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Differential *ACE* expression among tissues 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 disease, 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 twofold 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 pathophysiologic consequences of differences in *ACE* alleles in renal and cardiovascular disease.

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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 (Rigat et al. 1990). The D allele of the *ACE* genotype has been associated with cardiovascular and renal disease (Kunz et al. 1998; Ruggenenti et al. 2008; Staessen et al. 1997). However, the results are not uniform and analysis of both its pathophysiologic significance and the underlying mechanisms is complicated by multiple interactions with other genetic and environmental factors (Boonstra et al. 2001; van der Kleij et al. 2002). 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 (Hilbert et al. 1991; Jacob et al. 1991) 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 (Challah et al. 1998; Hilbert et al. 1991).

Challah et al. (1998) demonstrated that ACE activity in cultured vascular cells and in neointima formation in the carotid artery after balloon injury was influenced by ACE levels. Moreover, Ocaranza et al. (2002, 2004) showed that there is an enhanced hypertensive response and more fibrosis in response to myocardial infarction in the Brown Norway rat with higher ACE levels. 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 (Rook et al. 2005). 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 and 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. (1991). 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 14-h light/10-h dark cycle. Food and water were available *ad libitum*. After 6 weeks, 14 rats (seven of each line) were anesthetized using isoflurane/O_2/N_2O mixture. 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 24-h urine samples were collected. Afterward the rats were anesthetized and the abdomen was opened through midline incision. A 2-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. Midcoronal 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 (Korstanje et al. 2004). To determine the *ACE* genotypes, primers were used as described by Hilbert et al. (1991). 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, Düren, Germany) according to the manufacturer's protocol. Samples were diluted and 2 μg was used for cDNA synthesis using the Omniscript RT kit (Qiagen, Valencia, CA), with random hexamer primers. *ACE* mRNA levels were determined using a custom-designed primer-probe set (Applied Biosystems, Foster City, CA) with the primers 5′-CACCGGCAAGGTCTGCTT-3′, 5′-CTTGGCATAGTTT CGTGAGGAA-3′, and the probe 6-FAM 5′CAACAA GACTGCCA 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 microglobin housekeeping gene (*B2M*), since the geNorm VBA applet for Microsoft Excel (Vandesompele 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 a 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 demiwater 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 min. 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, bipeptide 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 min. Primary antibodies (Chemicon International, Billerica, MA; CD143, MAB4051, clone 9B9) were incubated for 60 min 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% bovine serum albumin (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 containing 0.03% H_2O_2 for 10 min. Counterstaining was performed using Mayer's hematoxylin.

When looking with low magnification at the transverse section of the stained renal tissue, the ACE staining is presented as a band expanding from the corticomedullary region laterally back toward 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 corticomedullary 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 (Buikema et al. 1992). After the blood vessel was cleaned of connective tissue, eight rings of 2-mm length were cut with a sharp razor blade; 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 was connected to a displacement transducer to determine isotonic changes (Buikema et al. 1993). 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 µM phenylephrine (PE). Then a second response with 10 µM PE was evoked which served as a control contraction. These contractions between the two rings studied in parallel or between different groups were not significantly different (data not shown). After washout and renewed stabilization, rings were incubated with 100 µM N^G-mono-methyl-L-arginine (L-NMMA) for 45 min. Then, with L-NMMA still present, parallel rings were stimulated with either AngI or AngII (0.1 nM-1 µM bath concentrations). Finally, AngI-induced response was studied in the presence of Lisinopril. These rings were preincubated 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 interquartile range or mean and SEM for the response curves. Data were analyzed using the statistical program SPSS v12.0.2 for Windows (SPSS, Inc., Chicago, IL) and Prism v4.03 (GraphPad Software, La Jolla, CA). Statistical differences were determined using the Mann-Whitney U test. Significance was accepted at $p < 0.05$.

