have shown that the length of the microsatellite marker determines splicing efficiency between B and L alleles. However, there was no direct correlation between CA repeat length and efficiency. Additionally, we have identified two new rat *Ace* alleles (an *f* allele first found in F344/NHsd strain, and an a allele found in ACI/EUR strain) and two mouse *Ace* alleles that differ in the length of the CA repeat. Since rat and mouse strains are widely used in the RAAS research, genotyping for the different *Ace* alleles should be strongly taken into consideration when formulating the research question and planning the experimental setup, as well as during results analysis. These findings in general offer great possibilities to study differences within the RAAS.

Effect of ACE inhibition beyond proteinuria reduction

ACEi is the first line therapy in treatment of hypertension and proteinuric renal disease. The renoprotection is predominantly focused on the clinical parameters like decrease in proteinuria and blood pressure regulation. Etiologically, most of the hypertensions and CKDs are idiopatic. One of the main hypotheses behind it is that the RAAS under certain conditions becomes over-activated spinning into the vicious circle, leading to damage. Thus, blocking of the RAAS would be beneficial. And it is. It is mostly by means of ACE inhibitors which are sometimes combined with ARBs, or some novel drugs like renin inhibitors, trying to block the RAAS on multiple levels. Additionally, volume depletion, by either low salt diet or diuretics, is beneficial since it has a potential to enhance renoprotection when combined with drug interventions. It is amazing that even after decades of ACEi being on the market their full potency is still not entirely understood. How come that even with the angiotensin and aldosteron escape, ACE inhibition remains renoprotective? One could argue that even if the increased AngII was initiator of damage, ACEi could influence the function of AngII, or be involved in other renoprotective pathways, particularly anti fibrotic pathways within the kidney. In line with this, in chapter 5 we were set to investigate if inhibition of local renal ACE could add to the renoprotection. We used the adriamycin nephrosis model of renal damage and treated the animals with ACEi Lisinopril, AngII type 1 receptor antagonist (AT1RA) or a vehicle. We found that residual proteinuria and renal ACE independently predicted renal damage at the end of the study (measured by FGS). In the AT1RA group only proteinuria predicted renal damage. Thus, ACEi have renoprotective properties that strech beyond proteinuria reduction, which most

probably act via inhibiting the intrarenal ACE. Here, potential anti-fibrotic properties of ACE inhibitors should not be overlooked especially since there are reports that ACE inhibitors can limit complement activation, interstitial fibrosis and inflammation [3,4].

ACE2 is not involved in monogenic and polygenic hypertension in rat

Shortly after the discovery of ACE2, an enzyme acting as a counterpart of ACE, a very attractive idea emerged that next to blocking of ACE, stimulating ACE2 might add therapeutic effect in renal and CV disease. Theoretically it should be working but in the recent decade a vast majority of studies has shown that it is not that simple. A decrease in ACE2 has been reported in diabetic renal disease and in hypertension, but on the other hand blood pressure in ACE2 knock out mice was normal or only modestly increased, and they had no renal damage [5,6]. To study the involvement of ACE2 in hypertension, in chapter 6 we have looked into the ACE2 mRNA expression and ACE2 enzyme activity in the rat models of monogenic and polygenic hypertension. We used TGR(mREN2)27 rat strain as model for monogenic and stroke-prone spontaneously hypertensive rat (SHRSP) for polygenic hypertension. We compared them to their normotensive reference strains, i.e. the Spraque Dawley (SD) and Wistar Kyoto (WKY). What we found was somewhat different than expected and previously described, and that is no difference in ACE2 expression and activity despite increase in blood pressure in our hypertensive models. We used histology to localize the ACE2 in the kidney and found ACE2 protein primarily in glomeruli and endothelium. Proximal tubuli were negative for ACE2 staining while distal tubuli showed weak presence of ACE2. This was, again, different than previously reported but our finding was consistent in all four rat strains and on both frozen and paraffin stained kidney tissue samples. This data indicate that ACE2 does not seem to be directly involved in adult hypertension. Also, differences between laboratories should be still taken into account when assessing ACE2 protein localization.

