

Transgenic Angiotensin-Converting Enzyme 2 Overexpression in Vessels of SHRSP Rats Reduces Blood Pressure and Improves Endothelial Function

Brit Rentzsch, Mihail Todiras, Radu Iliescu, Elena Popova, Luciana A. Campos, Marilene L. Oliveira, Ovidiu C. Baltatu, Robson A. Santos, Michael Bader

Abstract—Rat models of hypertension, eg, spontaneously hypertensive stroke-prone rats (SHRSP), display reduced angiotensin-converting enzyme 2 (ACE2) mRNA and protein expression compared with control animals. The aim of this study was to investigate the role of ACE2 in the pathogenesis of hypertension in these models. Therefore, we generated transgenic rats on a SHRSP genetic background expressing the human ACE2 in vascular smooth muscle cells by the use of the SM22 promoter, called SHRSP-ACE2. In these transgenic rats vascular smooth muscle expression of human ACE2 was confirmed by RNase protection, real-time RT-PCR, and ACE2 activity assays. Transgene expression leads to significantly increased circulating levels of angiotensin-(1-7), a prominent product of ACE2. Mean arterial blood pressure was reduced in SHRSP-ACE2 compared to SHRSP rats, and the vasoconstrictive response to intraarterial administration of angiotensin II was attenuated. The latter effect was abolished by previous administration of an ACE2 inhibitor. To evaluate the endothelial function in vivo, endothelium-dependent and endothelium-independent agents such as acetylcholine and sodium nitroprusside, respectively, were applied to the descending thoracic aorta and blood pressure was monitored. Endothelial function turned out to be significantly improved in SHRSP-ACE2 rats compared to SHRSP. These data demonstrate that vascular ACE2 overexpression in SHRSP reduces hypertension probably by locally degrading angiotensin II and improving endothelial function. Thus, activation of the ACE2/angiotensin-(1-7) axis may be a novel therapeutic strategy in hypertension. (*Hypertension*. 2008;52:967-973.)

Key Words: ACE2 ■ angiotensin-(1-7) ■ angiotensin II ■ SHRSP ■ endothelial function

The renin-angiotensin system (RAS) is a major regulator in human physiology. It plays a key role in blood pressure regulation, controls volume and electrolyte balance, and mainly affects the heart, vasculature, and kidney.¹ The effects are not only achieved through the vasoconstrictor angiotensin II (Ang II) but also through its metabolites, eg, angiotensin-(1-7) [Ang-(1-7)].

Ang-(1-7) exerts actions opposite to those of Ang II, which are mediated by its receptor Mas.² In blood vessels this heptapeptide mainly acts as a vasodilator and antiproliferative hormone.³ Ang-(1-7) was shown to cause a vasodilatory response in canine and porcine coronary arteries, rat aortas, and rabbit renal arterioles.^{4–6} Many studies have shown that these vascular actions of Ang-(1-7) could be accomplished in different ways: by antagonism of AT₁ receptors,⁷ by release of NO or other vasorelaxing factors,^{8–10} or by affecting other biological active peptides. Accordingly, a bradykinin potentiating action of Ang-(1-7) was reported.^{9,11} Furthermore, a crosstalk between the Ang-(1-7) receptors and other recep-

tors, such as the kinin B2 and AT₂ receptor, may also be involved.^{12,13}

The angiotensin-converting enzyme (ACE) homologue ACE2 is supposed to be the main responsible enzyme for Ang-(1-7) formation. ACE2 is a membrane-bound carboxypeptidase which is expressed in heart, kidney, lung, small intestine, and testis.¹⁴ It can be released into the circulation by shedding, however its activity in plasma is very low to undetectable, probably because of the presence of an endogenous inhibitor.^{15–17} ACE2 can directly form Ang-(1-7) through hydrolysis of Ang II or indirectly through hydrolysis of Ang I to Ang-(1-9) with subsequent conversion to Ang-(1-7) by ACE.^{18,19} Consequently, ACE2 activity may counterbalance the effects of ACE to increase Ang II levels by preventing the accumulation in tissues where ACE2 and ACE are both expressed.^{20,21}

In model systems of hypertension, eg, spontaneously hypertensive stroke-prone (SHRSP) and SABRA rats, it was shown that ACE2 mRNA and protein expression was reduced

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From the Max-Delbrück-Center for Molecular Medicine (B.R., M.T., R.I., E.P., L.A.C., O.C.B., M.B.), Berlin-Buch, Germany; the Department of Biology, Chemistry, and Pharmacy (B.R.), Free University Berlin, Germany; and the Department of Physiology and Biophysics (M.L.O., R.A.S.), Federal University of Minas Gerais, Belo Horizonte, Minas Gerais, Brazil.

