Targeting the Degradation of Angiotensin II With Recombinant Angiotensin-Converting Enzyme 2

Prevention of Angiotensin II–Dependent Hypertension

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Abstract—Angiotensin (Ang)-converting enzyme 2 (ACE2) cleaves Ang II to form Ang-(1-7). Here we examined whether soluble human recombinant ACE2 (rACE2) can efficiently lower Ang II and increase Ang-(1-7) and whether rACE2 can prevent hypertension caused by Ang II infusion as a result of systemic versus local mechanisms of ACE2 activity amplification. rACE2 was infused via osmotic minipumps for 3 days in conscious mice or acutely in anesthetized mice. rACE2 caused a dose-dependent increase in serum ACE2 activity but had no effect on kidney or cardiac ACE2 activity. After Ang II infusion (40 pmol/min), rACE2 (1 mg/kg per day) resulted in normalization of systolic blood pressure and plasma Ang II. In acute studies, rACE2 (1 mg/kg) prevented the rapid hypertensive effect of Ang II (0.2 mg/kg), and this was associated with both a decrease in Ang II and an increase in Ang-(1-7) in plasma. Moreover, during infusion of Ang II, the effect of rACE2 on blood pressure was unaffected by a specific Ang-(1-7) receptor blocker, A779 (0.2 mg/kg), and infusing supraphysiologic levels of Ang-(1-7) (0.2 mg/kg) had no effect on blood pressure. We conclude that, during Ang II infusion, rACE2 effectively degrades Ang II and, in the process, normalizes blood pressure. The mechanism of rACE2 action results from an increase in systemic, not tissue, ACE2 activity and the lowering of plasma Ang II rather than the attendant increase in Ang-(1-7). Increasing ACE2 activity may provide a new therapeutic target in states of Ang II overactivity by enhancing its degradation, an approach that differs from the current focus on blocking Ang II formation and action. **(*Hypertension*. 2010;55:90-98.)**

Key Words: ACE2 ■ soluble ■ recombinant ■ angiotensin II ■ angiotensin-(1-7)

Angiotensin (Ang)-converting enzyme 2 (ACE2) is the only known enzymatically active homologue of Ang-converting enzyme (ACE).[1–3] ACE2 is a monocarboxypeptidase that removes single amino acids from the C terminus of its substrates.[1–3] ACE, by contrast, is a peptidyl dipeptidase that removes C-terminal dipeptides. ACE promotes Ang II formation from Ang I, whereas ACE2 converts Ang I to Ang-(1-9) and Ang II to Ang-(1-7), respectively.[1–3] The catalytic efficiency of human ACE2 is 400-fold higher with Ang II than with Ang I as a substrate.[3] Moreover, because the product of Ang I cleavage by ACE2, Ang-(1-9), has no known biological action, it seems logical to postulate that cleavage of Ang II to Ang-(1-7) is a major action of ACE2.

There is increasing interest in the possible renoprotective effects of ACE2.[4–9] A protective effect of ACE2 against acute lung injury[10,11] and cardiovascular disease[12,13] has also been proposed. Ang-(1-7) is a blood-vessel dilator identified as an endogenous ligand for a G protein–coupled Mas receptor.[14–16] Ang II, among its many other known biological effects, is a potent vasoconstrictor and promotes renal sodium retention, both of which lead to hypertension.

The blockade of steps leading to Ang II formation using ACE inhibitors and renin inhibitors or blocking the action of Ang II on the Ang II type 1 receptor using specific antagonists has provided a rationale for modern antihypertensive and cardiovascular therapies.[17] We reasoned that enhancing the degradation of Ang II likewise may provide an effective approach to lower Ang II levels and, thus, provide the basis for novel therapies on the basis of reducing Ang II overactivity. Currently there are no in vivo studies, to our knowledge, showing that amplification of ACE2 activity results in lowering of Ang II and increasing Ang-(1-7) levels. There-

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fore, it has been difficult to examine the relative biological importance of the 2 anticipated effects of ACE2 on Ang II: lowering Ang II versus increasing Ang-(1-7).

Increasing ACE2 activity could provide an effective approach to reduce high blood pressure, particularly in situations when levels of ACE2 may be reduced, as has been documented in models of rat hypertension.[12,18,19] Indeed, in one of those models, the spontaneously hypertensive stroke-prone rats, transgenic ACE2 overexpression in vascular smooth muscle led to attenuation of hypertension.[20] Moreover, in ACE2-deficient mice, the infusion of Ang II has been shown to increase blood pressure above the level of WT control.[21]

The purpose of this study was, therefore, to examine whether the administration of soluble human recombinant ACE2 (rACE2) can effectively degrade Ang II in vivo and whether rACE2 can prevent Ang II–induced hypertension. We sought also to unravel the relative contributions of lowering Ang II versus increasing Ang-(1-7) by rACE2 on blood pressure regulation during Ang II administration both acutely and chronically.

Methods

Soluble Human rACE2

Details on the production and characterization of rACE2 are provided in the expanded Materials and Methods section in the online Data Supplement (please see http://hyper.ahajournals.org).

Chronic Studies

All of the studies were approved by the Institutional Animal Care and Use Committee. To study the dose response of rACE2, 3 groups of male mice (C57BL/6J) of 10 weeks of age were given rACE2 for 3 days (at doses 0.1, 1.0, and 5.0 mg/kg per day) using osmotic minipumps (model 1003, ALZET). Male ACE2 knockout (KO) mice also on C57BL/6J background (donated by Drs S. Gurley and T. Coffman, Duke University, Durham, NC[21]) were either sham operated or infused for 3 days with rACE2 (1 mg/kg per day), Ang II (40 pmol/min), or concurrently with rACE2 (1 mg/kg per day) and Ang II (40 pmol/min). An additional group of mice received rACE2 (1 mg/kg per day) by minipumps for 14 days (please see the online Data Supplement).

Pumps were implanted subcutaneously on the back between the shoulder blades and hips while animals were anesthetized by inhalation of isoflurane anesthetic. Control mice were sham operated the same way as mice that were implanted with osmotic minipumps containing rACE2.

In separate experiments, hypertension was also induced in 10-week–old C57BL/6J mice by the subcutaneous infusion of Ang II (Sigma-Aldrich; 40 pmol/min; 1000 ng/kg per minute).[21] Two additional groups were infused simultaneously with either Ang II (40 pmol/min) and rACE2 (1 mg/kg per day) or concurrently with Ang II (40 pmol/min), A779 (100 ng/kg per minute; Bachem), and rACE2 (1.0 mg/kg per day) through separate osmotic minipumps. The compound A779 has been shown to selectively block the Ang-(1-7)/Mas receptor in several studies both in vitro[14] and in vivo.[22,23] The dose that we used in this study (100 ng/kg per minute), moreover, has been reported to reverse the antifibrotic effect of Ang-(1-7) in vivo.[24]

Systolic blood pressure (SBP) was measured noninvasively in conscious and anesthetized mice by determining the tail blood volume with a volume-pressure recording sensor and an occlusion tail-cuff using a computerized system (CODA System, Kent Scientific); mice were conditioned previously to the blood pressure monitoring procedure for ≥4 consecutive days before the day of the experiment. The volume-pressure recording system has been validated and provides a high correlation with telemetry and direct arterial blood pressure measurements.[25]

Acute Studies

To study the acute effect of rACE2 on SBP and Ang II degradation, male C57BL/6J mice also 10 to 13 weeks old were anesthetized with an IP ketamine injection (200 mg/kg of body weight). Two hours before anesthesia, mice were pretreated with an IP injection of either sterile PBS or rACE2 (1 mg/kg). Immediately after inducing anesthesia, mice were placed on a heating platform for 10 minutes. SBP was monitored noninvasively every 30 seconds for a period of 25 minutes. After 5 minutes of baseline SBP recording, acute hypertension in anesthetized mice was induced with an IP bolus of Ang II (0.2 mg/kg), and the SBP was monitored for the remaining 20 minutes. In additional experiments, Ang II (0.2 mg/kg) was infused together with an ACE2 inhibitor (MLN-4760, Millennium Pharmaceuticals; 1 mg/kg) after rACE2 infusion 2 hours earlier, as described above. In separate experiments, mice pretreated with PBS were injected with A779 at 2 different doses (0.2 and 1.0 mg/kg) or concomitantly with Ang II and Ang-(1-7) (both at a dose of 0.2 mg/kg). A group of mice receiving rACE2 were also injected with Ang II and A779 (both at the dose of 0.2 mg/kg).

In a set of mice under ketamine anesthesia (pretreated with PBS or rACE2, as above), 5 minutes after Ang II injection, euthanasia was performed by cervical dislocation, and blood was rapidly drawn by cardiac puncture for measurements of ACE2 activity and Ang II and Ang-(1-7) levels.