Differences in renal global DNA methylation after ischemia influence renal damage

Finally, when it comes to the development of damage we still don't know what forces are determining the cellular outcome after an insult occurs. Many processes and cellular transitional states are involved, ultimately leading the cell to one of the two outcomes – repair or damage [7]. One of the mechanisms closely involved is DNA methylation. Increased methylation leads to gene silencing and thus directly influences the tissue reaction to injury [8]. Renal ischemic injury often occurs in the course of the renal damage. To analyze the role of DNA methylation in renal response to ischemia, in chapter 7 we have measured global DNA methylation after ischemia/reperfusion (I/R) injury in four different rat strains. The two strains with increased DNA methylation also had more increased tubulointerstitial damage, and vice versa. The normomethylated strains had less renal damage after I/R injury. These global DNA methylation levels corresponded to the expression

of damage markers KIM-1, IL-1b and MCP-1. This difference in methylation levels could not be explained by the enzymes responsible for DNA methylation, since we found no difference between them in correlation to the methylation levels. We measured global methylation, which is a sum of methylated levels of all genes that were responding to I/R injury (the upregulated and the down regulated ones). Further studies are necessary to closer elaborate the connection of single gene methylation to the tissue damage levels.

Future perspectives

As our knowledge of the RAAS expands, the practical utilization of that knowledge follows. Even though blockade of the RAAS has been used by practitians for decades for renoprotection, there are still pathways and mechanism to be explored and questions to be answered. Non ACE dependent AngII formation, primarily by chimase in type 2 diabetes, that can add to its profibrotic effect still needs to be evaluated [9,10]. Actually, the fact that every target in RAAS blockade approach develops an "escape mechanism", while the effect of therapy remains, points toward a possible underlying signaling between the known and until now unknown components of the RAAS. Also, the possible difference in effectiveness of plasma and tissue RAAS blockade in regard to the renal outcome might play a role. Either that, or the full pharmacodynamic of the drugs we effectively use in renoprotection still escapes us.

In regard to ACE, an attractive approach to be explored is that it has two separate catalytically active domains, a C- and an N-domain. There have been reports in human that the contributions of C- and N-domain differ between high ACE DD and low ACE II genotype in regard to the AngII generation. Namely, in DD genotype only C- domain is active, while both domains seem to be active in II genotype [11]. Moreover, earlier studies suggested that relative potency of different ACE inhibitors varies when it comes to blocking the two domains [12,13]. It would be interesting to investigate whether the clinical effectiveness of different ACE inhibitors differ between ACE alleles.

With the increasing body of data into the possible utilization of ACE2 axis components, renin inhibitors as well as therapeutic gene modulation, the field of RAAS research is expanding steadily. Especially the anti-inflammatory potential of the novel, cyclic form of Ang (1-7) which is more stable and has higher affinity to the Mas receptor [1]. Could potentiation of one axis have similar effects as blocking the other? Would the stimulation of the ACE2/Ang(1-7)/Mas receptor axis bring the missing beneficial effect needed for complete renoprotection? And in which patients? It will not be much longer before new practical guidelines for renoprotection are put into practice. Taking into the account the patient's *ACE* genotype, and the dynamic within the RAAS that goes with it, might provide additional information necessary for developing the best therapy strategy for CKD. At present, personally adjusted therapy for chronic diseases is far from common practice, it is however a promising prospect and its value awaits to be confirmed.