M.T. and R.I. contributed equally to this study.

Correspondence to Michael Bader, Prof PhD, Max-Delbrück-Center for Molecular Medicine (MDC), Robert-Rössle-Str. 10, Berlin-Buch, D-13125, Germany. E-mail mbader@mdc-berlin.de

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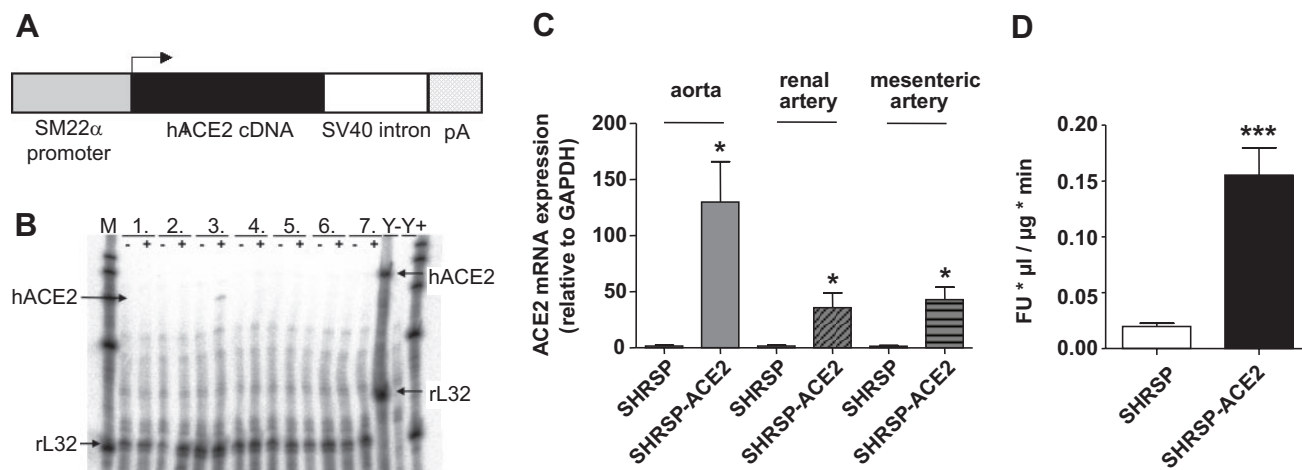


Figure 1. Generation of transgenic rats with vascular overexpression of human ACE2. **A**, Schematic representation of structural components of the DNA construct used for pronuclear microinjection. **B**, Ribonuclease protection assay of different organs using a specific probe (≈ 350 bp) for the transgene RNA (hACE2; protected fragment, 293 bp) and a probe (≈ 150 bp) for the housekeeping gene rL32 (protected fragment, 111 bp). On the left the protected fragments and on the right the probes are marked by arrows. Lanes: 1. stomach, 2. ileum, 3. aorta, 4. lung, 5. kidney, 6. heart, 7. skeletal muscle; –, SHRSP controls; +, SHRSP-ACE2 rats; Y+, yeast plus RNase; Y–, yeast without RNase. **C**, Real-time RT-PCR detection of total ACE2 mRNA in aorta, renal, and mesenteric artery. Expression of ACE2 was normalized to the respective GAPDH content for each sample and then expressed relative to a reference sample. **D**, ACE2 enzymatic activity of aorta in arbitrary fluorescence units (FU) $\times \mu\text{L}/\mu\text{g} \times \text{min}$ in SHRSP-ACE2 and SHRSP rats. Data are means \pm SEM of 3 animals in each group, * $P \leq 0.05$; *** $P \leq 0.001$.

compared with control animals.²² Another common important feature of these animal models is the early development of endothelial dysfunction, which is caused by increased oxidative inactivation of nitric oxide (NO)²³ or decreased NO synthase (NOS) activity and NO levels.²⁴

The vascular endothelium plays a crucial role in the maintenance of vascular tone and blood pressure through the release of vasoactive substances such as NO, prostaglandins, endothelin, or superoxide anions.²⁵ In pathological conditions the characteristics of the endothelium change leading to the development of endothelial dysfunction.²⁶ This is characterized by impaired endothelium-dependent relaxation and results in the failure of vasoactive, anticoagulant, and anti-inflammatory effects of the endothelium. Endothelial dysfunction is often the first step for the occurrence of many disease states in the cardiovascular system including atherosclerosis, diabetes, and heart failure.^{27–29} Therefore, the improvement of the endothelial function is a novel therapeutic strategy in the treatment of cardiovascular diseases.