To avoid nonspecific (interassay) 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 nonspecific differences between rings from the same rat (intraassay variance), contractile responses to AngI and AngII were first normalized by calculating the response as a percentage of the (reference) control contraction response to PE (%PE). 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 curves 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 given in Table 1. Body weights of WU-B and WU-L were similar, at both week 6 (time point used for aortic rings) and week 12 (time point used for ACE activity/mRNA/24-h urine

Table 1 Clinical characteristics of Wistar rats

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

Values are median (quartile range)

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 Fig. 1. Expression is shown as the number of *ACE* mRNA molecules per molecule of *B2M* mRNA. ACE activity was approximately two times higher in for WU-B in serum, kidney, and heart homogenates (all, $p < 0.05$, Fig. 1a, b, and d, left side). In the aorta homogenate, ACE activity was approximately two times higher in WU-L ($p < 0.05$, Fig. 1c). Relative *ACE* mRNA expression was higher for WU-B in kidney tissue ($p < 0.05$, Fig. 1b, right side), nonsignificantly higher in cardiac tissue ($p = 0.06$, Fig. 1d, right side), and significantly lower in aorta ($p = 0.02$, Fig. 1c, right side) compared with WU-L.

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-h). In WU-B there was a higher kidney ACE protein expression compared with WU-L, with the most remarkable difference in tubular ACE protein expression presented visually and calculated as a percentage of ACE staining of the corticomedullary 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. 2i, 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. 2k).

Vascular response

The contractile responses to equimolar concentrations of AngI and AngII in isolated parallel aortic rings are presented in Fig. 4. Figure 4a 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 curves of AngI and AngII was significantly higher for WU-L (Fig. 4c, $p < 0.05$), 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 (Fig. 4d, $p =$ ns).

Discussion

Genetically, deletion in intron 16 of the human *ACE* gene results in an increased ACE activity in both plasma and tissue level (Danser et al. 1995; Mizuiri et al. 2001; Rigat

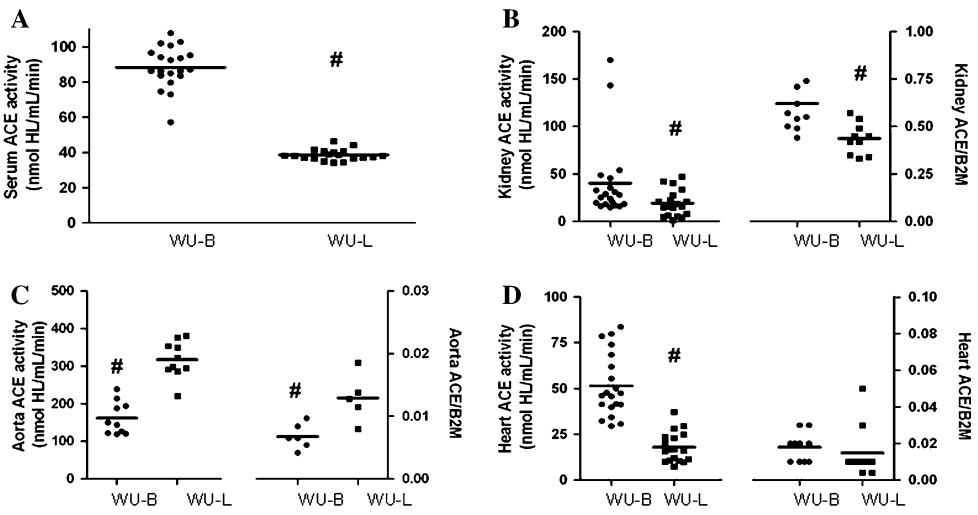


Fig. 1 ACE activity and relative ACE expression in Wistar WU-B vs. WU-L. Expression is shown as the number of *ACE* mRNA molecules per molecule of *B2M* mRNA. The WU-B has higher ACE activity in serum, kidney, and heart (**a, b, d**, left side, $^{\#}p < 0.05$). Relative *ACE* mRNA expression is higher in kidney tissue from WU-B (**b**, right side, $^{\#}p < 0.05$) and borderline significantly higher in heart tissue from WU-B (**d**, right side, $p = 0.06$). In the aorta, ACE activity and mRNA expression is higher in WU-L (**c**, $^{\#}p < 0.05$)