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List of abbreviations

α-SMA	alpha-smooth muscle actin
ACE	angiotensin converting enzyme
ACEi	angiotensin converting enzyme inhibitor/inhibition
ACE2	antiotensin converting enzyme 2
AngI	angiotensin I
AngII	angiotensin II
ARB	angiotensin II type 1 receptor blocker
AT1	angiotensin II type 1 receptor
AT1RA	angiotensin II type 1 receptor antagonist
AT2	angiotensin II type 2 receptor
bp	base pair
BP	blood pressure
CKD	chronic kidney disease
CrCl	creatinine clearance
CV	cardiovascular
DMNT	DNA methyltransferase
ESRD	end stage renal disease
FGS	focal glomerulosclerosis
GFR	glomerular filtration rate
I/R	ischemia/reperfusion
KIM-1	kidney injyry molecule-1
PAS	periodic acid-Schiff
PBS	phosphate buffered saline
PE	phenylephrine
RAAS	renin-angiotensin aldosterone system
SBP	systolic blood pressure
TIF	tubulo-interstitial fibrosis

Nederlandse samenvatting

Inleiding

Het aantal mensen met een chronische nierziekte (CKD) neemt jaarlijks toe. Dit is een wereldwijd probleem dat zowel leidt tot hogere sterfte en daling van de kwaliteit van het leven van patiënten, als tot hoge zorgkosten. Ondanks de huidige behandelingsmethoden bereiken veel patiënten het punt waarop dialyse en niertransplantatie de enige opties zijn. Nieuwe preventieve en therapeutische strategieën zijn daarom noodzakelijk. Bekende klinische risicofactoren zoals hypertensie, eiwit in de urine, diabetes, zwaarlijvigheid en roken spelen daarbij een belangrijke rol, en zijn onderwerp van veel onderzoek. Bij de mechanismen die uiteindelijk leiden tot nierschade speelt regulatie van genexpressie in de nier hoogstwaarschijnlijk een belangrijke rol. Door betere inzichten in de intrarenale factoren die betrokken zijn bij de vatbaarheid voor nierschade en de mechanismen van progressief nierfunctie-verlies, komen we dichter bij de oplossing voor de ultieme uitdaging, namelijk het voorkómen van het ontstaan van chronische nierziekten en het verhinderen van ziekte progressie na de diagnose.

Het Renine-Angiotensine Aldosteron systeem (RAAS) is een belangrijk regulatie systeem voor bloeddruk, zoutbalans en nierfunctie. In de nieren wordt inactief angiotensinogeen (geproduceerd door de lever) omgezet in angiotensine I (AngI) door het enzym renine. AngI wordt door het angiotensin converting enzyme (ACE) omgezet in angiotensine II (AngII). De meeste biologische effecten van AngII komen tot stand door binding aan de AngII type 1 en AngII type 2 receptoren (AT1 en AT2). De vaatvernauwende eigenschappen van AngII spelen een rol bij regulatie van de bloeddruk regulatie en van de bloeddoorstroming van de nieren. Echter, een overactief RAAS met teveel productie van AngII leidt tot weefselschade in hart, bloedvaten en nieren. Daardoor speelt het RAAS een centrale rol bij progressieve nierschade. Remming van het RAAS, met medicijnen zoals ACE-remmers vormt dan ook een belangrijke behandelwijze tegen nierschade. Naast de bovenomschreven ACE/AngII/AT1 arm is recent een nieuwe arm binnen het RAAS beschreven, waarin een homoloog van ACE, het ACE2, zorgt voor generatie van angiotensine (1-7) (Ang (1-7), dat aangrijpt op de Mas receptor: de ACE2/Ang (1-7)/Mas receptor arm. Recent onderzoek suggereert dat de twee armen tegengestelde effecten hebben, en elkaar in balans houden (zie figuur hoofdstuk 1.). Bovendien wordt gesuggereeerd dat het evenwicht tussen deze twee enzymen de spil vormt van de uiteindelijke effecten van het RAAS systeem en dat het stimuleren van ACE2 gunstig zou kunnen zijn voor het verloop van nier- en hartziekten.