The purpose of the present study was to investigate the role of ACE2 in the vascular system. Therefore transgenic rat lines were generated on a SHRSP genetic background expressing human ACE2 in vascular smooth muscle cells (VSMCs). We analyzed the effects on blood pressure and evaluated the role of ACE2 in endothelial function in this hypertensive rat model.

Materials and Methods

Generation of Transgenic Rats

A 2.8-kb fragment of the smooth muscle 22 α (SM22 α) promoter and the cDNA for human ACE2 gene (hACE2, RZPD-Clone DKFZp434A014, 2.5 kb) were subcloned into pBluescript II SK (Stratagene). The missing ATG start codon was added, and a polyadenylation cassette of SV40 virus was inserted 3' of the stop codon. The resulting 6.5-kb transgene fragment was released from

the vector by *KpnI* and *NotI* digestion, purified with QIAquick Gel Extraction (Qiagen), microinjected into the pronuclei of fertilized SHRSP zygotes, which were transferred into foster mothers using established methods.³⁰ The offspring was analyzed for genomic integration of the transgene by PCR of DNA obtained from tail biopsies, using a primer set specific for the SV40 DNA fragment (SV40-F: 5'-GAAGGAACCTTACTTCTGTGG-3', SV40-R: 5'-TCTTGTATAGCAGTGCAGC-3').

Phenotypic Analysis of Transgenic Rats

The methods for the phenotypic analysis of the transgenic rats are described in the Extended Materials and Methods section in the data supplement, available online at <http://hyper.ahajournals.org>.

Results

Generation of Transgenic Rats With Vascular Overexpression of Human ACE2 [TGSHRSP(SM22ACE2)]

The construct depicted in Figure 1A was used to generate the transgenic rat strain TGSHRSP(SM22ACE2) (SHRSP-ACE2). A 2.8-kb fragment of the SM22 gene was cloned upstream to the human ACE2 (hACE2) cDNA. This promoter was already shown to drive vascular smooth muscle–specific transgene expression.³¹ As genetic background for transgene expression we used SHRSP rats. We obtained 3 founder animals identified by PCR genotyping, 6973, 6918, and 6915. For each founder, transgenic lines were established and characterized with regard to the expression pattern of the transgene. The transgene expression detected by RNase protection assay (RPA) was almost identical in the 3 lines with strongest expression in the aorta (Figure 1B). We also analyzed the endogenous expression of ACE2 which was mainly detected in the ileum, but hardly in aorta, as described previously,¹⁴ and not different between transgenic and control rats (data not shown). For the following experiments we used the transgenic line 6918. In this line we also confirmed ACE2

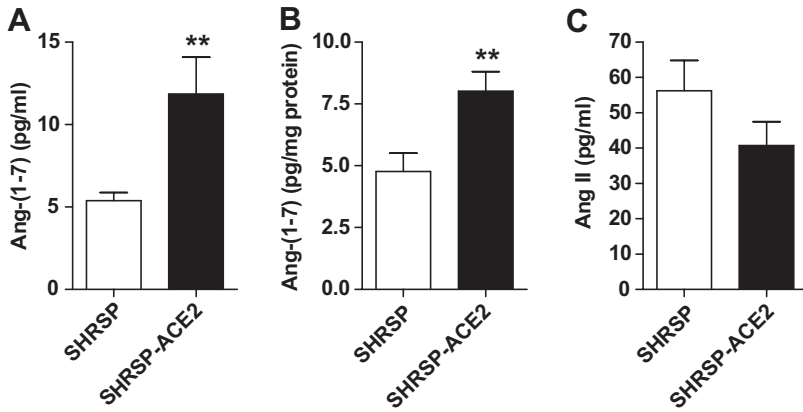


Figure 2. Ang-(1-7) (A) or Ang II (C) plasma levels as well as Ang-(1-7) aorta levels (B) were determined by radioimmunoassay in C_{18} extracted samples of arterial plasma and aorta homogenate, respectively. Data are means \pm SEM, ** $P \leq 0.01$ SHRSP vs SHRSP-ACE2 ($n \geq 7$ to 13).

overexpression in smaller vessels, such as renal and mesenteric arteries, which are more important for blood pressure regulation, using a quantitative real-time PCR that detects endogenous and transgenic ACE2 mRNA (Figure 1C).