ACE2 Activity

ACE and ACE2 activities were measured by an enzymatic assay that uses a fluorogenic peptide substrate 7-Mca-YVADAPK(Dnp; R&D Systems), as described previously.[26]

Measurements of Plasma and Whole Kidney Ang II and Ang-(1-7)

Please see the online Data Supplement for details.

Determination of Plasma Antihuman rACE2 Antibodies

Please see the online Data Supplement for details.

Statistical Analysis

Results are presented as mean±SEM. Differences in the means among multiple groups were compared using 1-way ANOVA. The blood pressure curves were compared with the use of general linear model multivariate analysis. Pairwise multiple comparisons were made with the Bonferroni post hoc analysis to detect significant differences between groups. For data exhibiting nonnormal distribution, a nonparametric Kruskal-Wallis test was used, followed by a Mann–Whitney test for pairwise comparisons. $P<0.05$ was considered statistically significant. SPSS version 17.0 for Windows was used for statistical analyses.

Results

Enzymatic Properties of rACE2

The ability of rACE2 to cleave Ang II and Ang I was evaluated in vitro (Figure S1, available in the online Data Supplement). By high-performance liquid chromatography analysis at 0, 30, and 60 minutes, Ang-(1-7) and phenylalanine were identified as the only products of Ang II cleavage by rACE2. rACE2 was also capable of cleaving Ang I in vitro with the emergence of a very small peak that corresponds with Ang-(1-9) (Figure S1B). The effect of rACE2 on Ang I cleavage is modest as compared with the marked effect on Ang II (compare Figure S1A and S1B).

The cleavage of Ang II during incubation with rACE2, measured by Ang II disappearance over time, was blocked by

a specific ACE2 inhibitor, MLN-4760 (Figure S2A). By contrast, the disappearance of Ang I over time was very slow and not significantly affected by MLN-4760 (Figure S2B). Altogether, these findings show that rACE2 effectively digests Ang II, whereas it has only a modest effect on Ang I digestion.

Chronic Studies

Effect of rACE2 Administration on Serum and Tissue ACE2 Activity and Blood Pressure in Normotensive Mice

Infusion of rACE2 for 3 days to 3 groups of animals at 3 different doses, 0.1 mg/kg per day (n=11), 1 mg/kg per day (n=13), and 5 mg/kg per day (n=10), resulted in a dose-dependent increase in serum ACE2 activity (1.30±0.34, 6.56±0.84, and 22.21±1.67 relative fluorescence units [RFU]/μL per hour, respectively; $P<0.001$ by ANOVA; Figure 1A). In contrast to serum ACE2 activity, there was no significant increase in ACE2 activity in the kidney cortex after rACE2 administration (Figure 1B). In heart tissue, ACE2 activity was much lower than in the kidney, as reported previously,[26] and also ACE2 activity did not increase in animals infused with the 3 different doses of rACE2 (Figure 1C).

Recombinant ACE2 had no significant effect on SBP in conscious animals infused with 0.1, 1.0, or 5.0 mg/kg per day for 3 days (Figure 1D). In a subgroup of these animals, SBP was also measured under short-duration anesthesia. Under these conditions, SBP was lower than in conscious animals, but it was also not significantly affected by the infusion of either 1 or 5 mg/kg per day of rACE2 (Figure S3).

Serum ACE activity was not significantly affected by any of the doses of rACE2 (Figure S4A). Plasma Ang II decreased modestly but significantly with the 2 highest doses, whereas Ang-(1-7) levels also increased modestly but not significantly (Figure S4B and S4C).

Effect of rACE2 on Blood Pressure and Plasma Ang II and Ang-(1-7) Levels During Sustained Infusion of Ang II

In conscious mice that had been infused with Ang II for 3 days, SBP was significantly higher than in sham-operated controls not infused with Ang II (150.7±5.2 mm Hg, n=12, versus 132±2.2 mm Hg, n=8, respectively; $P<0.05$). In mice infused with Ang II and rACE2 (1 mg/kg per day), SBP was significantly lower than in animals infused with Ang II alone (132.4±4.3 mm Hg, n=12 versus 150.7±5.2 mm Hg, n=12; $P<0.05$; Figure 2A). When A779 was given to block the Ang-(1-7)/Mas receptor,[14] together with Ang II and rACE2, blood pressure was reduced to the same extent as in animals infused with rACE2 and Ang II (130.5±4.4 mm Hg, n=9, versus 132.4±4.3 mm Hg, n=12, respectively; Figure 2A).

The infusion of Ang II alone for 3 days resulted in plasma Ang II levels ≈3-fold higher than those of controls (173.1±38.3 versus 54.8±21.6 fmol/mL, respectively; $P<0.05$). The combination of rACE2 and Ang II resulted in a reduction of plasma Ang II to the level observed in controls (52.7±27.8 and 54.8±21.6 fmol/mL, respectively). In the group infused concomitantly with rACE2, Ang II, and A779, plasma Ang II was reduced to 47.9±19.4 fmol/mL, a value also similar to that of controls (54.8±21.6 fmol/mL). Figure 2B and 2C summarizes plasma Ang II and Ang-(1-7) levels, respectively, in the 3 groups of Ang II–infused animals. Plasma Ang-(1-7) tended to be higher in mice infused with Ang II and rACE2 as compared with the mice infused with Ang II only, but this difference was not statistically significant. In the Ang II–infused group that received rACE2 and A779,

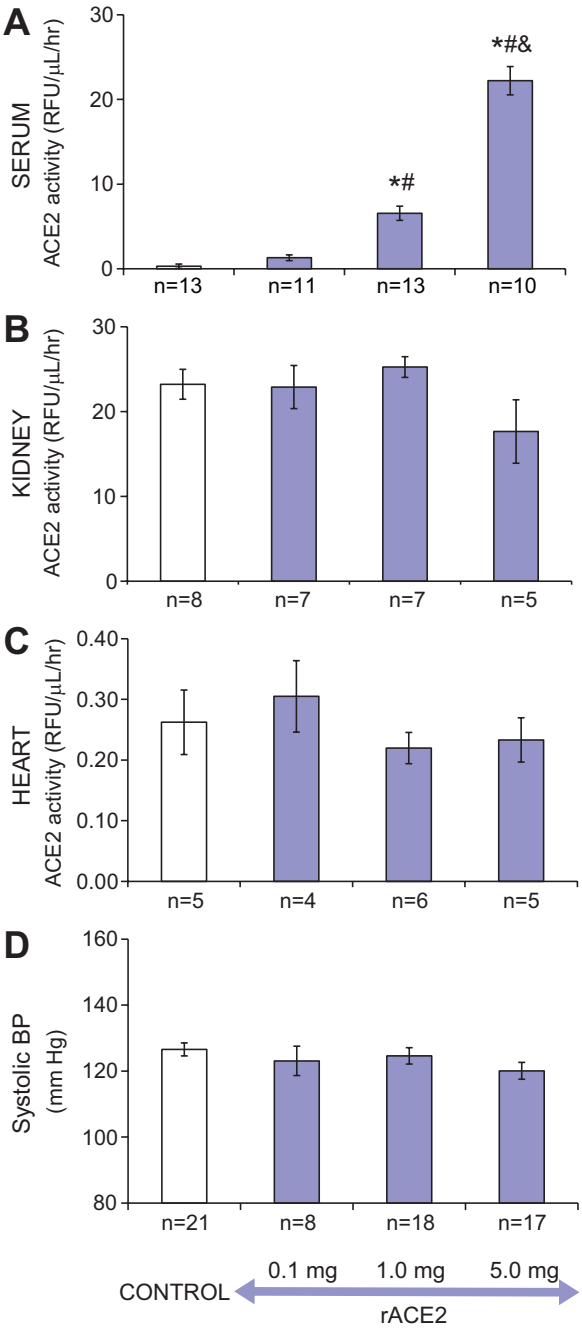

Figure 1. Serum (A), kidney cortex (B), and heart tissue ACE2 activity (C), as well as SBP (D) in sham-operated mice (□) and mice infused for 3 days SC with recombinant ACE2 at doses of 0.1, 1.0, or 5.0 mg/kg per day (rACE2; ■). A through C, After rACE2 administration, a dose-dependent increase in serum ACE2 activity was observed (A), whereas there was no increase in either kidney cortex (B) or heart ACE2 activity (C); *$P<0.001$ vs control, #$P<0.001$ vs 0.1 mg/kg per day, &$P<0.001$ vs 1 mg/kg per day. D, rACE2 did not lower SBP significantly at any of the doses used.

