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Murine Recombinant ACE2: Effect on Angiotensin II Dependent Hypertension and Distinctive ACE2 Inhibitor Characteristics on rodent and human ACE2

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Abstract

A newly produced murine recombinant ACE2 was characterized *in vivo* and *in vitro*. The effects of available ACE2 inhibitors (MLN-4760 and two conformational variants of DX600 –linear and cyclic) were also examined. When murine ACE2 was given to mice for 4 weeks, a marked increase in serum ACE2 activity was sustainable. In acute studies, mouse ACE2 (1mg/kg) obliterated hypertension induced by angiotensin II infusion by rapidly decreasing plasma angiotensin II. These effects were blocked by MLN-4760 but not by either form of DX600. *In vitro*, conversion from angiotensin II to angiotensin-(1–7) by mouse ACE2 was blocked by MLN-4760 (10^{-6}M) but not by either form of DX600 (10^{-5}M). Quantitative analysis of multiple angiotensin peptides in plasma *ex vivo* revealed formation of angiotensin-(1–9) from angiotensin I by human but not by mouse ACE2. Both human and mouse ACE2 led to the dissipation of angiotensin II with formation of angiotensin-(1–7). By contrast, mouse ACE2-driven angiotensin-(1–7) formation from angiotensin II was blocked by MLN-4760 but not by either linear or cyclic DX600.

In conclusion, sustained elevations in serum ACE2 activity can be accomplished with murine ACE2 administration thereby providing a strategy for ACE2 amplification in chronic studies using rodent models of hypertension and cardiovascular disease. Human, but not mouse ACE2, degrades angiotensin I to form angiotensin-(1–9). There are also species differences regarding rodent and human ACE2 inhibition by known inhibitors such that MLN-4760 inhibits both human and mouse ACE2 whereas DX600 only blocks human ACE2 activity.

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Disclosure Statement

Daniel Batlle has a pending patent application entitled “Methods for achieving a protective ACE2 expression level to treat kidney disease and hypertension”.

Manfred Schuster — ownership and income in Apeiron Biologics. Dr. Schuster has Apeiron stocks and receives his salary from Apeiron as COO of the company.

Marko Poglitsch receives his salary from Apeiron Biologics.

Keywords

ACE2; Recombinant; Mouse; Human; Hypertension; Angiotensin; Inhibitor

INTRODUCTION

Current therapeutic approaches to decrease angiotensin II activity are based on reducing its formation using either ACE inhibitors or renin inhibitors, or inhibiting its action using AT1 receptor antagonists[1]. Angiotensin-converting enzyme 2 (ACE2) can effectively degrade angiotensin (Ang) II[2, 3] and therefore may provide a new potential strategy to reduce Ang II activity[2, 4, 5, 6]. The potential therapeutic value of ACE2 activity amplification has been suggested for hypertension[7–9], cardiovascular disease[8, 10–13], pulmonary hypertension[14] and diabetic kidney disease[15–18]. This potential therapeutic effect of ACE2 amplification, however, can only be inferred from studies overexpressing this enzyme via adenovirus delivery[19] or using a brain-targeted ACE2 transgene driven by a synapsin promoter[20] but has been limited by the lack of pharmacological tools to amplify ACE2 activity in a sustained manner.

The availability of a human form of recombinant (r) ACE2 allowed us to demonstrate its value in acute studies in mice but is of limited value for chronic studies in rodent models because of the development of neutralizing antibodies that decrease ACE2 activity after two weeks[7]. Therefore, we reasoned that the production and characterization of mouse ACE2, which should not elicit formation of neutralizing antibodies when given to mice, would be helpful to examine its potential therapeutic value in chronic studies using rodent models. In this study, we report the production of mrACE2 and the characterization of its effects in vitro as well as when infused to mice acutely and chronically.

Enzymatic properties of ACE2, such as substrate hydrolysis and specificity towards enzymatic inhibitors have only been tested on purified human recombinant ACE2[2–5, 7, 21], crude tissue extracts[22–24] or cultured cells[25]. Just recently, crude extracts from mouse ACE2 over-expressing cells has been employed[26]. Since cell culture extracts may contain contaminants of multiple other enzymes that are specific to the host cell used, results of such studies need to be confirmed and expanded using highly purified rACE2 protein from mouse.

Previous studies have examined the hydrolysis of various peptides including Ang II, Ang I, apelin, dynorphin A, des- Arg bradykinin, and ghrelin by human rACE2[2, 3,4, 9]. In the present study, we concentrated on the analysis of Ang II and Ang I degradation by mouse ACE2 because of the key involvement of these peptides within the renin-angiotensin system (RAS). Previous animal studies have provided conflicting results regarding substrate preference of the ACE2 enzyme[22, 23]. For instance, studies using human recombinant ACE2[2, 4, 5, 7] or unpurified ACE2 from sheep tissue lysates[23] suggested that ACE2 cleaves mainly Ang II, whereas others suggested that in rat circulation[27] and proximal tubules[22] ACE2 largely hydrolyzes Ang I but has no effect on Ang II hydrolysis. In this study, we examined the effect of mouse and human rACE2 on the hydrolysis of both Ang II and Ang I, the two main peptides within the RAS that are cleaved by ACE2.

In the process of our work with murine rACE2 we found striking species differences between the two currently used ACE2 inhibitors (MLN-4760 and DX600) that we think are critically relevant to the proper interpretation of studies with these inhibitors. Accordingly, we examined the ability of MLN-4760 and two conformational variants of DX600 (a linear and a disulfide bridged cyclic from) to inhibit human and mouse ACE2 *in vitro* and using a

novel ***ex vivo*** approach to concomitantly measure several peptides within the RAS system in plasma. The effect of mrACE2 on blood pressure and Ang II plasma levels in the presence and in the absence of these ACE2 inhibitors was also studied in mice.