ACE2 Activity Assay

To further characterize the transgene expression we measured ACE2 activity in different organs. In comparison to the SHRSP controls, ACE2 activity in the aorta of SHRSP-ACE2 was 7.8 times increased (Figure 1D). ACE2 activity was detectable in other organs, such as ileum, lung, kidney, and heart, as well as in very low amounts also in plasma, but it did not show significant differences between SHRSP and SHRSP-ACE2 (Figure S1).

Ang-(1-7) and Ang II Peptide Levels

As a result of increased ACE2 expression and activity we expected an increased degradation of Ang II and an augmentation of Ang-(1-7) peptide levels. Therefore, we determined the concentrations of Ang-(1-7) and Ang II in plasma using specific radioimmunoassays. The measurements revealed that transgene expression leads to a 2.2-fold higher plasma Ang-(1-7) concentration in transgenic rats compared to controls (SHRSP-ACE2: 11.8 ± 2.2 versus SHRSP: 5.4 ± 0.5 pg/mL plasma, $n \geq 10$, $P = 0.002$, Figure 2A). We further determined the Ang-(1-7) concentration in aorta and found a similar significant increase in the transgenic rats in comparison to controls (SHRSP-ACE2: 8.0 ± 0.8 versus SHRSP: 4.8 ± 0.7 pg/mg protein, $n \geq 13$, $P = 0.006$, Figure 2B). However, ACE2 overexpression does not lead to a significant reduction of the plasma Ang II concentration in transgenic animals (SHRSP-ACE2: 40.7 ± 6.8 versus SHRSP: 56.2 ± 8.6 pg/mL plasma, $n \geq 7$, $P = 0.206$, Figure 2C). These changes led to a significant increase of the Ang-(1-7)/Ang II ratio in SHRSP-ACE2 transgenic rats.

Mean Arterial Pressure in Conscious Rats

Ang II is one of the most important vasoconstrictors and Ang-(1-7) is thought to have vasodilatory functions. Consequently, changes in relative levels of these peptides should induce changes in basal blood pressure. Thus, we measured mean arterial pressure (MAP) in conscious rats by intraarterial catheter. Basal blood pressure was significantly decreased in the transgenic rats in comparison to the SHRSP control

animals (134 ± 1.8 versus 149 ± 3.2 , $P < 0.001$, $n \geq 12$, Figure 3A). In addition, the heart rate data did not show a significant change in the SHRSP-ACE2 rats in comparison to the SHRSP controls (342 ± 6.7 versus 333 ± 5.1 , $P = 0.314$, $n = 12$, Figure 3B).

Cardiac Hypertrophy

SHRSP rats develop cardiac hypertrophy early in development because of the exposure to high blood pressure.^{32,33} SHRSP-ACE2 rats exhibiting decreased blood pressure

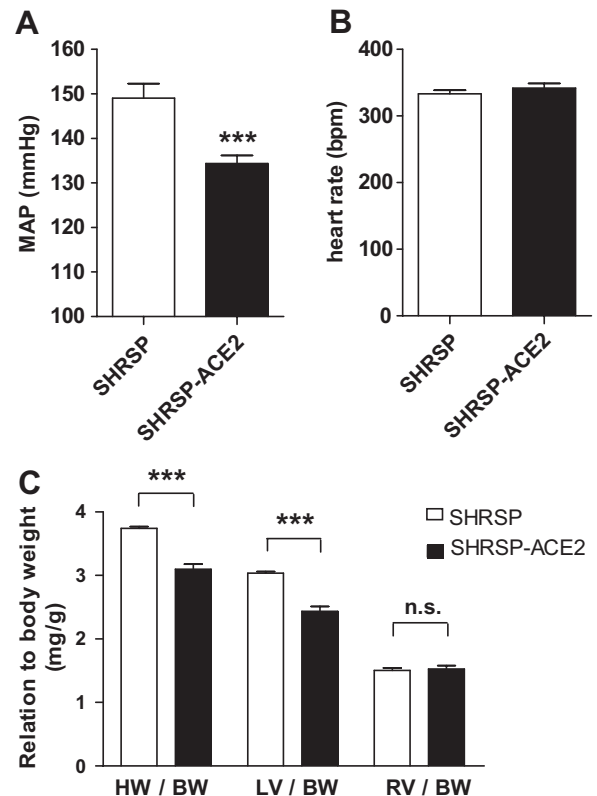


Figure 3. Mean arterial blood pressure (MAP) (A), heart rate (B), and cardiac hypertrophy (C) in SHRSP and SHRSP-ACE2. C, Cardiac chambers of 5-month-old rats were dissected and weighed to determine the heart weight/body weight (HW/BW), left ventricular weight/body weight (LV/BW), and right ventricular weight/body weight (RV/BW) ratios. Values are expressed as means \pm SEM of 11 to 13 animals in each experimental group. *** $P \leq 0.001$; n.s., not significant.