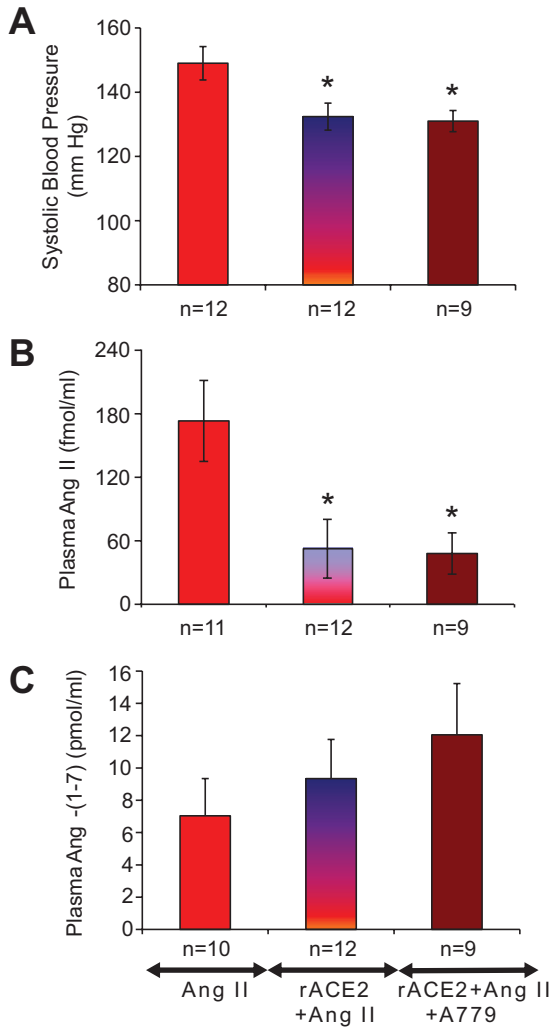

Figure 2. SBP (A), plasma Ang II (B), and Ang-(1-7) levels (C) after 3 days infusion of either Ang II alone or simultaneously with Ang II and rACE2 or concurrently with Ang II, rACE2, and A779 (a selective antagonist of the Ang-[1-7] receptor). A, The administration of rACE2 to mice receiving Ang II resulted in a significantly lower blood pressure as compared with mice infused with Ang II alone. The administration of A779 concomitantly with Ang II and rACE2 was associated with a blood pressure similar to that seen in animals infused with rACE2 and Ang II; *$P<0.05$ vs Ang II only. B, Plasma Ang II levels in mice receiving the combined infusion of rACE2 and Ang II were markedly lower as compared with Ang II infusion alone. In the group infused concomitantly with rACE2, Ang II, and A779, plasma Ang II levels were similarly reduced, as compared with Ang II alone; *$P<0.05$ vs Ang II only. C, Plasma Ang-(1-7) levels in mice receiving the combined infusion of rACE2 and Ang II were higher as compared with Ang II infusion alone, but the difference did not reach statistical significance. In the group receiving A779 and rACE2, plasma Ang-(1-7) levels were even higher, but the difference was not statistically significant as compared with the other 2 groups.

plasma Ang-(1-7) levels were higher than in the other groups, but the difference also did not reach statistical significance (Figure 2C).

Effect of rACE2 on Kidney Ang II and Ang-(1-7) Levels and ACE2 Activity After Infusion of Ang II

In previous studies, it has been shown that the systemic infusion of Ang II results in intrarenal accumulation of Ang II.[21,27] We sought to determine whether rACE2 infusion decreases intrarenal Ang II accumulation in mice infused with Ang II.

The infusion of Ang II alone was associated with a marked increase in kidney Ang II levels as compared with noninfused sham controls (71.6±7.7 versus 9.5±1.4 fmol/mg, respectively; $P<0.001$). The concomitant infusion of rACE2 and Ang II resulted in a reduction of renal Ang II as compared with mice infused with Ang II only (42.5±5.0 and 71.6±7.7 fmol/mg, respectively; $P<0.005$), but it remained higher than in controls not infused with Ang II (9.5±1.4 fmol/mg; $P<0.001$). Kidney Ang-(1-7) levels were not significantly different between the groups infused with Ang II alone and Ang II and rACE2 (15.8±3.5 versus 10.3±2.0 pg/mg of protein, respectively).

Renal ACE2 enzymatic activity in mice infused concurrently with Ang II and rACE2 (20.1±1.3 RFU/μg of protein per hour) was not significantly different from either sham-operated controls (23.2±1.8 RFU/μg of protein per hour) or mice receiving Ang II alone (25.9±1.6 RFU/μg of protein per hour). Accordingly, increases in kidney ACE2 activity could not account for the observed decrease in intrarenal Ang II levels after rACE2 administration in animals infused with Ang II.

Effect of rACE2 on Kidney Ang II Levels and rACE2 Activity in an ACE2 KO After Infusion of Ang II

In ACE2-deficient mice receiving rACE2 (1 mg/kg per day), serum ACE2 activity was significantly higher from ACE2 KO mice not infused with rACE2 (3.75±0.70 versus 0.05±0.23 RFU/μL per hour, respectively; $P<0.005$). By contrast, in kidney cortex from the ACE2 KO mice infused with rACE2, ACE2 activity was undetectable and remained not significantly different from ACE2 KO mice not infused with rACE2 (−0.70±0.22 versus 0.13±0.19 RFU/μL per hour). This is consistent with the studies in kidneys from WT C57BL/6 mice when ACE2 activity was high but also unaffected by rACE2 (see Figure 1).

The infusion of Ang II alone to ACE2-deficient mice was associated with a marked increase in kidney Ang II levels as compared with ACE2 KO mice not infused with Ang II (100.8±16.4 versus 23.05±4.8 fmol/mg, respectively; $P<0.005$; Figure 3). The concomitant infusion of rACE2 and Ang II resulted in a significant reduction of Ang II as compared with mice infused with Ang II only (55.2±9.3 and 100.8±16.4 fmol/mg, respectively; $P<0.05$), but it remained higher than in KO not infused with Ang II (55.2±9.3 versus 23.05±4.8 fmol/mg; $P<0.05$; Figure 3).

The concordant behavior of kidney Ang II levels after rACE2 infusions in wild-type (WT) and ACE2 KO mice (Figure 3) further suggests that the observed effect of rACE2 on intrarenal Ang II levels is the result of degrading circulating Ang II as a result of increased serum ACE2 activity, because tissue ACE2 activity is completely unaffected by rACE2 in both ACE2 KO and WT mice.

Recombinant ACE2 Infusion for 2 Weeks

To examine whether a more prolonged human rACE2 infusion can produce a sustained effect on serum ACE2 activity, mice were infused with rACE2 (1 mg/kg per day) for 14 days. Because of the development of antibodies against human

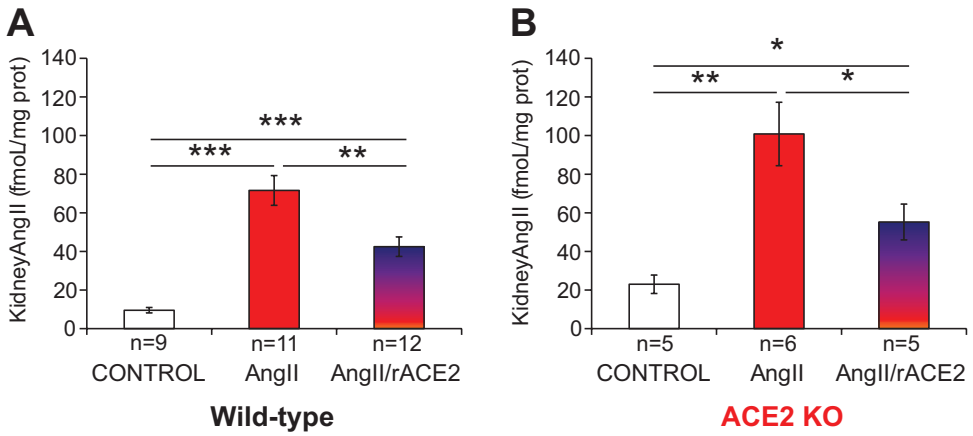

Figure 3. Kidney Ang II levels in WT (A) and ACE2 KO (B) mice. Mice were either sham-operated (control) or infused for 3 days with either Ang II (40 pmol/min) alone or simultaneously with Ang II and rACE2 (1 mg/kg per day). A, Kidney Ang II levels in WT mice receiving rACE2 concurrently with Ang II were significantly lower than in mice infused with Ang II only but were still higher than in controls not infused with Ang II. B, In ACE2 KO mice infused with Ang II, rACE2 administration resulted in a similar partial reduction in kidney Ang II as compared with ACE2 KO mice receiving Ang II alone; $^{*}P<0.05$, $^{**}P<0.005$, $^{***}P<0.001$.

rACE2 after 14 days of its administration, the initial increase in serum ACE2 activity was not sustained (Figure S5). Because of the observed immunogenicity effect of human rACE2 on mouse antibody titers and the attendant loss of serum ACE2 activity at 14 days, the maximal duration of infusion in this study was limited to the 3-day experiments described above, where there was no evidence of antihuman rACE2 antibodies.