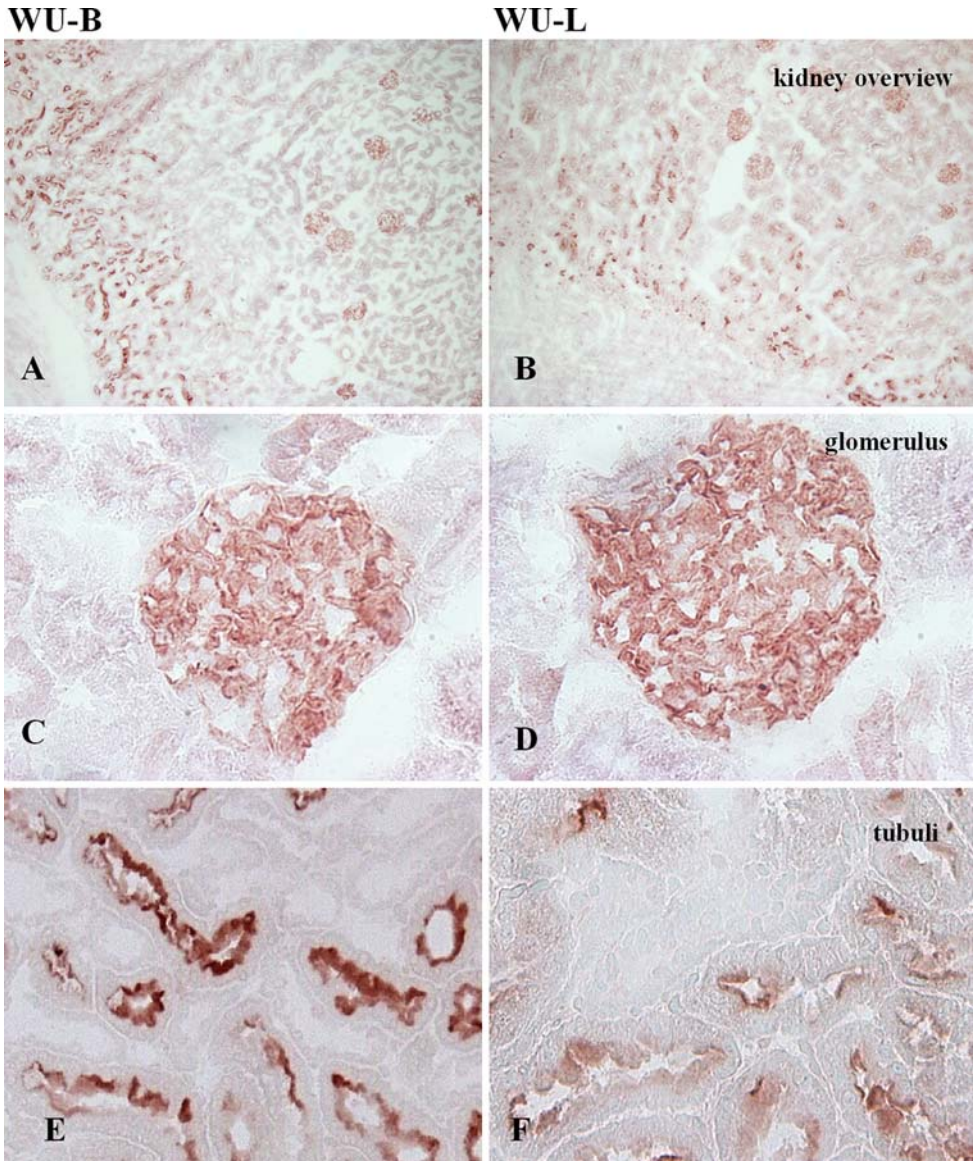
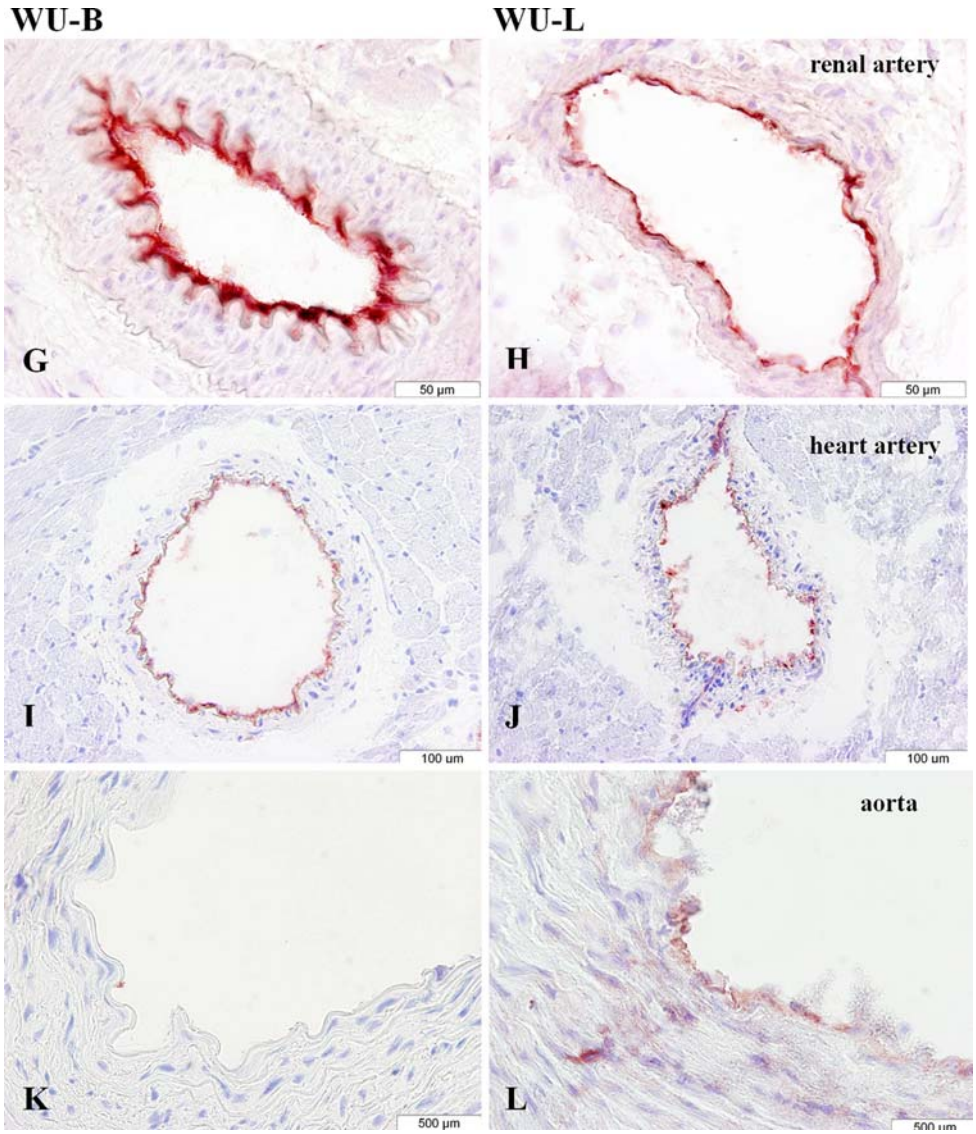