METHODS

Design of peptide tags and amplification of cDNA for soluble mouse ACE2

Gene-specific primers were designed based on the published mouse *ACE2* sequence (GenBank accession no. NM_001130513). *BamHI* and *NotI* restriction sites were placed on N- and C-terminus, respectively. Primers containing those restriction sites were used to amplify cDNA of soluble ACE2 (amino acid residues 1–740) from mouse kidney cDNA library (Clontech, Palo Alto, CA) which was accomplished by using KOD DNA polymerase (Novagen). The cDNA encoding sequence for soluble mouse ACE2 was cloned into *BamHI* and *NotI* sites of the pcDNA6V5/His and pcDNA4 Max vectors (Invitrogen, Carlsbad, CA). The cDNA sequence was verified by DNA sequence analysis on a 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Large Scale Expression and Purification of soluble mouse ACE2

For large-scale production, the extracellular domain of mouse rACE2 (amino acid residues 1–740) with C-terminal 10-His tag was expressed in transiently transfected FreeStyle™ 293F cells (Invitrogen Custom Services). Supernatant from cells maintained under protein-free conditions was harvested and the expression product was purified to high purity using a 3-step enrichment process. Conditioned media (25 L) were first subjected to tangential flow filtration for concentration and simultaneous buffer exchange against PBS. ACE2 protein was then enriched by affinity chromatography using Ni-Sepharose followed by size-exclusion chromatography (Superdex-300). Protein containing fractions were analyzed by SDS-PAGE followed by Sypro Red staining and Western blotting (anti-c-His antibody; Invitrogen) and (anti-mouse ACE2 antibody; R&D Systems). Protein pool was subjected to concentration using Millipore concentrators 30K MWCO. Protein estimation was performed by the Bradford's method, using BSA as a standard.

ACE2 Activity Measurements

ACE2 activity was determined following incubation with the intramolecularly quenched synthetic ACE2-specific substrate Mca-APK-Dnp (Anaspec). The measurements were performed in black microtiter plates with a 100 ul total volume. Briefly, 2ng rACE2, 2uL serum, 1 ug of mouse total protein or 4 ug of rat total protein from renal cortex homogenate was added to wells containing a buffer (50 mmol/l 4-morpholineethanesulfonic acid, 300 mmol/l NaCl, 10 umol/l $ZnCl_2$, and 0.01% Triton-X-100, pH 6.5), containing EDTA-free tablets (Roche) and 10^{-5}M substrate. Reactions were in duplicates (one of two wells constituted a blank). Blank wells contained the same components, but 10^{-5}M of a specific ACE2 inhibitor, MLN-4760, (a gift from Millenium Pharmaceuticals, Cambridge, MA; currently licensed by Ore Pharmaceuticlas under the name Ore 1001) was also added.

For ACE2 activity measurements *in* kidney cortex from mice that received an injection of MLN-4760 or DX600 (1ug/kg), homogenates were assayed in as a low dilution as possible (1/2 dilution − 20 uL homogenate in 40 uL total volume/well). This was necessary because we have established in pilot studies that after ip injections the inhibitory effect of MLN-4760 on kidney tissue is markedly decreased with dilution. After incubation at ambient temperature for 30 min (kidney cortex from mice receiving ACE2 inhibitors), 1 hr (kidney cortex, serum of mice infused with rACE2) or 24 hr (serum of mice not infused with rACE2), fluorescence was measured using an FLX800 microplate fluorescence reader (BIOTEK Instruments Inc., Winooski, VT, USA) at 320 nm excitation and 420 nm emission

wavelength. In mice injected with mrACE2, we found out that serum activity plateaues long before the 24 hour measurement time point. In mrACE2 infused mice, measurements at 24 hours, when the substrate is depleted, were avoided because ACE2 activity would have been underestimated. Accordingly, serum samples from rACE2-infused mice we measured at an early time point (1 hour).

Total fluorescence was corrected for volume (in serum samples) or protein content (in kidney homogenates) after subtracting blank values. For inhibition curves, MLN-4760 and DX600 [two conformational forms: linear (L-DX600, Phoenix Pharmaceuticals) or disulfide bridged cyclic variant (Bachem, C-DX600)] were incubated with rACE2 or kidney lysates in dilutions from 10^{-5}M to 10^{-11}M.

To examine the effect of redox state modification on inhibitory properties of L-DX600 or C-DX600 against ACE2, both conformational forms of DX600 were pre-incubated in either buffer or in 1% 2-mercaptoethanol for 2 hours. Afterwards, the inhibitors were added to triplicate wells (10^{-5}M end-concentration) containing either hrACE2 or mrACE2 (2ng/well) and fluorescence was measured and compared to wells without DX600.

Measurements of Plasma Ang II and Ang-(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)[28]. The plasma was saved and stored at −80°C until further processing.

Angiotensin peptides were extracted from plasma 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. Results were reported in fmol/mL plasma. The levels of plasma Ang-(1–7) were measured by radioimmunoassay (Hypertension Core Lab, Wake Forest University School of Medicine).

Inhibitor and Substrate Specificity of Mouse and Human Recombinant ACE2

Human rACE2 is known to hydrolyze both Ang II and Ang I[2]. Enzymatic properties of mrACE2 were therefore evaluated by its ability to cleave these two natural ACE2 substrates using two approaches. One approach involved a new technique called "RAS-fingerprinting" as described below. The other approach was based on the disappearance of exogenous Ang II and Ang I over time measured quantitatively with an antibody-based method using commercially available EIA kits. Reaction conditions were: for Ang II (10^{-9}M) degradation, 0.2 nM rACE2; for Ang I degradation (10^{-8}M), 40.0 nM rACE2, in PBS pH 7.4 supplemented with 10^{-5}M $ZnCl_2$. Cleavage reaction was stopped after different periods of time (1–24 hours) by the addition of 10^{-3} M EDTA (final concentration). To examine whether the cleavage of Ang II and Ang I by rACE2 is inhibited by two specific ACE2 inhibitors, MLN-4760 and DX600, mouse and human rACE2 were incubated with Ang II (10^{-9}M) or Ang I (10^{-8}M) (Sigma-Aldrich) in the presence or absence of MLN-4760 (10^{-6}M) or DX600 (10^{-5}M and 10^{-6}M) at 37°C for up to 24 hours. Ang II and Ang I were measured quantitatively using commercially available EIA kits (SPIBio, Cayman Chemicals and Bachem, respectively). Ang-(1–7) formed from Ang II after rACE2 was measured at the latest time of incubation of rACE2 with Ang II because at that time point accumulation of the generated Ang-(1–7) was expected to be the highest and thus more easily measurable by the EIA assay (Bachem) used for the experiments.

Liquid chromatography–tandem mass spectrometry (LC-MS/MS) of angiotensin peptides

In blood plasma, all RAS enzymes, except renin, are present in excess compared to their substrates. Therefore 100pg/ml of recombinant renin (Sigma) was added to isolated plasma, as recently described[29]. Then plasma was divided into smaller samples and mrACE2 or hrACE2 (both-at a final concentration 5ug/mL) was added to the samples. After 10 min. incubation at 37°C, in the presence or absence of L-DX600, C-DX600 or MLN-4760 (all at concentrations 10^{-7} – 10^{-5}M), protease inhibitor cocktail was added to stop the reaction and samples were subjected to LC-MS/MS analysis.