Karakterisatie van ACE b/l polymorfisme

Bij de mens leiden twee ACE gen varianten (polymorfismen), de I en de D variant, tot verschillen in plasma en weefsel ACE. Genetisch bepaalde hoge ACE levels zijn op die manier gerelateerd aan de ontwikkeling en progressie van nierziekten. In **hoofdstuk 2** beschrijven en karakteriseren we twee gelijksoortige ACE polymorfismen (b/l polymorfisme) in outbred Wistar ratten. We vergeleken

ratten met twee dezelfde b allelen (WU-B) en ratten met twee dezelfde l allelen (WU-L). In WU-B ratten waren serum ACE activiteit, ACE gen expressie in de nieren en ACE activiteit in de nieren en het hart significant hoger dan in WU-L ratten. Dit bevestigt eerdere studies, waarbij de b ACE variant was geassocieerd met hogere ACE niveaus. ACE aankleuring in de nier volgde het patroon van mRNA expressie en activiteit met een duidelijk verschil tussen WU-B en WU-L ratten. Interessant is dat in WU-B ratten de ACE expressie en activiteit in de aorta juist lager was dan in WU-L. De bevinding van het b/l polymorfisme in Wistar ratten is om twee redenen van belang. Ten eerste worden Wistar ratten veel gebruikt in studies naar nier- en hartziekten, waar RAAS regulatie een belangrijke rol speelt. Bij de planning van experimenten en tijdens de analyse van de resultaten moet dus rekening worden gehouden met het bestaan van dit b/l ACE polymorfisme. Ten tweede is dit een nieuw diermodel dat ons de gelegenheid geeft om het RAAS systeem te bestuderen onder aangeboren hoog/laag ACE condities. Dit hebben we gedaan in **hoofdstuk 3**. Aan de WU-B en WU-L ratten hebben we gedurende 3 weken AngII met een minipomp toegediend. Na stimulatie met het schadelijke AngII ontwikkelden ratten met genetisch bepaalde hoge ACE levels (WU-B) glomerulaire en interstitiële nierschade, terwijl ratten met genetisch bepaalde lage ACE levels (WU-L) beschermd waren (zie hoofdstuk 3, figuur 2). Opvallend was dat WU-B ratten een lagere basale ACE2 activiteit hadden dan WU-L ratten. De genetisch bepaalde RAAS balans kan dus een rol spelen in de reactie van de nier op schadelijke stimuli. Aangezien soortgelijke ACE polymorfismen bestaan in de mens, zou het interessant zijn om te onderzoeken of het hebben van een bepaald ACE polymorfisme bepalend is voor de reactie op therapie. In **hoofdstuk 4** gaan wij dieper in op de achtergrond van ACE b/l polymorfisme. We laten zien dat er verschil bestaat in de lengte van een deel van het ACE gen tussen de b en l variant. Dit verschil bevindt zich in de intronic CA-repeat, een deel van het gen dat niet wordt vertaald in eiwit. Verder onderzoek is nodig om het exacte mechanisme te achterhalen.

Nieuwe inzichten in de ontwikkeling en behandeling van nierziekten

Angiotensine converting enzym inhibitors (ACEi) zijn de eerste keus bij de behandeling van nierziekten. ACEi verlaagt de bloeddruk en vermindert de uitscheiding van eiwit in de urine, waardoor de progressie van nierziekten wordt geremd. Combinatie therapie van ACEi en Angiotensine II type I receptor blockers (ARBs) geven een beter resultaat dan de monotherapie. Volume depletie middels een laag zout dieet bewerkstelligt extra gunstige effecten tijdens de monotherapie en de combinatie therapie. Het is nog niet precies duidelijk hoe de gunstige effecten van ACEi worden bewerkstelligd. Zo daalt vlak na het begin van de therapie weliswaar het AngII, maar na enige tijd keert de circulerende AngII spiegel terug naar de normale waarde, terwijl de therapeutische effecten voortduren. Het is daarom waarschijnlijk dat ACEi niet uitsluitend werken door een effect op de aanmaak van AngII in de circulatie, maar ook betrokken zijn bij andere