Acute Studies in Anesthetized Mice

Effect of rACE2 on Blood Pressure

The effect of rACE2 on blood pressure was further examined in studies in anesthetized mice in response to a bolus of Ang II. Mice that were pretreated with rACE2 2 hours before Ang II infusion showed a marked increase in serum ACE2 activity when compared with animals pretreated the same way with PBS (21.7±1.2 versus 0.28±0.09 RFU/μL per hour, respectively; $P<0.001$). Baseline SBP in rACE2-infused mice, however, was not significantly different from mice not pretreated with rACE2 (111±4.4 versus 108±3.0 mm Hg, respectively; P value not significant), which is consistent with the 3-day rACE2 infusion protocol (see Figure 1).

Administration of a bolus of Ang II (n=14; time: 0 minutes in Figure 4A), resulted in a rapid increase in SBP. The SBP peak in the first minute was markedly higher than in the group of Ang II–infused animals pretreated with rACE2 (Δ_{SBP} 68.3±3.9 versus Δ_{SBP} 29.2±3.8 mm Hg; $P<0.05$). The difference in blood pressure between the 2 groups persisted throughout the continuous monitoring at 30-second intervals for 20 minutes (Figure 4A). After 20 minutes, the SBP remained significantly higher than baseline in the group infused with Ang II alone (Δ_{SBP}=19.7±3.4 mm Hg; $P<0.001$), whereas it was not significantly different from baseline in the group pretreated with rACE2 (Δ_{SBP}=−3.3±4.7 mm Hg; P value not significant). In similar experiments in a group of animals infused with Ang II and an ACE2 inhibitor (MLN-4760, 1 mg/kg), rACE2 failed to lower blood pressure such that the peak increase was not significantly different from that in mice infused with Ang II alone (Δ_{SBP} 81.8±6.4 versus Δ_{SBP} 73.8±7.8 mm Hg, respectively; P value not significant; Figure 4B).

SBP was unchanged by the Ang-(1-7) receptor blocker, A779, at 2 different concentrations (0.2 and 1.0 mg/kg) as compared with control (saline-infused) animals (Figure 5A). The administration of A779 also did not alter the peak increase in SBP caused by Ang II bolus as compared with mice that received bolus of Ang II only (Δ_{SBP}=62±6.5 versus Δ_{SBP}=63.6±4.5 mm Hg, respectively; P value not significant; Figure 5B).

In mice pretreated with rACE2 that received A779, the Ang II bolus produced a significantly lower peak increase in blood pressure than in mice pretreated with PBS (Δ_{SBP}=19.8±8.5 versus 62±6.5 mm Hg; $P<0.001$; Figure 5B). SBP returned to the baseline values within 5 minutes after Ang II administration and was kept at this level throughout the 20 minutes of observation (Figure 5B). Thus, the Mas receptor blocker, A779, did not further potentiate the increase in blood pressure induced by Ang II or interfere with the recovery from Ang II–induced hypertension.

To further examine any possible effect related to Ang-(1-7) on blood pressure, mice pretreated with PBS were administered a supraphysiologic dose of Ang-(1-7) (0.2 mg/kg) in addition to Ang II (Figure 5C). When blood pressure was measured in mice administered concomitantly with Ang-(1-7) and Ang II, there was an identical pattern of blood pressure as compared with that in animals given only the Ang II bolus (Figure 5C). The peak blood pressure increase induced by Ang II was similar in the presence and absence of Ang-(1-7) (Δ_{SBP}=79.4±9.0 versus 71.4±9.0 mm Hg, respectively). Moreover, Ang-(1-7) did not enhance the blood pressure recovery after Ang II bolus, which was incomplete in both groups as compared with baseline (Figure 5C). This suggests that, under these experimental conditions, any increases in Ang-(1-7) related to rACE2 infusion did not have a detectable effect on blood pressure.

Effect of rACE2 on Plasma Ang II and Ang-(1-7) Levels in Ang II–Infused Mice

Because in rACE2-infused mice SBP normalized within 5 minutes after Ang II bolus (see Figures 4 and 5), we chose

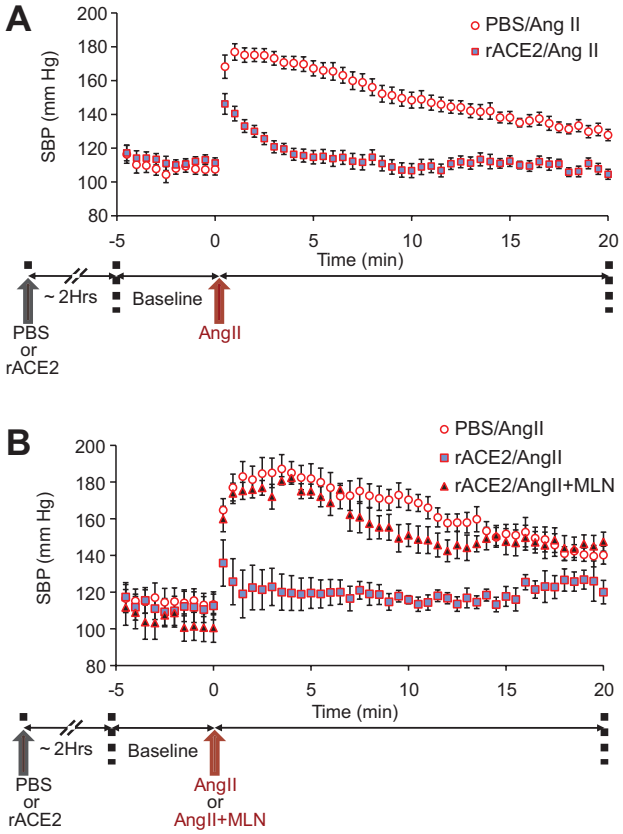

Figure 4. Continuous SBP measurement in anesthetized mice. Mice received PBS or rACE2 (1 mg/kg) in a single IP injection 2 hours before blood pressure measurement. After an IP bolus of Ang II (0.2 mg/kg) or Ang II (0.2 mg/kg) along with an ACE2 inhibitor, MLN-4760 (1 mg/kg; arrow, time point 0 minutes), SBP was recorded at 30-second intervals for the following 20 minutes. A, There was no difference in baseline SBP between rACE2 and PBS-infused animals before Ang II bolus. A bolus of Ang II to mice pretreated with PBS (n=14) was associated with a rapid increase in SBP. In mice administered with rACE2 before Ang II injection (n=11), the SBP increase was blunted and normalized within the first 5 minutes after Ang II injection. B, In a separate group of experiments, in mice pretreated with rACE2 (n=6) a bolus of Ang II (arrow) was associated with a blunted SBP increase as compared with mice pretreated with PBS (n=7) or mice that were rACE2-pretreated and received a bolus of Ang II with MLN-4760 (n=6).