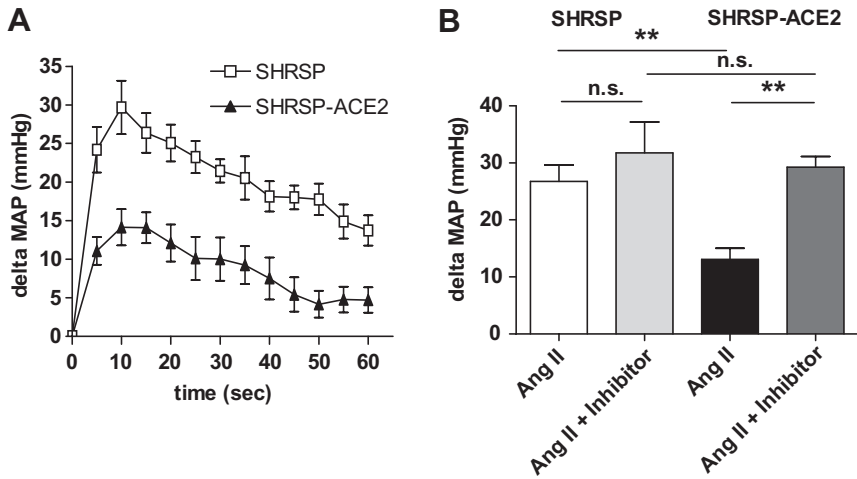


Figure 4. Time-dependent vasoconstriction response of SHRSP vs SHRSP-ACE2 rats after administration of Ang II (100 ng/kg) via intraarterial catheter (A). The bar graph (B) represents the first 15 seconds of the Ang II response with and without ACE2 inhibitor NAAE pretreatment (100 μg/kg). Results are expressed as mean ± SEM of 9 rats in each experimental group for Ang II treatment and of 3 rats in each group for the inhibitor pretreatment. ***P* ≤ 0.01, ****P* ≤ 0.001, n.s., not significant.

should consequently show an attenuated hypertrophy. To evaluate this possibility, cardiac chambers of 5-month-old rats were dissected and weighed to determine the ratio of heart weight (HW) to body weight (BW), the weight of the left ventricle (LV) to BW, and the weight of the right ventricle (RV) to BW (Figure 3C). The HW/BW ratio was decreased by 16% from 3.7 ± 0.03 in SHRSP to 3.1 ± 0.08 in SHRSP-ACE2 (*P* ≤ 0.0001, n ≥ 11). The LV/BW ratio was also significantly decreased in SHRSP-ACE2 rats (SHRSP-ACE2: 2.4 ± 0.08 versus SHRSP: 3.0 ± 0.03, *P* < 0.0001, n ≥ 11). In contrast, the RV/BW ratio did not show a significant difference (SHRSP-ACE2: 1.5 ± 0.05 versus SHRSP: 1.5 ± 0.04, *P* = 0.74, n ≥ 11), as expected for a cardiac hypertrophy induced by systemic hypertension.

Blood Pressure Response to Ang II

Furthermore, we investigated the vasoconstrictive response to Ang II in vivo. Increased ACE2 expression may cause faster degradation of Ang II, and thus vasoconstriction may be attenuated in the transgenic animals after Ang II treatment. We administered 100 ng/kg Ang II intraarterially and found a significantly attenuated vasoconstrictive response in SHRSP-

ACE2 in comparison to controls (13.1 ± 1.9 versus 26.8 ± 2.9, *P* ≤ 0.01, n = 9, Figure 4A and 4B). To verify the role of ACE2 overexpression in this effect, we repeated the experiment in the presence of the ACE2 inhibitor NAAE³⁴ (Figure 4B). As expected, this inhibitor abolished the decreased Ang II response (29.3 ± 1.9 versus 13.1 ± 1.9 without inhibitor pretreatment, *P* ≤ 0.01, n = 3 to 9). In SHRSP control rats, the response to Ang II was not changed by the ACE2 inhibitor (31.8 ± 5.4 versus 26.8 ± 2.9 without inhibitor pretreatment, *P* = 0.41, n = 3 to 9).