beschermende mechanismen, in het bijzonder anti-fibrotische mechanismen in de nier. In lijn met deze gedachte hebben we daarom in **hoofdstuk 5** onderzocht of remming van lokaal ACE in de nieren een toegevoegde waarde in de nierbeschermende effecten van ACEi. Eerst hebben wij in ratten nierschade geïnduceerd door injectie van adriamycine. Daarna werden de ratten behandeld met ACEi, of ARB, of een placebo. In ratten waar het lokale ACE in de nier sterker geremd was, was de uitkomst voor wat betreft nierschade het gunstigste, los van andere effecten van de ACEi zoals verlaging van bloeddruk en vermindering van de eiwituitscheiding. In hoeverre dit effect verklaard kan worden door de potentiële anti-fibrotische eigenschappen van ACEi moeten nog verder onderzocht worden.

ACEi kunnen de progressie van nierziekten afremmen, maar kunnen deze vaak niet helemaal stoppen. Er worden daarom voortdurend nieuwe therapeutische strategieën onderzocht. Een aantal daarvan is gericht op andere componenten van het RAAS systeem, hetzij door remming van de óvergeactiveerde schadelijke componenten hetzij door het induceren van mogelijk beschermende componenten. Één van de mogelijke beschermende componenten is de tegenhanger van ACE, het ACE2 enzym. Er wordt op basis van eerder onderzoek verondersteld dat ACE2 een rol speelt bij regulatie van de bloeddruk, een belangrijke factor bij nierschade. In een poging om de rol van ACE2 in de bloeddrukregulering op te helderen, hebben wij in **hoofdstuk 6** ACE2 expressie en activiteit onderzocht in de nieren van ratten met polygene en monogene hypertensie, dat wil zeggen: ratten met verschillende vormen van een genetisch aanleg voor hoge bloeddruk. De ACE2 expressie en activiteit in de nieren van deze ratten met hypertensie hebben we vergeleken met die van ratten met een normale bloeddruk. We vonden echter geen verschil tussen de groepen. ACE2 lijkt dus, niet betrokken te zijn bij de hypertensie in deze ratte-stammen.

In **hoofdstuk 7** hebben wij in een ander model voor nierschade, het ischemie/reperfusie (I/R) model, de regulatie van gentranscriptie bestudeerd. De codering voor alle genen is vastgelegd in het DNA molecuul en het eindproduct van een bepaald gen is een specifiek eiwit. Twee hoofdonderdelen in dit proces zijn gentranscriptie (afschrift van gen codering in een mRNA molecuul) en gentranslatie (het gebruik van de mRNA code om de juiste aminozuren samen te stellen tot een eiwit). De regulatie van dit proces is zeer complex. Bij gentranscriptie is toevoeging van een methyl groep (-CH_3) in het begin van het gen (DNA methylatie) één van de belangrijkste regulerende mechanismen. Veel studies hebben al aangetoond dat verhoogde DNA methylatie geassocieerd is met ongeplande gen silencing (verminderde gentranscriptie) en dit kan direct invloed hebben op de weefselreactie na schade. Wij hebben DNA methylatie in de nier gemeten in vier verschillende ratten stammen na ischemie/reperfusie schade. De twee stammen die een verhoogde nier DNA methylatie hadden, vertoonden ook meer nierschade. Met andere woorden, we vonden dat de DNA methylatie status geassocieerd is met de mate van nierschade. Methylatie speelt dus een belangrijke rol bij de individuele vatbaarheid voor nierschade.

Met de steeds toenemende kennis komen we steeds dichter bij het begrijpen van de ontwikkeling van nierziekten en krijgen we beter inzicht in de mogelijkheden voor preventie, vroege identificatie en behandeling. Onze resultaten ondersteunen het belang van intrarenale processen van gen-regulatie bij het ontstaan van nierschade. Het zal waarschijnlijk niet lang meer duren voordat meer persoonlijke therapie benadering de realiteit wordt.