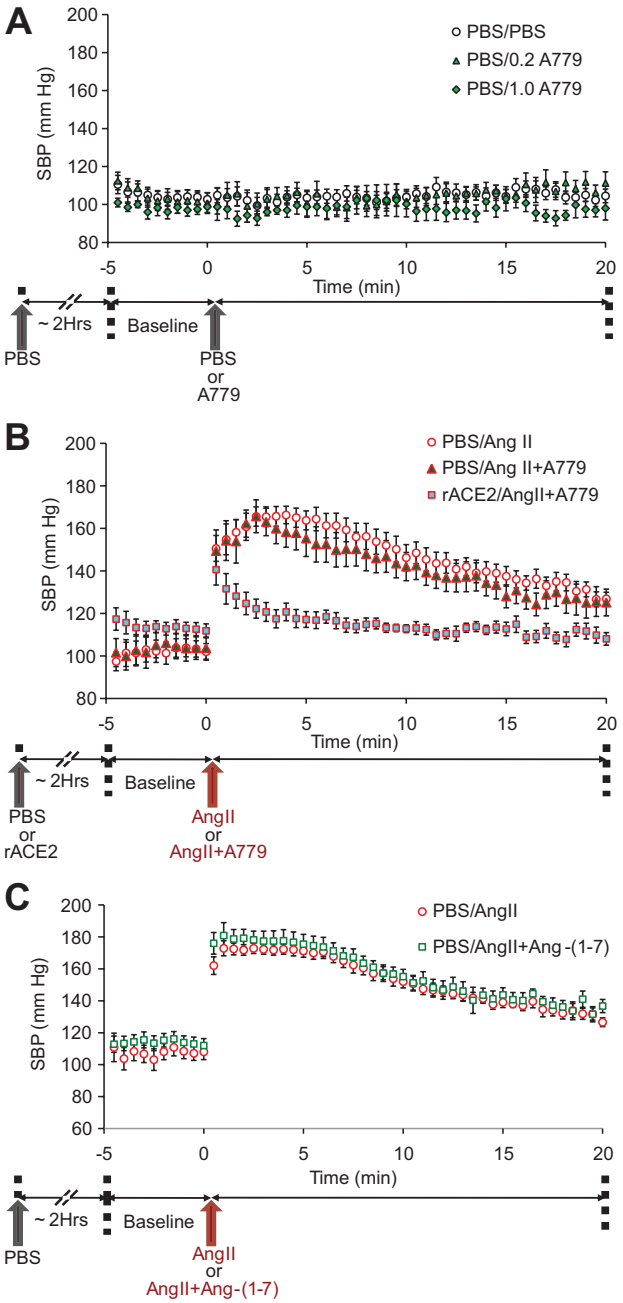

Figure 5. Mice received PBS or rACE2 (1 mg/kg) in a single IP injection 2 hours before blood pressure measurement. A, A saline IP bolus (PBS, time point: 0 minutes) did not alter SBP in anesthetized mice (n=8). An IP bolus of Mas receptor blocker, A779, at 2 doses (0.2 mg/kg; n=8) and (1.0 mg/kg; n=4) to mice pretreated with PBS was also not associated with an alteration in SBP. B, A bolus of Ang II (0.2 mg/kg, arrow) to mice pretreated with PBS (n=11) was associated with a rapid increase in SBP. In mice pretreated with PBS, the concomitant injection of Ang II and A779 (n=9) caused a similar SBP increase as in PBS pretreated mice injected with Ang II only. In mice administered with rACE2 (1 mg/kg IP) before a bolus of Ang II and A779 (n=9), the SBP increase was blunted and normalized within 5 minutes after Ang II injection. C, In PBS-pretreated mice (n=7) after IP injection of Ang II (0.2 mg/kg, arrow), SBP increased rapidly and remained elevated for the entire 20-minute period of the blood pressure measurements. The administration of Ang-(1-7) concurrently with Ang II (both peptides at the dose of 0.2 mg/kg; n=7) did not alter the pattern of blood pressure increase caused by Ang II administration.

this time point to examine plasma Ang peptides (Figure 6). In rACE2-pretreated mice (for 2 hours) plasma Ang II levels were markedly reduced as compared with animals infused with Ang II that had not received rACE2 (6.1±0.9 versus 76.7±39 fmol/mL, respectively; $P<0.001$). The concomitant administration of Ang II and A779 to mice pretreated with rACE2 did not affect plasma Ang II levels, which were similar to mice infused with Ang II and rACE2 (6.8±1.0 and 6.1±09 fmol/mL, respectively; Figure 6).

In the group pretreated with rACE2 and given a bolus of Ang II, plasma Ang-(1-7) levels were significantly higher than in the group infused with Ang II without previous rACE2 pretreatment (1.52±0.32 versus 0.74±0.08 pmol/mL; $P<0.05$). In the group also pretreated with rACE2 that received Ang II and the Mas receptor blocker, A779, plasma Ang-(1-7) increased further (1.93±0.44 and 1.52±0.32

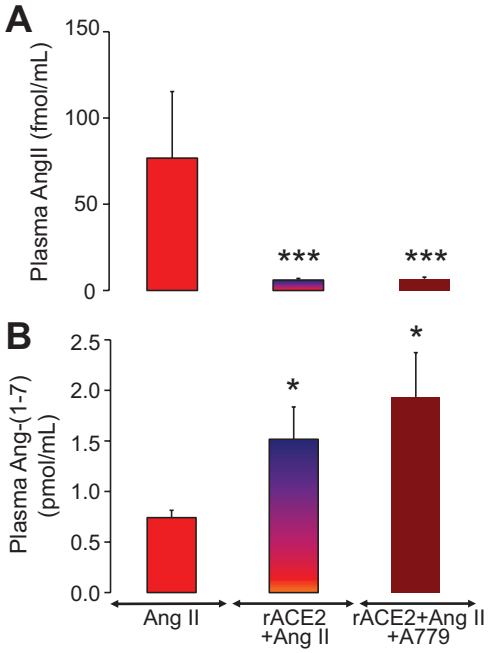

Figure 6. Plasma Ang II (A) and plasma Ang-(1-7) levels (B) measured 5 minutes after administration of Ang II bolus to anesthetized mice pretreated with either PBS or rACE2 in a manner similar to the groups described in Figures 4 and 5. The administration of Ang II alone (n=8) in mice pretreated with rACE2 was associated with markedly lower plasma Ang II levels and significantly increased plasma Ang-(1-7) levels as compared with mice pretreated with PBS (n=7). Injection of Ang II along with A779 (n=8) to mice pretreated with rACE2 resulted in a similar reduction in plasma Ang II and an increase in Ang-(1-7) levels; ***$P<0.001$, *$P<0.05$ vs Ang II only.

pmol/mL, respectively), although this difference did not reach statistical significance (see Figure 6).

Discussion

This study shows that the increase in blood pressure triggered by Ang II infusion can be completely prevented by the administration of soluble human rACE2. We used a highly purified soluble human rACE2 produced in the Chinese hamster ovary cell line, which has a calculated half-life in vivo of 8.5 hours (please see the supplementary Methods section). Our protocols involved acute studies in anesthetized animals and studies where rACE2 was given by osmotic minipumps for 3 days to conscious animals. Studies of long duration with human rACE2 administration were precluded because we found that mouse antihuman rACE2 antibodies developed over time, and this resulted in a decrease in serum ACE2 activity despite continued rACE2 infusion (please see the supplementary Results section).

As expected from the known effect of ACE2 on Ang II,[2,3] human rACE2 was shown in vitro to cleave a single amino acid phenylalanine from Ang II, which led to the formation of Ang-(1-7). Recombinant ACE2 also acted on Ang I to form Ang-(1-9), albeit with a much lower affinity than for Ang II to form Ang-(1-7) (Figures S1 and S2). We were able to show that plasma Ang II levels, after Ang II infusion, can be lowered, whereas Ang-(1-7) levels can be increased in mice infused with rACE2. This directly demonstrates the importance of ACE2 as a pathway in the degradation of Ang II in vivo because the plasma levels of Ang II were completely normalized after rACE2 administration (Figure 6).

Ang-(1-7), a peptide with vasodilatory properties,[16] is the only known product of ACE2-driven Ang II cleavage.[2,3] Increasing ACE2 activity could lower blood pressure, by lowering Ang II levels, by the generation of Ang-(1-7), or both. In the face of blockade of the Ang-(1-7) receptor with A779, the administration of rACE2 still completely abrogated the blood pressure increase caused by Ang II infusion and to the same extent as in the absence of A779 (Figures 2, 4, and 5). A significant increase in plasma Ang-(1-7) was seen in the acute studies, such that 5 minutes after a bolus of Ang II, plasma levels of Ang-(1-7) increased after rACE2 pretreatment (Figure 6). Neither the administration of A779 nor the administration of Ang-(1-7) at pharmacological doses had an effect on blood pressure (Figure 5).

This lack of effect of Ang-(1-7) on blood pressure should not be interpreted against the role of this peptide in circulatory control. This peptide has been shown to decrease resistance of peripheral blood vessels but also increases cardiac output.[28] The latter effect possibly counterbalances any blood pressure–lowering effect of Ang-(1-7) derived from its vasodilatory action. Our findings showing a lack of effect of Ang-(1-7) on reducing blood pressure are consistent with the results of long-term Ang-(1-7) infusion studies in a rat model of Ang II–dependent hypertension[24] but contrary to an earlier article in centrally denervated animals, where Ang-(1-7) appeared to have blood-lowering effect.[29] Because Ang-(1-7) significantly decreased cardiac fibrosis and prevented cardiac remodeling,[24] one may expect a potential benefit of increasing Ang-(1-7) by rACE2 administration from this action rather than from any blood pressure–lowering effect of this peptide.

Our data not only show that, during Ang II infusions, administration of rACE2 lowers blood pressure by a mechanism independent of Ang-(1-7), but also show that this blood pressure–lowering effect is attributable to an increase in circulating ACE2 activity. The specific ACE2 inhibitor, MLN-4760, completely abrogated the effect of rACE2 on Ang II–mediated hypertension, thereby demonstrating the ACE2 dependency of its effect on blood pressure during Ang II infusion. rACE2 had no effect on tissue ACE2 activity but effectively normalized plasma Ang II levels. This shows an effect on Ang II degradation attributable to an increase in circulating ACE2 activity. Moreover, plasma Ang II levels could not have been influenced by ACE-driven formation, because rACE2 did not result in changes in serum ACE activity (Figure S4).

It is noteworthy that rACE2 has only a partial effect on kidney Ang II levels (Figure 3) despite complete normalization of plasma Ang II levels (Figure 2). This shows that only the amount of Ang II that was circulating could be degraded by rACE2 present in serum, because at the kidney level there was no change in ACE2 activity after rACE2 infusions. The failure of rACE2 to increase tissue ACE2 activity was confirmed in ACE2 KO mice, where it should have been easier to demonstrate any detectable increases in ACE2 activity attributable to the lack of any baseline activity. The

soluble form of ACE2 differs from the full-length form in that it lacks the anchoring site, which normally would retain ACE2 on the cell plasma membrane.[30] This may explain, in part, why neither kidney nor heart ACE2 activity was increased with different doses of rACE2, whereas serum ACE2 activity increased markedly (Figure 1).