Endothelium-Dependent Vasorelaxation in Aortic Rings

Knowing about the early development of endothelial dysfunction in SHRSP rats,³² we tried to dissect out the role of ACE2 in this process. To this purpose, we used aortic rings to perform vascular reactivity experiments. As summarized in Figure 5, aortic rings isolated from SHRSP-ACE2 rats exhibit a stronger relaxation response to the endothelium-dependent vasodilator, carbachol, compared with SHRSP controls. Endothelium-independent relaxation by SNP was only moderately decreased in aortic rings of SHRSP-ACE2 animals.

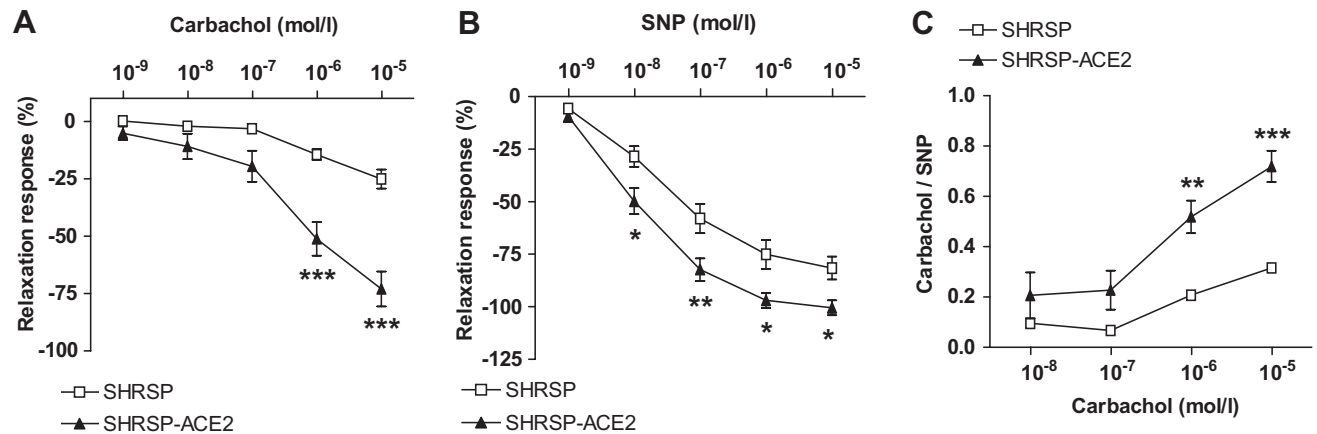


Figure 5. Concentration-dependent relaxation response of rat aortic rings to the endothelium-dependent vasodilator, carbachol (A), and to the endothelium-independent vasodilator, sodium nitroprusside (SNP; B). The rings were precontracted with phenylephrine (1 μmol/L). At the plateau of contraction, cumulative concentrations of carbachol or SNP (1 nmol/L to 10 μmol/L) were added, and the vasorelaxation to each concentration of vasodilator was calculated as a percentage of the maximal vasoconstriction. C, Vascular response to carbachol normalized by SNP. Results are expressed as mean ± SEM of 3 to 8 rats in each experimental group. ****P* ≤ 0.001.