Kratak rezime

Uvod

Broj ljudi sa hroničnim bubreznim bolestima se iz godine u godinu povećava. To je problem svetskih razmera, koji s jedne strane vodi do značajnog smanjenja kvaliteta života pacijenata, a s druge strane do visokih troškova lečenja. I pored terapije, velika većina pacijenata stigne u situaciju kada su dijaliza i transplantacija bubrega jedine opcije lečenja. Nove strategije u prevenciji i terapiji su neophodne. Pored priznatih faktora rizika kao sto su povišen krvni pritisak, proteinurijai, dijabetes, gojaznost i pušenje, regulacija ekspresije gena igra značajnu ulogu u razvoju bubrežne bolesti. Boljim razumevanjem faktora koji utiču na predispoziciju za razvoj bubrežnog oštećenja kao i mehanizama progresije bolesti, bliže smo krajnjem izazovu – prevenciji razvoja ali i zaustavljanju progresije bubrežne bolesti pre momenta kada su mere zamene funkcije bubrega veštačkim sredstvima neophodne.

Renin angiotenzin aldosteron sistem (RAAS) je najvažniji hormonski sistem za regulaciju krvnog pritiska, balansa soli u organizmu i ekstracelularnog volumena tečnosti. U početku RAAS kaskade, inaktivni angiotenzinogen (sekretovan u jetri) se pomoću bubrežnog enzima renina konvertuje u angiotenzin I (AngI). AngI preko centralnog RAAS enzima angiotenzin konvertujuceg enzima (ACE) se dalje konvertuje u angiotenzin II (AngII). Većina bioloških efekata AngII su posredovani vezivanjem za AngII tip 1 i AngII tip 2 receptore (AT1 i AT2). Vazokonstriktorne karakteristike AngII su bitne za dobru regulaciju krvnog pritiska i za dobar protok krvi kroz bubrege. Medjutim, prekomerno aktivan RAAS sa suviše AngII može voditi do oštećenja tkiva. Iz tog razloga dobra RAAS regulacija je u centru pristupa protiv progresije bubrežne bolesti. Pored ACE/AngII/AT1 osovine, nova ACE2/Ang(1-7)/Mas receptor osovina je skoro identifikovana u RAAS sistemu. Poslednja istraživanja sugerišu da su ove dve osovine u balansu i da je su efekti ACE2 enzima suprotni od efekata ACE enzima (slika poglavlje 1). Dodatno, predloženo je da je ravnoteža ova dva enzima u centru regulacije RAAS sistema, i da stimulacija ACE2 osovine moze imati pozitivan efekat u bubrežnim i srčanim bolestima.

Karakterizacija ACE b/l polimorfizma

Kod ljudi dve različite varijante (polimorfizimi) ACE gena, I i D varijanta, vode do različitog nivoa ACE enzima u plazmi i tkivima. Genetski determinisan visok ACE je povezan sa početkom i progresijom bubrežne bolesti. U **poglavlju 2** opisujemo i karakterišemo sličan ACE polimorfizam (b/l polimorfizam) u "outbred" Wistar pacovima. Linija sa dva ista b ACE gena je nazvana WU-B, a ona sa dva ista l ACE gena WU-L. U WU-B, serumska aktivnost ACE, bubrežna ekspresija ACE gena i bubrežna i srčana ACE aktivnost su bili statistički značajno viši nego u WU-L. Ovo je za očekivati, jer je b ACE varijanta ranije već bila povezna sa višim ACE nivoima. ACE bojenje u bubregu je pratilo obrazac ekspresije i aktivnosti sa jasnom razlikom izmedju WU-B i WU-L. Interesantno je da su ACE