Fig. 2 ACE staining in kidney (A-F) and blood vessels (G-L). **a-f** Kidney tissue of Wistar rat WU-B vs. WU-L (**a** and **b**, kidney overview; **c** and **d**, glomerulus; **e** and **f**, tubuli) showing more staining of ACE in the tubules of the WU-B rat. **g** and **h** Renal artery of WU-B (**g**) and WU-L (**h**) with more staining in WU-B. **i** and **j** Two coronary vessels with clear endothelial staining without differences between WU-B (**i**) and WU-L (**j**). **k** and **l** Aortic tissue with no staining in WU-B (**k**) and endothelial staining in the WU-L (**l**) rat

et al. 1990). Numerous studies have been performed analyzing the possible association of the human *ACE* I/D genotype with cardiovascular and renal disease (Ng et al. 2005; Staessen et al. 1997). Whereas meta-analyses confirm a role for the D allele as a renal and cardiovascular risk factor (Samani et al. 1996), the significance of genetically high/low ACE is still controversial (Staessen et al. 1997). 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 alleles (between the Brown Norway and Lou rats) (Challah et al. 1998) and identified these alleles in the outbred Wistar rat. We bred two lines homozygous for the two alleles (called WU-B and WU-L) and determined their phenotype related to ACE in serum, heart, and kidney tissue. On both the 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 (Mizuiri et al. 2001; Rigat et al. 1990). To identify the physiologic mechanisms of the genetic modulation of ACE activity, we measured the *ACE* mRNA expression. Our data had a similar tendency: higher *ACE* mRNA expression in kidney tissue but not significantly higher in heart tissue of the WU-B line ($p = 0.06$). These measurements showed clearly that both the ACE activity and mRNA expression