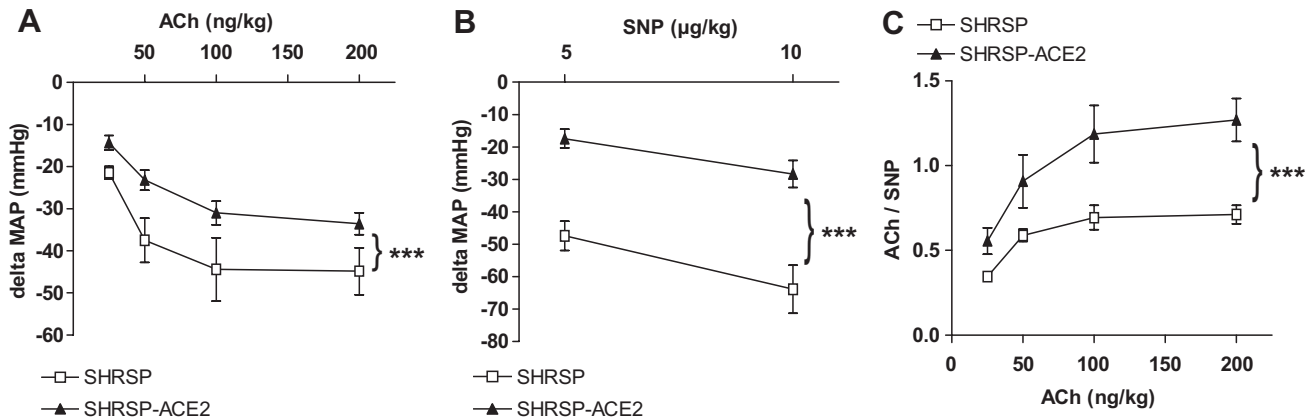


Figure 6. In vivo evaluation of endothelial function (EF). The vasodilatory response to cumulative concentrations of the endothelium-dependent vasodilator ACh (25 to 200 ng/kg; A) and the endothelium-independent vasodilator SNP (5 and 10 μ g/kg) were measured (B). EF was calculated with the formula $EF = \Delta MAP(ACh) / \Delta MAP(SNP)$, using the data of 10 μ g/ μ L SNP (C). Results are given as means \pm SEM of 6 rats in each experimental group. *** $P \leq 0.001$.

These results demonstrate that aortic endothelial function (EF) is improved by the overexpression of ACE2 in SHRSP rats.

In Vivo Evaluation of Endothelial Function

To confirm these in vitro data of vascular reactivity, we investigated additionally EF in vivo. To determine this parameter we measured the changes in blood pressure produced by administration of the endothelium-independent vasodilator sodium nitroprusside (SNP) as well as the endothelium-dependent vasodilator acetylcholine (ACh) into the descending thoracic aorta via a catheter inserted in the carotid artery. Notably, the direct administration of ACh into the arterial system did not cause any direct cardiac effects attributable to its rapid degradation. Finally, EF was calculated with the formula: $EF = \Delta MAP(ACh) / \Delta MAP(SNP)$.

Figure 6 shows the responses to SNP and ACh administration, demonstrating that the SHRSP control rats show a more pronounced vasodilatory response to both substances as the SHRSP-ACE2 rats. This can be explained by the already existing vasodilation in the transgenic animals. The calculated EF, in contrast, indicates a significant improvement in the SHRSP-ACE2 in comparison to the SHRSP rats (Figure 6C). Additionally, we determined the EF in response to the vasodilator bradykinin and obtained similar results (Figure S2).

In summary, the in vitro as well as the in vivo data show that vascular overexpression of ACE2 in SHRSP-ACE2 rats causes an improved EF, probably attributable to local degradation of Ang II to Ang-(1-7), and leads to a decrease in blood pressure.

Discussion

The carboxypeptidase ACE2 serves as a negative regulator of the RAS. It has emerged as a crucial enzyme to counterbalance the actions of ACE in determining the levels of the important vasoconstrictor Ang II in tissue.³⁵

The relevance of ACE2 becomes particularly apparent in model systems of spontaneous or diet-induced hypertension, eg, salt-sensitive SABRA rats, spontaneously hypertensive rats (SHR), and SHRSP rats. In these rat strains, it was shown

that ACE2 mRNA and protein expression was reduced compared with control animals.²² Furthermore, it could be demonstrated that ACE2 is a strong candidate gene for a defined quantitative trait locus (QTL) associated with hypertension on the X chromosome in these model strains.²² Additionally, polymorphisms in the ACE2 gene have also been linked to the development of pathological myocardial hypertrophy and heart disease in human.^{36,37}

The purpose of this study was to investigate the role of ACE2 in the pathogenesis of hypertension in the SHRSP rat model. Therefore, we generated transgenic SHRSP rats with overexpression of ACE2 in vascular smooth muscle cells. Using RPA, real-time RT-PCR, and ACE2 activity tests we could show that ACE2 overexpression was specific for the vascular system and included smaller vessels. Consequently, the persistent overexpression of ACE2 in resistance vessels may cause the observed attenuation of hypertension in SHRSP rats.

Furthermore, the findings reported here demonstrate that ACE2 overexpression leads to a reduced vasoconstrictive response to Ang II administration, because the ACE2 inhibitor NAAE abolished the difference in the Ang II response between SHRSP-ACE2 and SHRSP rats. The specificity of this inhibitor is not yet comprehensively clarified, but our results showing specific blocking of the ACE2 transgene effect contributes evidence that it preferentially inhibits this enzyme.