Regardless of the explanation for the lack of an effect of rACE2 on tissue ACE2 activity, the finding in itself sheds light on the mechanism of action of rACE2. We surmise, on the basis of this finding, that the therapeutic potential of rACE2 stems from its ability to increase serum ACE2 activity, which normally is very low, and, thus, enhanced degradation of circulating Ang II. Because the levels of this hormone are usually not elevated in normotensive mice, the administration of rACE2 would not be expected to have any major effect on blood pressure. In keeping with this prediction, blood pressure was unchanged by rACE2, even at very high doses (Figure 1). This is in contrast to its effect in Ang II–infused animals, where lowering of blood pressure was easily demonstrable when plasma Ang II levels were increased (Figures 2 and 6).

Amplification of ACE2 activity at the tissue level should have important effects that cannot be achieved by rACE2 administration, which has an action restricted to increasing serum ACE2 activity. In this regard, in a transgenic model of ACE2 overexpression in the vasculature of spontaneously hypertensive stroke-prone rats, blood pressure in this model of hypertension was reduced.[20] Other studies have also shown the potential of ACE2 amplification within the central nervous system in lowering blood pressure.[19,31] In addition, lentiviral overexpression of ACE2 reversed cardiac hypertrophy and fibrosis induced by Ang II in Sprague-Dawley rats.[32] The same group reported recently that chronic administration of xanthenone, a compound that has properties as ACE2 activator, reduced blood pressure and reversed myocardial fibrosis in the spontaneously hypertensive rat.[33]

Perspectives

Ang II–induced hypertension can be prevented by rACE2 as a result of ACE2-driven Ang II degradation within the circulation. It is possible that relative or absolute deficiency of ACE2 is involved in the pathogenesis of certain forms of hypertension by limiting the degradation of Ang II at certain tissues or systemically. Therapies aimed at amplifying ACE2 activity will hopefully be developed and will find its place in the management of forms of hypertension where Ang II may be overactive. Agents that increase ACE2 activity at the tissue level may be particularly suitable for conditions associated with local tissue ACE2 downregulation and overactivity of the renin-Ang system. Moreover, ACE2 amplification may provide a complement to existing therapies aimed at inhibiting Ang II formation and action, which are only partially effective in suppressing Ang II overactivity.

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Disclosures

M.S. has ownership in and receives income from Apeiron Biologics. He has Apeiron stocks and receives his salary from Apeiron as chief scientific officer of the company. H.L. has ownership in and receives income from Apeiron Biologics. He also has Apeiron stocks and receives compensation from Apeiron for chief executive officer activities. J.M.P. is founder of Apeiron Biologics and has Apeiron shares. D.B. has submitted an application for a patent titled, "Methods for Achieving a Protective ACE2 Expression Level to Treat Kidney Disease and Hypertension."

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Targeting the Degradation of Angiotensin II With Recombinant Angiotensin-Converting Enzyme 2: Prevention of Angiotensin II–Dependent Hypertension

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ONLINE SUPPLEMENT

Targeting the Degradation of Angiotensin II with Recombinant ACE2:

Prevention of Angiotensin II-dependent Hypertension

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EXPANDED MATERIALS AND METHODS

Production and characterization of soluble recombinant human ACE2

The cDNA encoding sequence for the soluble human ACE2 including its autologous signal sequence was cloned into a pIRES vector (Clontech). The extracellular domain of human rACE2 (amino acid residues 1-740) was expressed in stably transfected CHO-DHFR cells. Supernatant from cells maintained under protein-free conditions was harvested and the expression product was purified to homogeneity (>98% pure by size exclusion chromatography-HPLC; Zorbax GF250 column) by applying it sequentially to DEAE Sepharose, Source Q 15 anion exchanger and Superdex 200 resin. The purified rACE2 was compared to the commercially available ACE2 standard (R&D Systems) using a cleavage reaction of a fluorogenic substrate specific for ACE2 [Mca-APK(Dnp)-OH]. The calculated catalytic activity of the purified rACE2 was at the level of 106±7% of the commercial standard (range 94-117%).

As noted in the results section, the enzymatic properties of rACE2 were evaluated by its ability to cleave Ang II and Ang I (Figure S1A and S1B). The substrates and the cleavage products were identified and measured quantitatively by peak integration in reverse phase HPLC (Waters C18 μBondapak, 2.1x300 mm, 10 um, 125 Å, 10-60% CH_3CN in 20 min for Ang II; μRPC C2/C18 column, Amersham; 10-80% CH3CN in 20 min for Ang I). Reaction conditions were: 50 uM substrate (Ang II or Ang I) and 500 ng/mL enzyme in 50 mM MES, 300 mM NaCl, 10 uM $ZnCl_2$ and 0.01% Brij-30, pH 6.5. Cleavage reaction was stopped after 10-60 minutes by the addition of 100 mM EDTA (final concentration).

To examine whether the processing of Ang II and Ang I by rACE2 is inhibited by a specific ACE2 inhibitor (MLN-4760, Millennium Pharmaceuticals), rACE2 was incubated with 10 nmol/L of Ang II or Ang I (Sigma-Aldrich) in the presence or absence of MLN-4760 (1 umol/L) at 37°C for 8 hours (Figure S2A and S2B). Angiotensin II and Ang I were measured with an antibody-based method using commercially available EIA kits (SPIBio or Peninsula Laboratories, respectively).

Pharmacokinetics of human recombinant ACE2

To study the pharmacokinetics of rACE2, a bolus of rACE2 was administered i.p. to *Balb/c* mice at two different doses (250 ug/kg and 2.5 mg/kg). Resorption kinetics were monitored during 30 hours following the bolus injection by measuring serum ACE2 activity. Maximum values of rACE2 activity in the serum (corresponding to 18 ug/mL rACE2 concentration) were observed eight hours after bolus injection and decreased steadily during the following 22 hours until the end of the experiment. The calculated half-time of intraperitoneally administered rACE2 in mice was 8.5 hours.

Measurements of plasma and whole kidney Angiotensin II and Angiotensin-(1-7)

Blood samples were collected in tubes kept on ice containing ethylenediamine tetraacetic acid (25mM), o-phenanthroline (0.44mM), pepstatin A (0.12mM), and p-hydroxymercuribenzoic acid

(1mM), and then centrifuged (3000 g)[1]. The plasma was saved and stored at -80°C until further processing.
Kidneys were homogenized on ice-cold methanol in the presence of protease inhibitors [50 mmol/L ethylenediaminetetraacetic acid, 0.5-mmol/L *o*-phenanthroline, 1 mmol/L N-ethylmalemide, and 0.1 mmol/L pepstatin A][2]. A small sample of the homogenate was used to determine total protein content, using the BCA Protein Assay (Pierce, Rockford, IL). The homogenate was centrifuged at 16,000 rpm for 30 min, and the supernatant evaporated to dryness and stored at -80°C[3].
Angiotensin peptides were extracted from plasma and kidney homogenates using reverse phase phenyl silica columns (100 mg; Amprep Phenyl PH, Amersham Biosciences, Buckinghamshire, UK) as per manufacturer's instructions. The quantity of Ang II in the extract was determined using an EIA kit (SPIBio, Cayman Chemical, Ann Arbor, USA), as per manufacturer's instructions. The levels of plasma Ang-(1-7) were measured by radioimmunoassay (Hypertension Core Lab, Wake Forest University School of Medicine) [1]. Results were reported in fmol/mL or pmol/mL (plasma) and in fmol/mg or pmol/mg protein (kidney).

Determination of plasma anti-human rACE2 antibodies

To detect human rACE2-specific antibody, plasma samples from mice infused for three and 14 days with rACE2 at a dose of 1 mg/kg/day via minipumps were analyzed by an antigen based ELISA. Human rACE2 was diluted in coating buffer (NBK) at a concentration 10 µg/mL and coated onto Maxisorp® (NUNC) plates. Remaining active groups on the plate were blocked by incubation with 3% skim milk (Difco) in PBS. Mouse plasma was then added to the wells starting at a 1:10 dilution and further diluted serially 1:4. The dilutions were made in PBS supplemented with 0.5 % NP40. Mouse antibodies bound to ACE2 on the plate were detected using a goat anti-mouse IgG peroxidase labeled antibody (Zymed; 1:1000 in PBS supplemented with 2% skim milk powder). Detection was performed using ortho-phenylene diamine (OPD, Sigma) using H_2O_2 as a substrate according to the manufacturer's instructions. Absorbance was measured at 492 nm using as a reference wavelength 620 nm. Quantification was performed by comparison to a curve obtained with monoclonal mouse anti-ACE2 antibody at 1 µg/mL.