Plasma samples were spiked with 100pg/ml stable isotope-labeled internal standards and subjected to solid phase extraction using Sep-Pak cartridges (Waters) as recently described[29]. Following elution and solvent evaporation, samples were reconstituted in 50μl 50% acetonitrile/0.1% formic acid and subjected to LC-MS/MS analysis using a reversed phase analytical column (Luna C18, Phenomenex) using a gradient ranging from 10% acetonitrile/0.1% formic acid to 70% acetonitrile/0.1% formic acid in 9 minutes. The eluate was analyzed in line with a QTRAP-4000 mass spectrometer (AB Sciex) operated in the MRM mode using dwell times of 25 msec at a cone voltage of 4000 volts and a source temperature of 300°C. For each peptide and corresponding internal standards, two different mass transitions were measured[29]. Angiotensin peptide concentrations were calculated by relating endogenous peptide signals to internal standard signals provided that integrated signals achieved a signal-to-noise ratio above 10. The quantification limits for individual peptides were found to range between 1pg/ml and 5pg/ml undiluted plasma. Ten angiotensin peptides were simultaneously evaluated by this method: Ang I, Ang-(1–9), Ang II, Ang III, Ang IV, Ang-(1–7), Ang-(1–5), Ang-(2–7), Ang-(3–7), Ang-(2–10)[29].

ANIMAL STUDIES

Acute studies using mrACE2 and ACE2 inhibitors

Time course and Dose Response Studies of mrACE2—All of these studies were approved by the Northwestern University Animal Care and Use Committee. C57BL mice received mrACE2 in a single i.p. bolus (1.0mg/kg, n=3) and blood samples were collected at 15, 30, 60 min and 2 hours after mrACE2 injection. A separate group of mice received mrACE2 in a single i.p. bolus (0.2mg/kg, n=5) and blood samples were collected at 2, 4, 6 and 12 and 24 hours after mrACE2 injection. Serum ACE2 activity was measured from samples taken at different time points from tail vein bleeds.

To study dose response of mrACE2, a single i.p. injection of mrACE2 (at three different doses 0.01mg/kg, n=2; 0.1mg/kg, n=3; 1.0mg/kg, n=4) or PBS (n=3) was performed 2 hours before blood samples collection from the tail vein. Serum ACE2 activity was measured, as outlined above.

The Effect of rACE2 on Blood Pressure and Angiotensin II Plasma Levels— Blood pressure was measured non-invasively in anesthetized mice by determining the tail blood volume with a volume-pressure recording (VPR) sensor and an occlusion tail-cuff using a computerized system (CODA System, Kent Scientific, Torrington, CT), as previously described[7]. The VPR recording system has been validated and provides a high correlation with telemetry and direct arterial blood pressure measurements[30]. We chose the non-invasive VPR blood pressure recording to be able to acquire continuous BP data in a large number of animals from various experimental groups at the same time.

To study the acute effect of mrACE2 on systolic blood pressure (SBP), male *C57BL/6J* mice of 10–15 weeks of age were anesthetized with an i.p. ketamine injection (200 mg/kg BW). Two hours before anesthesia, mice were pre-treated with an i.p. injection of either sterile

PBS (0.4 mL) or mrACE2 at two doses (0.1 and 1mg/kg in 0.4 mL PBS). Immediately after inducing anesthesia, mice were placed on a heating platform for 10 minutes. Systolic BP was monitored non-invasively every 30 sec for a period of 25 minutes. After 5 minutes of baseline SBP recording, acute hypertension in anesthetized mice was induced with an i.p. bolus of Ang II (0.2 mg/kg) and the SBP was monitored for the remaining 20 minutes. In additional experiments, Ang II (0.2mg/kg) was infused together with an ACE2 inhibitor (either MLN-4760 or two conformational variants of DX600) (1mg/kg) following rACE2 infusion two hours earlier, as described above. In a set of mice under ketamine anesthesia (pre-treated with PBS or rACE2 as above), 5 min after Ang II injection euthanasia was performed by cervical dislocation and blood was rapidly drawn by cardiac puncture for measurements of serum ACE2 activity and plasma Ang II levels.

Effect of MLN-4760 and DX600 on Serum and Kidney ACE2 activity and Ang-(1–7) Levels—To study the acute effect of MLN-4760 and the two isoforms of DX600 on serum and kidney ACE2 activity, male *C57BL/6J* mice at 10–15 weeks of age were anesthetized with an i.p. ketamine injection (200 mg/kg BW). One hour before anesthesia, mice received an i.p. injection of sterile PBS (0.4 mL), MLN-4760 or either C-DX600 or L-DX600 (all at 1mg/kg in 0.4 mL PBS). Immediately after inducing anesthesia, euthanasia was performed by cervical dislocation and blood was rapidly drawn by cardiac puncture and kidneys removed for measurements of serum and kidney ACE2 activity and plasma Ang-(1–7) levels[28].

Chronic Studies using rACE2

A group of male mice (FVB) of 10 weeks of age was given mrACE2 (at a dose or 0.5 mg/kg per day) or hrACE2 (0.1 mg/kg per day) for 28 days using osmotic minipumps (ALZET). Another group of mice received mrACE2 (0.2 mg/kg per day) every day by intraperitoneal injections for 28 days.

Determination of plasma anti-rACE2 antibody levels—Details included in the Data Supplement.

Statistical analysis

Statistical analysis of two independent groups was performed using unpaired t-test. When more than two independent means were compared, ANOVA was used and followed by Bonferroni multiple comparison test. Significance was defined as $p<0.05$. Data were expressed as mean+/−SEM.

RESULTS

Large Scale Expression and Purification of Soluble Mouse ACE2

Please see Online Data Supplement (Table S1, Figure S1).

In Vitro Studies on the effects of ACE2 inhibitors on purified murine rACE2

A synthetic intramolecularly quenched fluorogenic Mca-APK(Dnp) substrate, which is hydrolyzed by ACE2[4], was used to test the effect of ACE2 inhibitors (MLN-4760[31], L-DX600[32], C-DX600) on enzyme activity of purified recombinant mouse and human ACE2 using dose-response curves. MLN-4760 effectively quenched cleavage of the Mca-APK(Dnp) substrate by both mouse rACE2 (Figure 1A) and human rACE2 (Figure 1B) at low picomolar concentrations. Both DX600 preparations (cyclic and linear), also inhibited cleavage of the Mca-APK(Dnp) substrate when incubated with human rACE2 (Figure 1B). When incubated with mouse rACE2, by contrast, linear DX600 exerted an inhibitory effect

on ACE2 activity only at high concentration (10^{-5}M) whereas cyclic DX600 did not inhibit mrACE2 at high concentrations (Figure 1A). Likewise, rat rACE2 was not inhibited by cyclic DX600 but was inhibited by MLN-4760 (Figure S2).