ekspresija i aktivnost u aorti bili niži u WU-B. Otkriće b/l ACE polimorfizma u Wistar pacovima je značajno iz dva razloga. Prvo, Wistar pacovi se generalno koriste u studijama o bubrežnim i srčanim bolestima, gde RAAS regulacija igra bitnu ulogu. Zbog toga znanje o postojanju b/I ACE polimorfizma u Wistar pacovima bi trebalo da se uzme u obzir kako kod planiranja eksperimenata tako i za vreme analize rezultata. Drugo, ovo je novi životinjski model koji pruža mogućnost istraživanja RAAS sistema pod urodjenim visokim/niskim ACE uslovima. To smo iskoristili u **poglavlju 3** gde smo WU-B i WU-L pacovima minipumpom dozirali AngII tri nedelje. Našli smo da su posle stimulacije ostećujućim AngII, pacovi sa genetski odredjenim visokim ACE (WU-B) su razvili glomerularno i intersiticijalno oštećenje bubrega, dok su pacovi sa genetski odredjenim niskim ACE (WU-L) bili zaštićeni (poglavlje 3, figura 2). Interesantno je da su WU-B imali nižu bazalnu ACE2 aktivnost od WU-L, tako da genetski predodredjena RAAS ravnoteža možda igra ulogu u reakciji bubrega na oštećujuće stimuluse. Pošto slična varijacija u ACE genu postoji kod ljudi, bilo bi interesantno videti da li postojanje odredjene ACE genetske varijante može da utiče na reakciju na različite terapije. U **poglavlju 4**, istraživali smo pozadinu ovog b/l ACE polimorfizma. Našli smo da se jedan deo ACE gena razlikuje u dužini izmedju b i l varijante. Ova razlika je u intronskom CA-ponavljanju. Pošto se ovaj deo gena u principu ne prevodi u protein, dalja istraživanja su neophodna da bi se došlo do preciznijeg mehanisma regulacije.

Nova saznanja o razvoju i terapiji bubrežnih bolesti

Prvi izbor u terapiji kod bubrežnih bolesti su inhibitori Angiotenzin konvertujućeg enzima (ACEi). ACEi kontrolišu visok krvni pritisak i snižavanju izlučivanje proteina u urinu, usporavajući progresiju bolesti. Kombinovana terapija ACEi sa blokatorima tip 1 angiotenzin II receptora (ARBs) daje bolji rezultat of monoterapije, a deplecija volumena preko dijete sa smanjenim unosom soli dodatno pojačavaja povoljne efekte bilo mono- bilo combinovane terapije. Mehanizam pozitivnih terapijskih efekata ACE inhibitora nije u potpunosti poznat. Na primer, ubrzo posle početka terapije nivoi AngII se vraćaju na preterapijske vrednosti dok je pozitivan terapijski efekat ACEi još uvek prisutan. Moguće je da su ACE inhibitori uključeni u neke dodatne zaštitne mehanizme, kao što su anti-fibrotični mehanizmi u bubrezima. U vezi sa tim, u **poglavlju 5** ispitivali smo da li inhibicija lokalnog ACE moze da doprinese renoprotekciji. Prvo smo pacovima adriamicinom indukovali oštećenje bubrega. Pacovi su posle tretirani ACE inhibitorom, ARBom ili placebom kao kontrolom. Našli smo da inhibicija lokalnog ACE enzima utice na progresiju bolesti i da pozitivni efekti ACE inhibitora najverovatnije prevazilaze efekte smanjenja izlučivanja proteina u urinu. Ove potencijalne anti-fibrotične karakteristike ACE inhibitora moraju jos biti istražene.