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ADDITIONAL FIGURES AND SUPPORTING INFORMATION

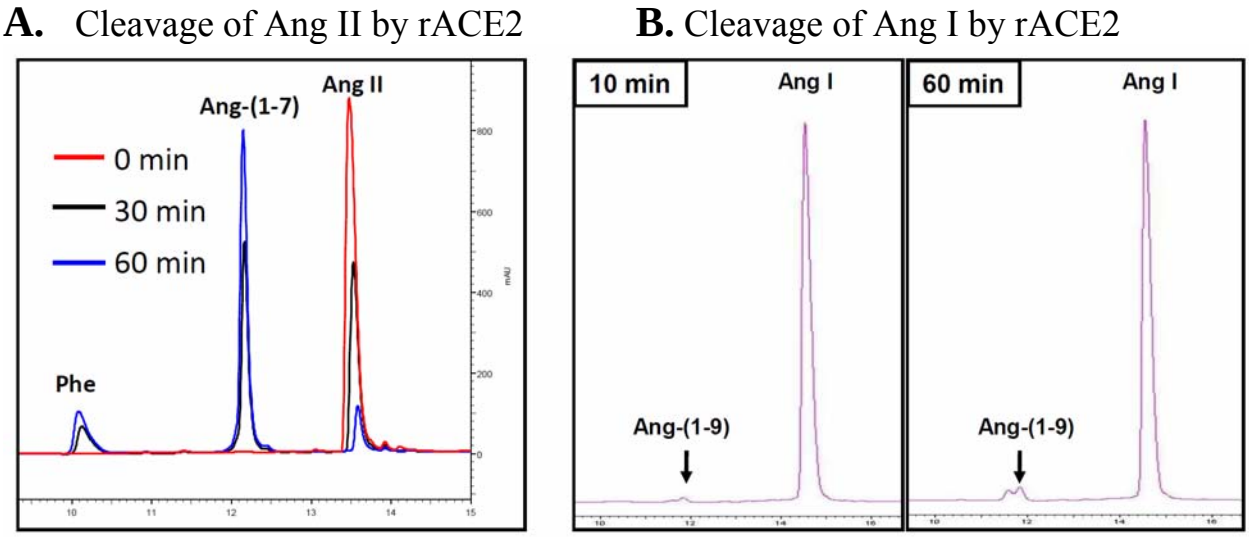

Figure S1. The *in vitro* cleavage of Ang II and Ang I by human rACE2 assessed by reverse-phase high performance liquid chromatography (RP-HPLC).

(Panel **A**) The cleavage of Ang II by human rACE2 was assessed by incubation of Ang II with rACE2 for 0, 30 and 60 min and a subsequent RP-HPLC analysis. The highest peak at 13.5 min elution time (on red) represents Ang II at the starting point of the reaction. A reduced Ang II peak is seen at 30 min (black) and 60 min (blue) consistent with a robust cleavage of Ang II by rACE2. The reaction products after 30 min and 60 min of incubation with human rACE2 show an emerging peak at 12.2 min elution time which corresponds to Ang-(1-7). A shallow, wide peak at 10.1 min elution time represents another breakdown product of Ang II, the single amino acid phenylalanine (Phe).

(Panel **B**). Angiotensin I cleavage by human rACE2.
(Left insert) After 10 minutes of incubation with rACE2, Ang I appears as a high sharp peak at a retention time of 15 min and Ang 1-9 appears as a small double peak at a retention time of 12 min (arrow).
(Right insert) Sixty minutes into the incubation with rACE2, the peak for Ang-(1-9) increases only modestly at the same time as Ang I peak decreases slightly as compared to 10 min incubation.
The above findings are consistent with a much lower catalytic efficiency of rACE2 for Ang I than with Ang II as a substrate.

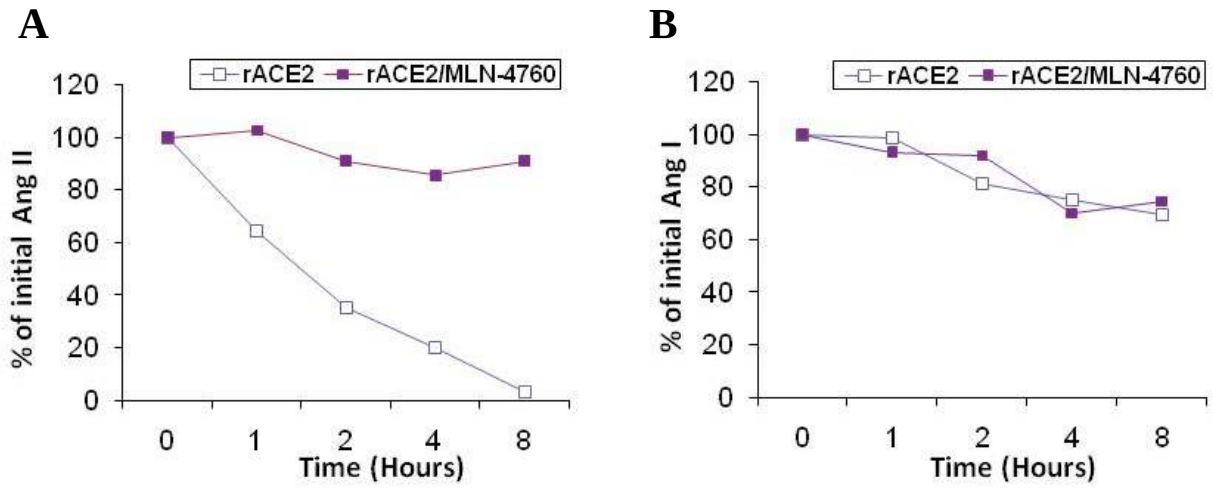

Figure S2. Disappearance of Ang II (Panel **A**) and Ang I (Panel **B**) during incubation of rACE2 (0.2 ng) with 10 nmol/L of Ang II or Ang I in the presence (filled squares) or absence (empty squares) of 1 μmol/L MLN-4760 (a specific ACE2 inhibitor).

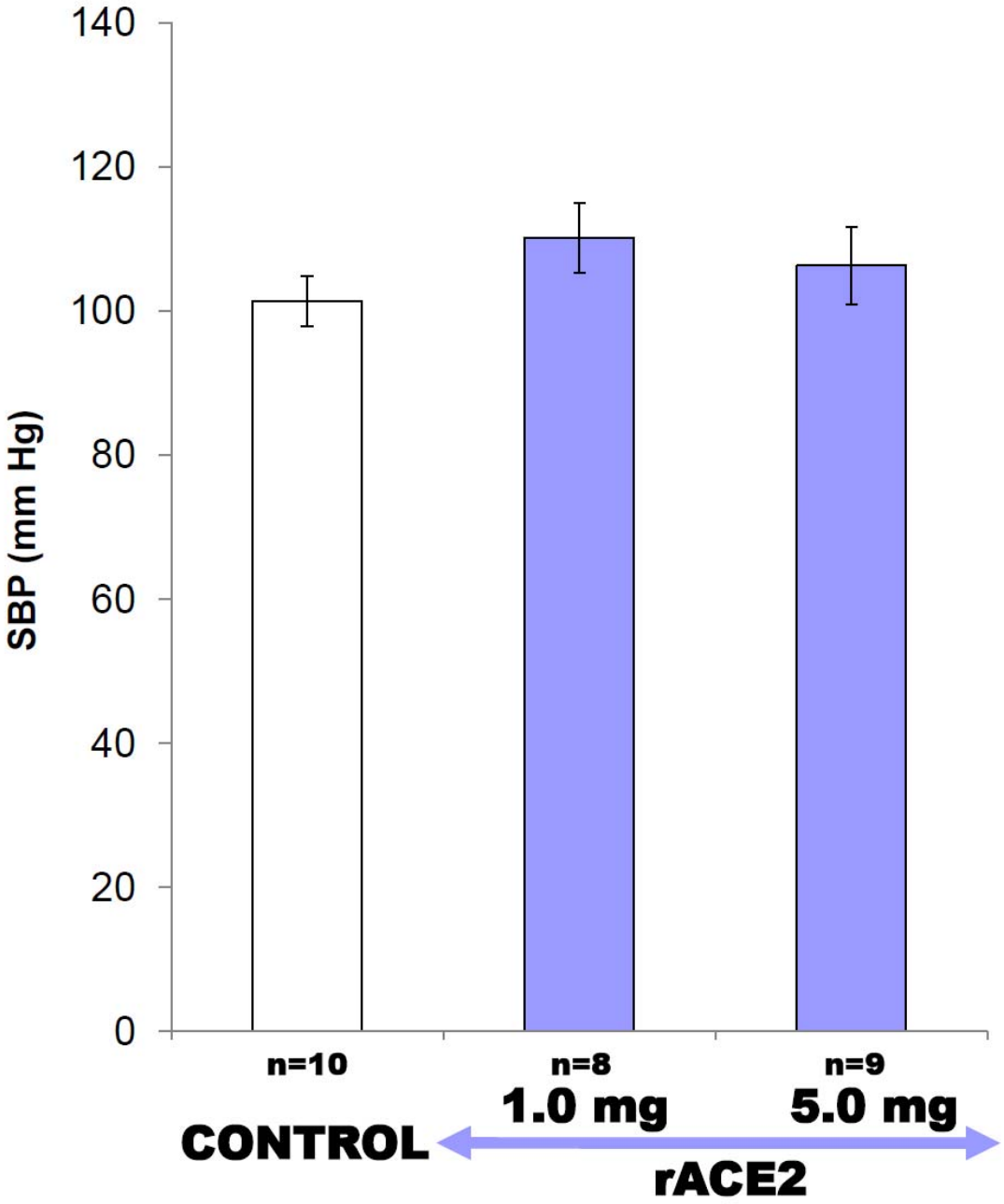

Figure S3. Systolic blood pressure measured under anesthesia in a subgroup of mice depicted in Figure 1 of the Results section. Systolic blood pressure in the control group (empty bar) did not differ significantly from mice infused for 3 days with rACE2 at doses 1 and 5 mg/kg/d (rACE2, filled bars).