Consistent with these findings on mouse and rat rACE2, cyclic DX600 showed a complete lack of inhibition of ACE2 activity in both mouse and rat kidney cortex whereas an inhibitory effect of linear DX600 was observed only at very high concentrations (Figure 2). Additional experiments showed that linear DX600 is only inhibitory of murine rACE2 activity in non-reduced state since pretreatment with 2-mercaptoethanol abolished this inhibitory action (Figure S3).

Hydrolysis of Angiotensin II and Angiotensin I *in vitro* by rACE2

The purified mrACE2 protein was also analyzed *in vitro* as to whether it can form Ang-(1–7) from exogenous Ang II (Figure 3C). For this, mrACE2 was incubated *in vitro* with Angiotensin II in a test tube and the samples at 4 hours of exposure were used to measure Ang-(1–7) formation from the degraded Ang II (Figure 3). The purified soluble mrACE2 protein (0.2 nM) degraded Ang II (10^{-9}M) as inferred from about a 60–70% decline in the levels of this peptide at 4 hours (from 100% to 32±12%, $p<0.05$, n=3 experiments). Concurrent with these experiments Ang-(1–7) levels were measured and taken as evidence of new Ang-(1–7) formation driven by rACE2. There was no Ang-(1–7) formation from Ang II in the absence of mrACE2, whereas Ang-(1–7) was generated from Ang II incubated with mrACE2 (Figure 3). MLN-4760 (10^{-6}M) blocked formation of Ang-(1–7) by both mouse rACE2 (Figure 3A) and human rACE2 (Figure 3B). DX600 (both linear and cyclic form, 10^{-5}M) did not prevent the formation of Ang-(1–7) by mouse rACE2 (Figure 3A) but effectively blocked the formation of Ang-(1–7) from Ang II driven by human rACE2 in a manner similar to MLN-4760 (Figure 3B). Therefore, purified mrACE2 degrades Ang II and forms Ang-(1–7) *in vitro* and the formation of Ang-(1–7) is inhibitable by MLN-4760 but not by linear or cyclic DX600.

Since human rACE2 has been reported to have a lower affinity to hydrolyze Ang I than Ang II as a substrate[4, 5], we used a higher ratio of rACE2 to Ang I, than in the Ang II experiments shown in Figure 3. Under these conditions, mouse rACE2 (4.0 nM) did not hydrolyze Ang I (10^{-8}M) effectively even after a prolonged incubation of 24 hours (Figure 4A). Human rACE2, by contrast, lowered the levels of Ang I rapidly within the first hour (Figure 4B). The degradation of Ang I by human rACE2 was prevented by the three ACE2 inhibitors (Figure 4B)

Effect of mrACE2 and hrACE2 *ex vivo* on Plasma Angiotensin Peptides

The effect of murine and human rACE2 on the levels of several endogenous Ang peptides in plasma was examined *ex vivo*. As compared to control (no rACE2), both mrACE2 and hrACE2 when added to blood plasma caused a complete disappearance of Ang II (1–8) and lead to the formation of Ang-(1–7) (Figure 5). As a result of the increase in Ang-(1–7) formation, both mrACE2 and hrACE2 lead also to the formation of Ang-(1–5) and in similar amounts (Figure 5 and Table S2). This reaction is driven by ACE which, like other peptidases, is present in plasma *ex vivo* assay (Figure 5).

The effects of human and murine rACE2 on the above mentioned Ang peptides were inhibited by MLN-4760 at 10^{-5}M (Figure 5). Lower concentrations of MLN-4760 were also inhibitory (Table S2). Both, linear and cyclic DX600 inhibited the effect of hrACE2 on Ang peptide formation (Figure 5) and this effect was also dose-dependent (Table S2). By contrast, neither L-DX600 not C-DX600, even at a high concentration (10^{-5}M), prevented the changes on Ang peptides effected by mrACE2 (Figure 5).

Human rACE2 decreased Ang I (Ang 1–10) levels by about 60% whereas mrACE2 did it only marginally, as reflected by the diameter of each corresponding sphere and the numerical value on the site of each sphere (Figure 5, upper panels). Of note, human rACE2 resulted in the formation of Ang-(1–9) from Ang I (1–10) whereas mouse rACE2 did not (Figure 5, lower panels).

The Effect of MLN-4760 and DX600 on in vivo Serum and Kidney ACE2 activity—To examine the effect of MLN-4760 and DX600 on the inhibition of endogenous mouse ACE2 *in vivo,* ACE2 activity was measured in kidneys and sera from mice that received MLN-4760 (n=8), C-DX600 (n=9) or L-DX600 (n=7) (1 mg/kg BW, i.p.) as compared to mice that received vehicle (PBS) (n=8) (Figure 6). One hour after the administration of MLN-4760, serum ACE2 activity was more than 80% lower ($p<0.001$) and kidney ACE2 activity was 60% lower ($p<0.001$) than in vehicle (PBS) control mice (Figure 6). In contrast, neither serum (Figure 6A) nor kidney ACE2 activity (Figure 6B) was significantly decreased by either C-DX600 or L-DX600 administration as compared to vehicle controls. Consistent with these findings, cyclic DX600 had no effect on plasma Ang-(1–7) levels as compared to vehicle (28.2±4.2 vs. 31.8±6.4 pg/mL) whereas MLN-4760 resulted in a substantial decrease in Ang-(1–7) levels in plasma as compared to vehicle treated mice (19.3±4.2 vs. 31.8±6.4 pg/mL). Although this difference did not achieve statistical significance this trend is consistent with the decrease in Ang-(1–7) levels observed *in vitro* and *ex vivo* with MLN-4760. Kidney Ang-(1–7) levels revealed values too low for detection (data not shown).

In Vivo Studies on purified Human and Mouse rACE2

Time Course and Dose Response—To establish the time point of the highest serum ACE2 activity in serum after an acute IP bolus of ACE2 (1mg/kg) blood was collected at 15 min, 30 min and 1, 2, 4 and 24 hrs after mrACE2 injection. After the bolus injection of mrACE2 serum activity was the highest at 2 hours (Figure S4A) and serum ACE2 activity declined steadily during the following hours until the end of the monitoring period (Figure S4A).

Since the peak values of serum ACE2 activity were observed at 2 hours after mrACE2 IP injection, dose response studies were performed for this time point. Two hours after mrACE2, ACE2 activity levels in serum increased in a dose-dependent manner (Figure S4B).