Fig. 2 continued



were related to the B and L alleles and suggested that ACE activity is controlled at the transcriptional level. Challah et al. (1998) described this polymorphism in inbred Brown Norway and Lou rats where the ACE transcription rate was nearly twofold higher in homozygote B rats compared to that in L rats. They also reported this difference in membrane ACE activity and mRNA expression in the lungs, while our investigation was more focused on renal and

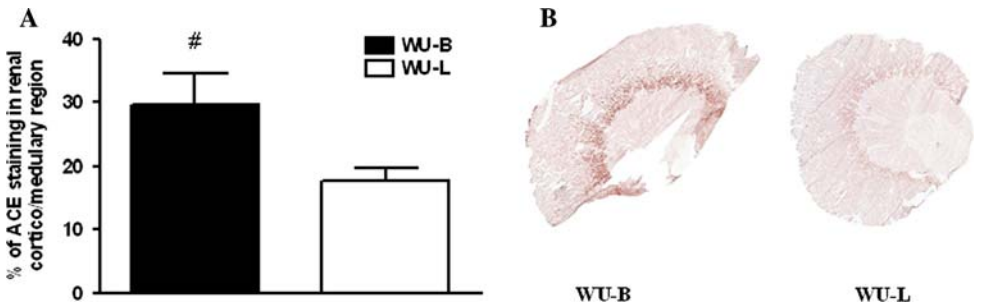


Fig. 3 Percentage of ACE staining in renal corticomedullary region. The ACE staining is presented as a band expanding from the corticomedullary region laterally back toward the cortex (**b**). We calculated the thickness of this band and presented it as a percentage to the length of the renal tissue from capsule to the end of corticomedullary section. The difference between WU-B and WU-L rats was statistically significant ($p < 0.001$), with more staining present in WU-B lines (**a**)

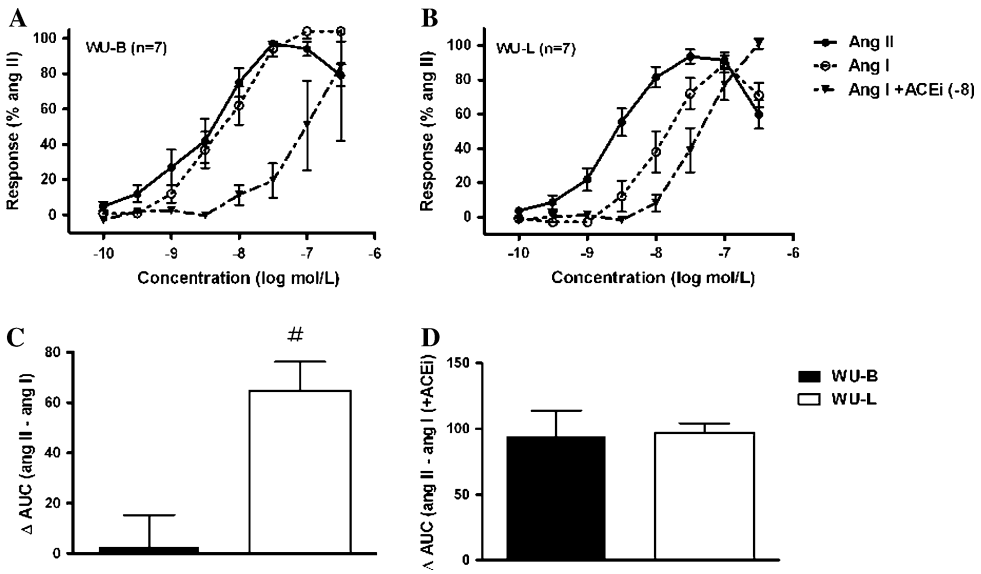


Fig. 4 Concentration-response relationship for AngI and AngII in WU-B (**a**) vs. WU-L (**b**). Two parallel rings were stimulated with either AngI or AngII and studied for contractile responses. The results are expressed as a percentage of maximal response to AngII for every pair of rings (mean ± SEM). The area under the curves was determined for the individual pairs and compared; WU-L had a significantly larger area, reflecting lower conversion from AngI to AngII (**c**, $^{\#}p < 0.05$). The effect of Lisinopril (ACEi) on the contractile response to AngI was studied; it showed a shift toward the right in the curves, with subsequently no differences between the conversion from AngI to AngII anymore (**d**)

cardiovascular tissue but with corresponding results. In follow-up studies between these two strains by Ocaranza et al. (2004), higher left ventricular ACE activity was reported in connection to B homozygous ACE allele.