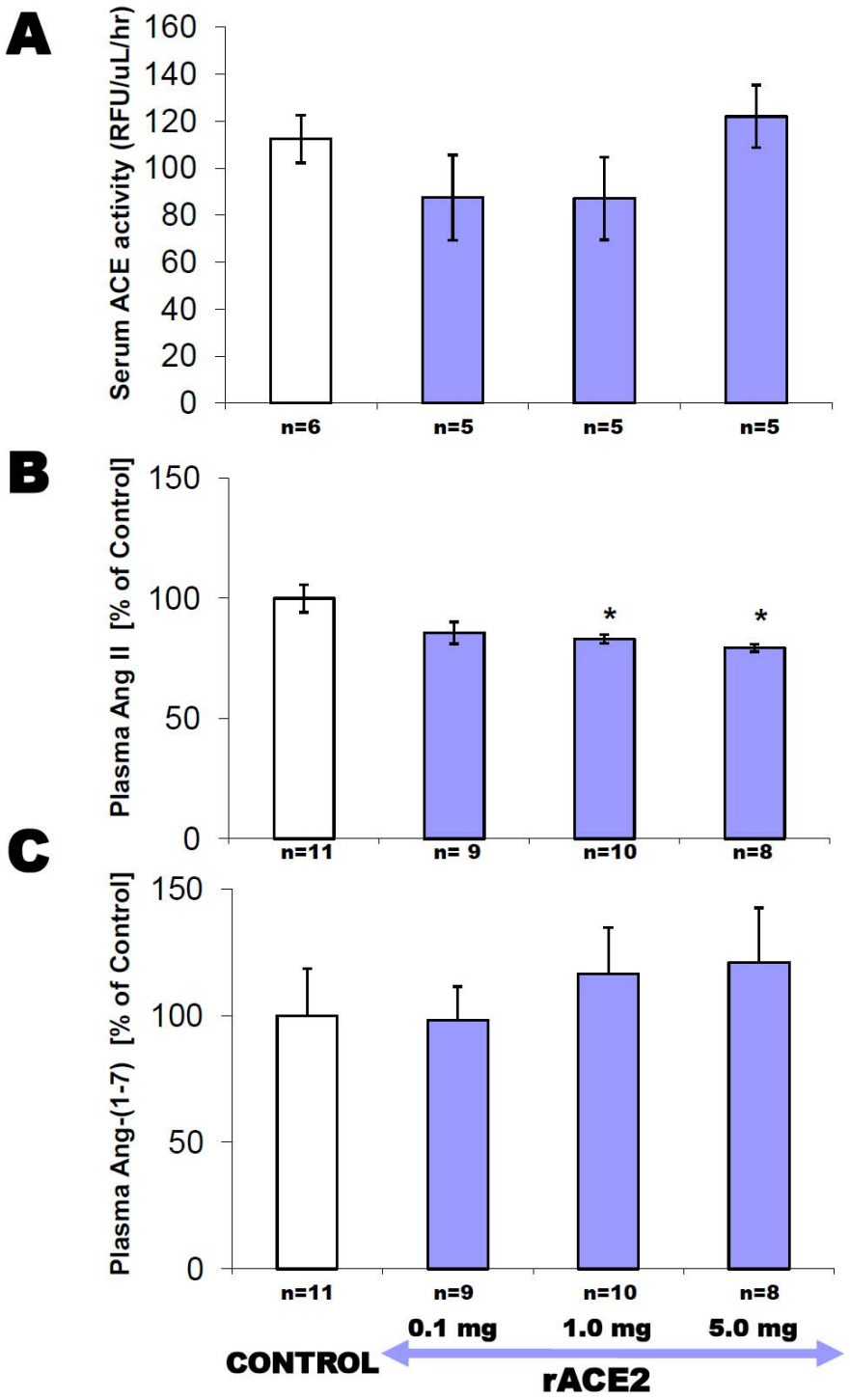

Figure S4. Serum ACE activity (Panel **A**) and plasma Ang II (Panel **B**) and plasma Ang-(1-7) levels (Panel **C**) in sham-operated mice (empty bar) and mice infused for 3 days subcutaneously with human recombinant ACE2 at doses 0.1, 1 or 5 mg/kg/d (rACE2, filled bars); *$p<0.05$ vs. sham controls.

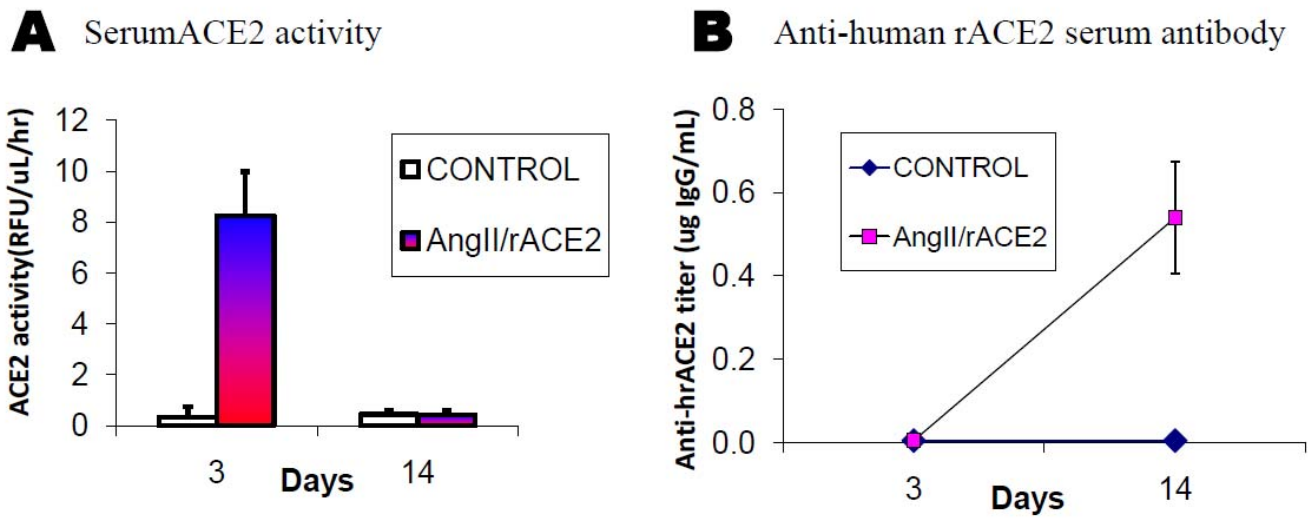

Figure S5. Serum ACE2 activity (Panel A) and anti-rACE2 antibodies (Panel B) in chronic studies with rACE2 infusion for three and fourteen days.

(Panel **A**) To examine whether a prolonged human rACE2 infusion can produce a sustained effect on serum ACE2 activity, mice were infused with rACE2 (1mg/kg/day) concurrently with Ang II (40 pmol/min) for three and 14 days. In mice infused with RACE2 and Ang II for three days, serum ACE2 activity increased markedly. In contrast to the 3 days infusion, serum ACE2 activity in mice infused with rACE2 and Ang II for 14 days (0.42±0.17 RFU/uL/hr) was as low as in sham operated control mice (0.44±0.17 RFU/uL/hr). The lack of a sustained effect of rACE2 on serum ACE2 activity when given to mice for 14 days suggested that neutralizing antibodies against human rACE2 had been formed thereby preventing a sustained increase in ACE2 activity.
(Panel **B**) ACE2 antibody titers were undetectable in sham operated controls and in mice infused for 3 days with human rACE2 and Ang II. In mice infused with human rACE2 and Ang II for 14 days, antibody titers were increased (0.54±0.13 ug/mL anti-hrACE2 IgG) as compared to sham operated controls (below the detection level).