Effect of rACE2 on blood pressure and plasma Ang II levels in Ang II–Infused Mice—The effect of mrACE2 on blood pressure was examined in acute studies in response to a bolus of Ang II in anesthetized mice pretreated either with a bolus of PBS or mrACE2 (1.0 mg/kg) 2 hours prior to Ang II administration. Two hours after mrACE2 administration, ACE2 activity in serum was markedly increased compared with animals not receiving mrACE2 (286±68 vs. 2.1±1.2 RFU/uL/hr, respectively, $p<0.001$). Baseline SBP in mice infused with mrACE2 (1 mg/kg), however, was not significantly different from mice not pretreated with mrACE2 (109±5.2 vs.102±3.1 mmHg, respectively; $p=0.238$) (see Figure 7).

Administration of a bolus of Ang II (n=8), resulted in a rapid increase in SBP (Figure 7). The peak increase in SBP observed at 30 sec. was markedly blunted in mice pretreated with mrACE2 (1.0mg/kg) (139±6.8 vs. 173±4.4 mmHg, $p<0.001$). Moreover, the SBP recovery was strikingly faster in mice pretreated with this dose of mACE2 compared with mice pretreated with vehicle (at 5 min, 106±8.2 mmHg vs. 168±3.9 mmHg, respectively, *$p<0.001$*). The difference in blood pressure between these two groups persisted throughout the continuous monitoring at 30-second intervals (Figure 7). A ten times lower dose of

mrACE2 (0.1mg/kg) had only a marginal effect in attenuating the maximal increase in BP after Ang II (162±11.9 mmHg, p=0.350) but also enhanced the recovery in blood pressure over time (Figure 7).

In similar experiments in a group of animals infused with Ang II and an ACE2 inhibitor (MLN-4760, 1.0 mg/kg n=6), mrACE2 (1 mg/kg BW) failed to lower blood pressure, i.e. the peak increase was not significantly different from that in mice infused with Ang II alone (n=14) (SBP 185±4.7 vs. 174±4.2 mmHg respectively; *p=0.253*). In mice pre-treated with murine rACE2 that received MLN-4760, blood pressure during Ang II infusion was markedly higher than in mice that received only mrACE2 (Figure 8A). In contrast, the linear DX600 had a minimal effect on SBP that was transient during murine rACE2 infusion (Figure 8B). Cyclic DX600 had no effect whatsoever on the mrACE2 blood pressure lowering effect during Ang II infusion (Figure 8C). These *in vivo* findings are consistent with the *in vitro* data on the effect of these inhibitors on ACE2 activity shown above.

Because in mrACE2-infused mice SBP normalized within 5 minutes after Ang II bolus (see Figure 7), we chose this time point to examine serum ACE2 activity and plasma Ang II following its infusion. Mice that were pretreated with mrACE2 2 hours before Ang II infusion showed a marked increase in serum ACE2 activity compared with animals pretreated with PBS (167±22 versus 0.8±0.4 RFU/uL/hr, respectively; p<0.001). The increase in serum ACE2 activity linked to mrACE2 administration was almost completely inhibited by MLN-4760 (2.7±0.5% of the ACE2 activity without ACE2 inhibitor, p<0.001), but not by linear (171±15% of the ACE2 activity without ACE2 inhibitor, p<0.05) or cyclic DX600 (125±21% of the ACE2 activity without ACE2 inhibitor, p=NS) (Figure 8D).

In mrACE2-pretreated mice (for 2 hours) plasma Ang II levels were markedly reduced as compared with animals infused with Ang II that had not received mrACE2 (78±30 versus 967±213 fmol/mL, respectively; p<0.001). The decrease in plasma Ang II levels associated with mrACE2 administration was inhibited by MLN-4760 (78±30 vs. 355±89 fmol/mL, respectively, p<0.05), but not by either linear (69±41 fmol/mL) or cyclic DX600 (77±54 fmol/mL) (Figure 9).

Recombinant murine ACE2 infusion for four Weeks—To examine whether an extended mouse rACE2 infusion can produce a sustained effect on serum ACE2 activity, mice were administered mrACE2 (0.5mg/kg/day) subcutaneously using minipumps or were injected i.p. (0.2 mg/kg/day) for 4 wks. The initial increase in serum ACE2 activity was sustained for 4 wks for both administration modes (Figure 10).

Antibodies against rACE2 were measured in mice that received mrACE2 or hrACE2 in osmotic minipumps for 28 days. Anti-rACE2 antibodies were not detectable in plasma from mice that received mrACE2 (0.5mg/kg/d; n=3) (OD_{490nm}=−0.010±0.001, n=3). In sharp contrast, mice that had been infused with human rACE2 (0.1 mg/kg/d; n=3) for the same period of time had strikingly elevated anti-rACE2 antibody levels (OD_{490nm} = 1.328±0.179; p<0.005 vs. mrACE2 group).

DISCUSSION

This study reports the characterization of mouse rACE2 and its effects on Ang II and Ang I degradation in a series of *in vitro* and *in vivo* studies. We produced a highly purified soluble mouse rACE2 over-expressed on large scale in human embryonic kidney cell line 293F (see Online Supplement). In acute studies, the administration of murine rACE2 markedly attenuated Ang II-induced hypertension and rapidly decreased plasma Ang II levels. The

prevention of Ang II-induced hypertension by mouse rACE2 was completely obliterated by MLN-4760 but essentially unaffected by linear DX600 and by cyclic DX600.

This discordant response between these available ACE2 inhibitors was further investigated in *in vitro* and *ex vivo* studies that confirmed fundamental species differences between the inhibitors. Specifically, MLN-4760 blocked ACE2 activity, measured by cleavage of the ACE2 substrate (Mca-APK-Dnp), using both murine and human rACE2 whereas both DX600 variants inhibited the enzymatic activity conferred by human rACE2 but only linear DX600 had an inhibitory effect on mrACE2 and only at very high concentrations. Similar results were found both in mice and rat kidney tissue. Recently, Pedersen et al.[26] reported an inhibitory effect of linear DX600 on inhibition of mouse and rat ACE2 over-expressed in cell lines. This inhibitory effect was seen at a relatively high concentration (10^{-5}M) which is consistent with our studies. These authors did not report on the effects of cyclic DX600 or MLN-4760 on ACE2 activity. Our studies moreover demonstrate that the inhibitory effect of linear DX600 on mouse rACE2 disappears in the reduced state. Of note, the redox state of DX600, however, does not affect the ability of this compound to effectively inhibit human rACE2 (see Figure S3).