To provide a functional assessment of differences in ACE activity in tissue AngI conversion, we included measurements of contractile responses to AngI and AngII in isolated aortic rings. These results show a consistently 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 that of earlier studies that evaluated the efficacy of ACEi using the difference in the dose-response curves for AngI and AngII as a functional index of ACEi (Buikema et al. 1996, 1997; Voors et al. 1998). Compared to the measurement of ACE activity in tissue homogenates, this setup has an advantage in that the normal vascular architecture is preserved. This is physiologically relevant since the conversion of AngI seems to occur at the site of action (Schuijt et al. 2002). However, this setup is limited in that responses to exogenously administrated AngI and AngII may be similar (Buikema et al. 1997), 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 to a functionally relevant difference between the B and L alleles. This difference is annihilated during preincubation 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 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 pathways, like chymase AngII forming pathways cannot be excluded. However, it should be noted that non-ACE pathways in rats are being debated and that chymase can cause breakdown of AngII in rats (Hollenberg 2000). The differences in AngI responsiveness were not present during preincubation with Lisinopril. This raises the hypothesis of there being another isoform of ACE present in the aorta which is inhibited by Lisinopril as well but does not cleave Hippuryl-His-Leu nor does it cross-react with our immunohistochemistry antibody. The local vascular RAS activity in aortic tissue has been demonstrated in rat by Igase et al. (2005) and distinguished from that in other vascular beds, namely, carotid arteries. Also, as noted above, in homogenates, in contrast to functional measurement in the aortic rings, the normal vascular

architecture is destroyed. This may affect the conversion of the substrate in ways that do not reflect the physiologic condition. Because ACE activity assay measures the conversion of Hippuryl-His-Leu to His-Leu, substances other than ACE, which cleave this product similarly, may have affected these measurements. Finally, a factor that could lead to discrepancies could be the difference in time points since the functional measurements of the aortic rings were done in 6-week-old rats and plasma and tissue measurements in 12–week-old rats. However, in our experience, AT1 receptor responsiveness in aortic rings gives the best comparable results in younger, 6–7-week-old animals (data not published).

To illustrate functional differences in ACE activity in tissue AngI conversion between the two alleles, we chose the experimental setup that we believed 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 animal aged 3 months (12 weeks) because this is the age most used by other investigators when studying the renal and cardiovascular systems. Also, complete inversion of tissue *ACE* expression and activity in a matter of weeks is highly unlikely and has 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, i.e., healthy congenic, transgenic, and Brown Norway vs. Lou rats, have shown no difference in blood pressure levels (Challah et al. 1998; Pueyo et al. 2004; Tian et al. 2004). A study by Kreutz at al. (1995) 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. Similarly, a majority of association studies in humans using the *ACE* I/D genotype did not show any significant relationship with blood pressure Harrap et al. 1993; Islam et al. 2006; Jeunemaitre et al. 1992; Sivakova et al. 2008).Therefore, it is not likely that there would have been a difference between the WU-B and WU-L rats. Wistar rats do not develop proteinuria without intervention. Phenotypic individual differences in renal ACE activity predict the severity of adriamycine-induced renal damage (Rook et al. 2005). 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 (Bos et al. 2003). In response to disease, ACE is upregulated (Hirsch et al. 1991; Largo et al. 1999). These results support the assumption that genetic differences in (tissue) ACE activity predispose to a less favorable course of renal damage and influence renal outcome. It would be very interesting in this respect to investigate whether this upregulation is influenced by the difference in *ACE* alleles.

Previous studies related pathophysiologic differences between the Brown Norway and Lou strains to variation in *ACE* B/L 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 for other genes influencing the phenotype between the two strains. 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 selected only 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. The model 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 AngI 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 that try to explain how the *ACE* gene polymorphisms interact with cardiovascular pathologies and define more exactly its molecular basis in animal studies as well as in humans.

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