ACE2 may attenuate the vasoconstrictive actions of Ang II by degradation or additionally by the generation of the vasodilator Ang-(1-7). Our data demonstrate a significant increase in the Ang-(1-7) peptide level in plasma and aortic tissue, but no significant decrease of Ang II in the plasma of SHRSP-ACE2 rats. The increased Ang-(1-7) levels in plasma probably result from a spillover of the peptide produced in the vascular wall and not from generation by circulating ACE2, because ACE2 was hardly detectable in plasma and not different between SHRSP and SHRSP-ACE2.

Ang-(1-7), through its recently identified receptor Mas,² may stimulate NO synthase and counteract the potentially detrimental actions of Ang II via the AT₁ receptor.³⁸ The

effects of Ang-(1-7) could also involve stimulation of prostaglandin (PG) synthesis, AT₂ receptor-dependent mechanisms, as well as bradykinin potentiation.^{8–11} In addition, besides the hydrolysis of angiotensin peptides, ACE2 can also cleave vasoactive peptides of other systems, like apelin-13 and -36, the kinin metabolites (des-Arg⁹)-BK and (des-Arg¹⁰)-kallidin, neurotensin, kinetensin, as well as the opioid peptide dynorphin A.¹⁹ Many of these substrates are relevant for cardiovascular regulations. Apelin, for instance, has vasoconstrictive and inotropic actions. Consequently, the cleavage of apelin could also play a role in the induced changes by ACE2 overexpression.^{39,40} Furthermore, ACE2 could modulate the expression of the AT₁ or the AT₂ receptor. ACE2 overexpression leads to a reduction in AT₁ receptors in the subfornical organ of mice.⁴¹ Such an AT₁ receptor downregulation in the vascular wall could account for the reduced vasoconstrictive response to Ang II in the SHRSP-ACE2 rats.

One prominent pathological feature of SHRSP rats is the development of a LV hypertrophy and fibrosis. Our data show an attenuated development of this phenotype in the SHRSP-ACE2 rats. The HW/BW as well as the LV/BW ratio are decreased in the transgenic rats compared to controls, but not the RV/BW ratio. This effect of the transgenic ACE2 is probably mainly attributable to the decreased blood pressure and consequently the reduced after load in the heart of the transgenic rats. Nevertheless, it may be reinforced by the increased circulating amount of Ang-(1-7). Several studies propose that ACE2, Ang-(1-7), and Mas have direct beneficial effects on myocardial function.^{42–44} We have shown that isoproterenol-induced heart hypertrophy was attenuated in transgenic rats with increased circulating Ang-(1-7) levels.⁴² Accordingly, Mas-deficient mice displayed an impaired cardiac function unmasking the key functional role of this receptor in the heart.⁴³

However, probably the most important observation in our new transgenic animal model was the protective effect of ACE2 overexpression on endothelial function. Hypertensive animal models like SHRSP rats exhibit a marked endothelial dysfunction already early in development.³² The key feature in this process is the inability of arteries and arterioles to dilate fully in response to an appropriate stimulus.²⁶

Our *in vitro* data show a clearly improved relaxation response of aortic rings to the endothelium-dependent vasodilator carbachol in ACE2-overexpressing rats compared to controls. In contrast, the vasodilatory response to ACh as well as to SNP *in vivo* was stronger in the SHRSP controls. This discrepancy may be explained by the already predilated vessels in the transgenic rats. Importantly in both cases, the calculated endothelial function was improved in the transgenic SHRSP-ACE2 rats.

There is increasing evidence that the ACE2/Ang-(1-7)/Mas axis plays an important role in maintaining endothelial function and vascular integrity. A recent publication of Peiró et al report about a pivotal role for its receptor Mas in preserving normal endothelium-dependent relaxation.⁴⁵ Furthermore, our group could show that Mas-deletion results in an imbalance between NO and reactive oxygen

species, endothelial dysfunction, and finally increased blood pressure.⁴⁶

Perspectives

In conclusion, our study shows that vascular overexpression of ACE2 reduces high blood pressure, attenuates the vasoconstrictive response to Ang II, and improves endothelial function on a long-term basis. Consequently, we reason that by improving endothelial function, ACE2 may play an important role in inhibiting atherosclerosis, a clinically relevant end point of progressive endothelial dysfunction. Furthermore, improving endothelial function by attenuating Ang II production as well as augmenting Ang-(1-7) generation may be a central mechanism through which ACE2 exerts cardiovascular and renal protection and which may be exploited for therapeutic purposes.

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Disclosures

None.

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Brit Rentzsch, Mihail Todiras, Radu Iliescu, Elena Popova, Luciana A. Campos, Marilene L. Oliveira, Ovidiu C. Baltatu, Robson A. Santos and Michael Bader

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