Important differences in inhibitor specificity were observed not only regarding blood pressure and ACE2 activity but also regarding Ang II degradation and Ang-(1–7) formation. That is, MLN-4760 prevented both human and mouse rACE2-driven Ang II degradation whereas the two variants of DX600 did not inhibit mouse rACE2-driven Ang II degradation. Consistent with the divergent effects on Ang II, the conversion from Ang II to Ang-(1–7) promoted by mouse rACE2 was prevented by MlN-4760 but not by either of the two conformational variants of DX600. The fact that linear DX600 inhibited mouse ACE2 activity using a synthetic fluorogenic substrate, although at high concentrations, and yet did not prevent the formation of Ang-(1–7) from Ang II deserves some comment. The affinity of linear DX600 for mrACE2 may be much lower than that of Ang II and thus in the presence of this peptide DX600 cannot bind effectively to mouse rACE2 and yet it may bind to human ACE2 owing to conformational differences in the enzymatic binding sites. Regardless of the mechanism, DX600 is not an effective inhibitor of rodent ACE2.

Our *in vitro* findings were corroborated *ex vivo* using a newly developed and highly sensitive procedure for simultaneous and absolute quantification of up to 10 angiotensin peptides in a single 1 ml of blood plasma sample[29]. This approach termed “RAS fingerprinting” yields a comprehensive profile of several endogenous angiotensin peptides within blood plasma. These ex vivo studies showed that mouse rACE2 resulted in a complete disappearance of Ang II in plasma and in the attendant formation of Ang-(1–7) and Ang-(1–5) peptides while other measured angiotensin peptides were not affected (Figure 5).

The results with mouse rACE2 are similar with those obtained with human rACE2 except that hrACE2 Ang-(1–9) formation from Ang I (1–10) is not effectively promoted by mouse rACE2. This lack of effect of mouse rACE2 on Ang I degradation is in contrast with the promoting action of hrACE2. This finding, moreover, is concordant with the lack of effect of mrACE2 to cleave Ang I in our in vitro experiments. It has previously been reported that human rACE2 hydrolyzes Ang II and, although with less efficiency, Ang I. As expected from the known effect of human rACE2 on Ang II[2, 4, 5, 7], our highly purified mouse rACE2 was shown *in vitro* to hydrolyze Ang II, which led to the formation of Ang-(1–7). Whereas mrACE2 degraded Ang II with similar efficiency as hrACE2, mrACE2 had only a negligible effect on Ang I degradation. That was fully consistent with the *ex vivo* studies in plasma (Figure 5). This difference between human and mouse ACE2 regarding their efficiency on Ang peptide degradation suggests that mouse rACE2 may be more selective for Ang II

degradation than human rACE2. The availability of this form of rACE2 will therefore simplify the interpretation of the action of mouse rACE2 within the RAS to the extent that it effectively degrades Ang II but not Ang I.

Differences in inhibitor sensitivity were further shown in in *ex vivo* experiments in plasma incubated in the presence of mouse rACE2 and the various ACE2 inhibitors by measuring levels of various Ang peptides. Linear and cyclic DX600 inhibited the effects of human rACE2 dose-dependently but they did not affect at all the changes exerted by mouse rACE2 on the Ang peptides even at a high concentration (10^{-5}M). MLN-4760, by contrast, had all the anticipated effects on the studied Ang peptides in the presence of both human and mouse rACE2: **1)** accumulation of Ang II and decrease in Ang-(1–7) and Ang-(1–5) levels and **2)** accumulation of Ang I and a decrease in Ang-(1–9) formation (Figure 5). While Ang I has no known biologic effects, it is a key substrate for the formation of Ang II. Moreover, it is cleaved by ACE2 to form Ang-(1–9), a peptide recently shown to have antifibrotic action[33].

In vivo the administration of DX600 to mice had no significant effect in either serum or kidney ACE2 activity. The precise mechanism as to why rodent ACE2 is resistant to DX600 inhibition *in vivo* and *in vitro* whereas it is effective in inhibiting human ACE2 is not elucidated by these studies. One possible explanation is that murine ACE2, unlike human ACE2, does not bind DX600 and Ang I effectively because of differences in the three-dimensional molecular structure between the human and mouse ACE2 substrate binding domains. Conformational instability of the DX600 polypeptide or its rapid degradation by peptidases could also contribute to the lack of effect of this inhibitor on ACE2 activity *in vivo*.

To examine whether a prolonged mouse rACE2 infusion can produce a sustained increase in serum ACE2 activity, mice were infused with mrACE2 (1mg/kg per day) for 4 weeks. Two approaches were done, daily IP injections and osmotic minipumps. With the daily IP injections, higher levels of serum ACE2 activity were documented than those observed with minipumps (Figure 10). The highest level with IP doubtless reflected the peak level as they were measured 2 hours post-injection. The lower levels with mini-pump administration are more representative of the steady state. Importantly, the high levels of serum ACE2 activity measured weekly were sustainable over time as there was no evidence of antibody formation using mrACE2. Therefore, mouse rACE2 circumvents the problem with immunogenicity and development of neutralizing ACE2 antibodies that we had previously reported when human rACE2 was given to mice by minipumps[7]. This resulted in almost complete loss of ACE2 activity after 2 weeks of administration[7]. The availability of mouse rACE2 will therefore facilitate the examination of potential therapeutic effects of rACE2 amplification in chronic mouse models of diabetic kidney disease and cardiovascular disease where the administration of human rACE2 clearly loses efficiency over time owing to its immunogenicity.

Perspectives

There are major differences between the known specific inhibitors of ACE2 on human and murine recombinant ACE2 that should be taken into account when designing and interpreting *in vivo* and *in vitro* studies. MLN-4760 inhibits both human and murine ACE2 whereas DX600 inhibits human ACE2 preferentially. Although linear DX600 at high concentrations inhibits mouse ACE2 activity measured with a synthetic fluorogenic substrate, it does not prevent the formation of Ang-(1–7) from Ang II. Mouse rACE2 effectively degrades Ang II (1–8) forming Ang-(1–7) whereas it has no effect on the formation of Ang-(1–9) from Ang I (1–10). RAS “fingerprinting” is a powerful novel approach to simultaneously evaluate quantitatively several angiotensin peptides in plasma and can be used to demonstrate profound differences on the effect of available ACE2

inhibitors on the formation of various angiotensin peptides driven by human and mouse ACE2. The availability of mouse recombinant ACE2 with well-defined properties will provide a new tool to examine the potential therapeutic benefit of increasing ACE2 activity and fostering the formation of Ang-(1–7) from Ang II in mouse models of hypertension, cardiovascular and diabetic kidney disease.

Acknowledgments

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Novelty and Significance

What is New

- This study reports the characteristics of a newly produced and highly purified form of soluble murine recombinant Angiotensin Converting Enzyme 2 (ACE2).
- It is shown that this form of murine rACE2 effectively degrades Ang II with the formation of Ang (1–7). This process, driven by murine ACE2, is inhibited by MLN-4760, but not by DX600.