Koliko su ACE inhibitori važni i korisni u usporavanju progresije bubrežne bolesti, oni ne mogu potpuno da zaustave progresiju bolesti. Nove terapijske strategije se konstantno istražuju. Najveći

broj je usmeren na ostale delova RAAS sistema, bilo prema blokadi prekomerno aktivnih ostećujućih komponenti ili preko stimulacije mogućih povoljnih zaštitnih komponenti. Jedan od obećavanjucih zaštitnih substanci je ACE2 enzim, koji se u najnovijoj literaturi pominje kako funkcionalni antagonista ACE-u. U pokušaju da razjasnimo ulogu ACE2 u regulaciji krvog pritiska u **poglavlju 6** smo istraživali ekspresiju i aktivnost ACE2 u poligenom i monogenom životinjskom modelu hipertenzije. Dobijene rezultate smo uporedjivali sa kontrolnom grupom koja je imala normalan krvni pritisak. Suprotno očekivanjima, nismo nasli da postoji razlika izmedju grupa. ACE2 enzim izgleda da nije uključen u regulaciju hipertenzije kod odraslih.

U **poglavlju 7** smo koristili model bubrežne ishemije/reperfuzije da ispitujemo regulaciju transkripcije gena. Naime, svi geni su upisani u DNK molekulu, i završni produkt odredjenog gena je specifični protein. Dva glavna segmenta ovog procesa su transkripcija gena (prepisivanje genetskog koda u iRNK molekul) i translacija (korišćenje ovog iRNK koda tako da se odgovarajuće aminokiseline povežu u protein). Regulacija ovih procesa je izuzetno kompleksna. Na nivou genetske transkripcije, dodatak metil grupe ($-CH_3$) prednjem delu gena (DNK metilacija) je jedan on značajnih regulatora. Velika većina studija je pokazala da je povećana DNK metilacija povezana sa neplaniranim "uspavljivanjem gena" i da može da utiče na reakciju tkiva na ostećujući stimulus. Mi smo merili metilaciju DNK kod četiri različite linije pacova posle ishemije/reperfuzije. Dve linije koje su imale povećanu DNK metilaciju su takodje imale više bubrežnog oštećenja. Drugim rečima nasli smo da je status DNK metilacije povezan sa nivoom bubreznog ostećenja i to je sigurno faktor koji treba uzeti u obzir pri odredjivanju individualne osetljivosti na bubrežno oštećenje.

Konstantnim širenjem znanja bliži smo razumevanju razvoja bolesti bubrega i boljim smernicama za prevenciju, rano prepoznavanje i lečenje. Neće proći još dugo vremena do momenta kada će individualni pristup terapiji postati realnost.

Dankwoord

Thank you

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Doing a PhD is much more than just producing a book. It is a quite personal journey with challenges, doubts, failures, egos, administration nightmares and for many of us cultural adjustments. But most of all, it is a journey of learning and determination. I do believe that after taking this road, one will never look at a set of data, or a graph with the same eyes. Somewhere along the way we become scientists. And the skills we acquired, mostly out of necessity, will prove to be very useful in any future road that lies ahead.
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Jelena
Groningen, 2011

Curriculum Vitae

Jelena Kamilić was born in Belgrade, on 21st of December 1977. When she was six she got her first library card and her mom inroled her to learn English language. She finished primary school "Branko Radičević" in Blok 45, and the "10th Belgrade Gimnasium". She continued to study medicine at School of Medicine at the University of Belgrade, Serbia. Her first scientific project was about polycystic ovaries syndrome (PCOS) on the Clinic for Endocrinology under supervision of Dr. Djuro Macut. After a poster presentation at ISCOMS (International Student Congress of Medical Sciences) in the UMCG in summer of 2005, she got invited to an interview for a PhD position. From October 2005 until March 2010 she performed doctoral research described in this thesis on the Department of Pathology and Medical Biology and the Department of Nephrology in the University Medical Center Groningen (UMCG) in the Netherlands. Currently she is working as a postdoctoral research fellow at the Department of Liver, Digestic and Metabolic Diseases in the UMCG, where she is working on the mechanisms of regulation of intestinal permeability.

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