What is Relevant

- The availability of murine rACE2 provides a strategy to study *in vivo* the effect of Ang II degradation and Ang (1–7) formation in animal models.
- Human rACE2, which was previously used, results in the formation of neutralizing antibodies when given to rodent models and therefore its use for chronic studies is limited.

Summary

A newly developed murine recombinant ACE2, when given to mice, greatly attenuates angiotensin II-dependent hypertension and effectively lowers plasma angiotensin II levels. When given to mice for several weeks, it results in sustained elevation in serum ACE2 activity. The study also shows that there are fundamental species differences regarding murine and human ACE2 inhibition by known inhibitors.

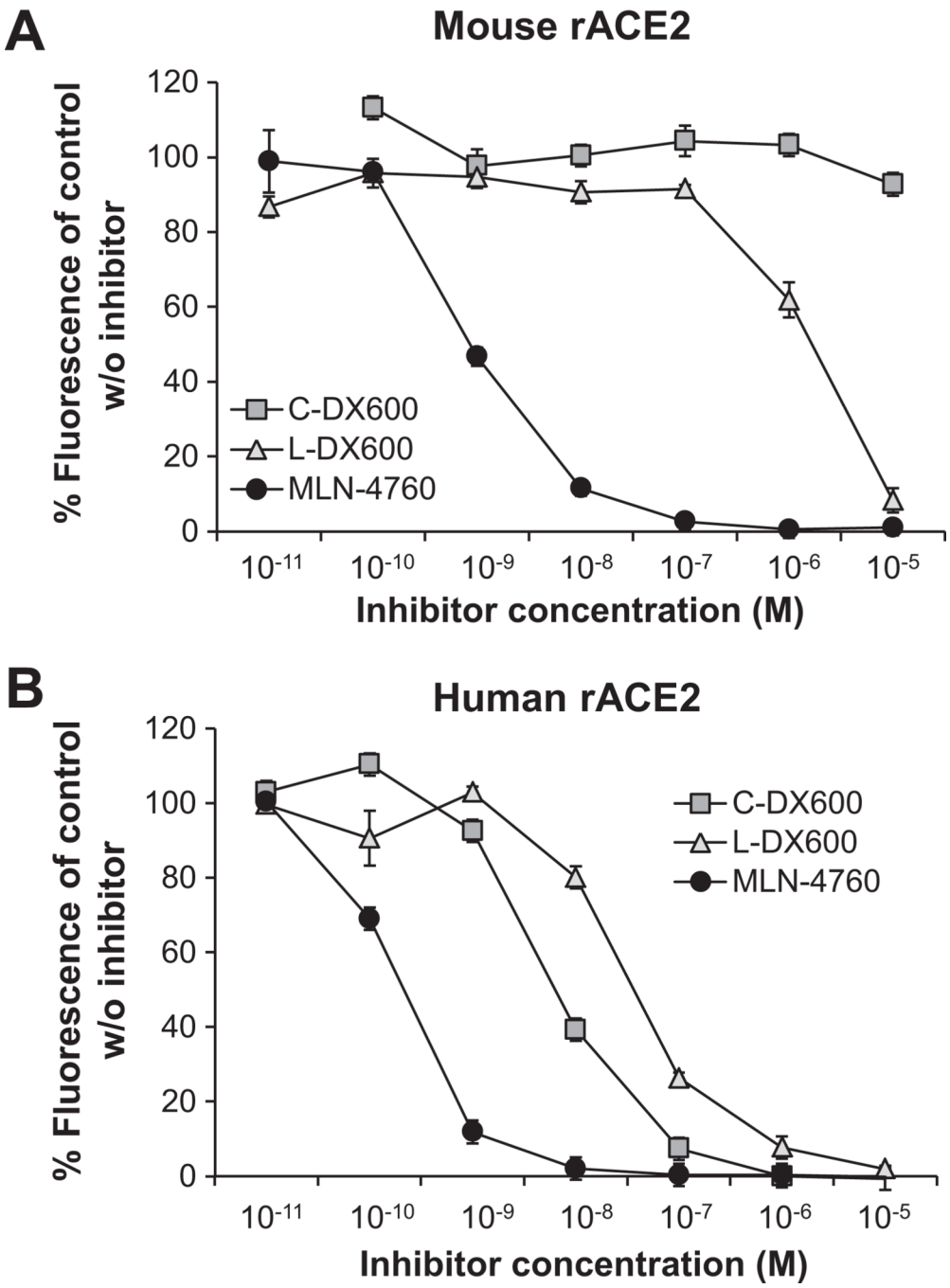

Figure 1.

The effect of MLN-4760 and two conformational DX600 isoforms (C-cyclic; L-linear) at concentrations from 10^{-11}M to 10^{-5}M on ACE2 activity of mouse rACE2 (Panel A) and human rACE2 (Panel B). ACE2 activity was measured by recording fluorescence formed by cleavage of a synthetic intramolecularly quenched substrate, Mca-APK-(Dnp) (10^{-5}M). MLN-4760 inhibited both mouse and human rACE2 and at relatively low concentration. Linear DX600 showed an inhibitory effect on mouse rACE2 activity but only at a high concentration (near maximal inhibition at 10^{-5}M), whereas cyclic DX600 had no inhibitory effect on mouse rACE2 at any concentration studied (Panel A). Both DX600 isoforms

effectively inhibited human rACE2 (near maximal inhibition 10^{-6}M-10^{-7}M) (Panel B). Each data point represents mean±SE of triplicate wells (n=3).

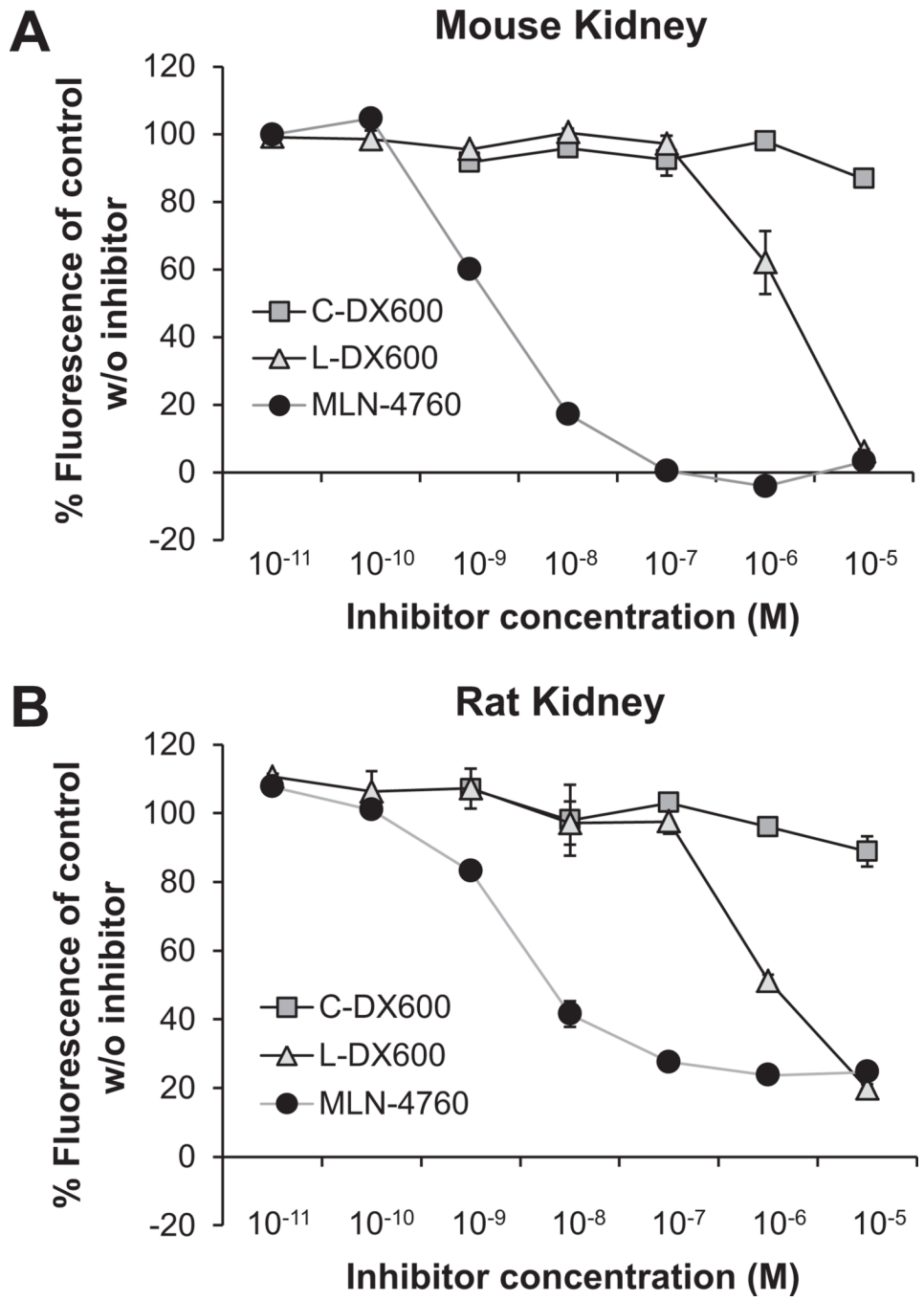

Figure 2.

The effect of ACE2 inhibitors: MLN-4760 (black circles) and DX600 (two conformational variants: cyclic - gray squares; and linear - gray triangles), at concentrations 10^{-5}M to 10^{-11}M on activity of endogenous ACE2 from mouse kidney cortex (Panel A) and rat kidney cortex (Panel B). ACE2 activity was measured using a synthetic intramolecularly quenched fluorogenic substrate, Mca-APK(Dnp) (10^{-5}M). MLN-4760 effectively inhibited mouse and rat ACE2-mediated cleavage of the substrate and the associated fluorescence formation. Cyclic DX600 had no inhibitory effect on mouse and rat kidney ACE2 activity whereas linear DX600 inhibited ACE2 activity but only at high concentrations. Each data point represents mean±SE of triplicate wells (n=3).

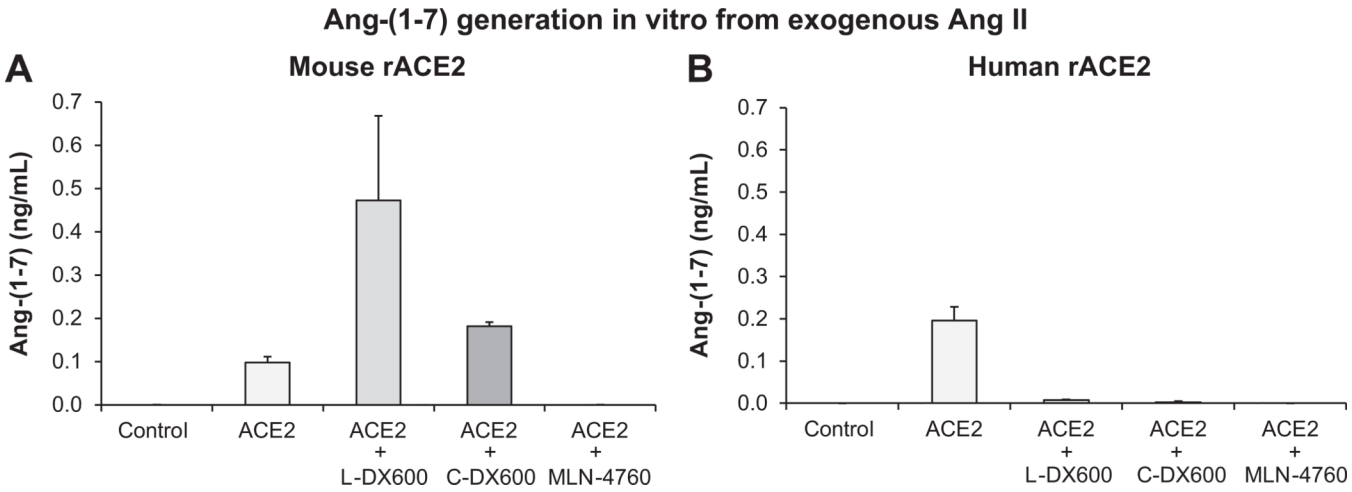

Figure 3.

In vitro Ang-(1–7) formation from exogenous Ang II. Ang II was incubated *in vitro* with mouse rACE2 (Panel A) or human rACE2 (Panel B) in the presence or absence of MLN-4760 (10^{-6}M), linear (L) DX600 (10^{-5}M) or cyclic (C) DX600 (10^{-5}M). Control depicts Ang II incubated without rACE2. The amount of formed Ang-(1–7) (pg/mL) was measured after 4 hour incubation with Ang II. There was no spontaneous formation of Ang-(1–7) from Ang II without rACE2. Angiotensin-(1–7) increased after incubation with either mouse (Panel A) or human rACE2 (Panel B). No formation of Ang-(1–7) from Ang II by either mouse rACE2 or human rACE2 was detectable in the presence of MLN-4760 (Panels A and B). DX600 (linear and cyclic) did not abolish mouse rACE2-mediated Ang-(1–7) formation (Panel A). By contrast, either form of DX600 prevented human rACE2-mediated Ang-(1–7) formation (Panel B).

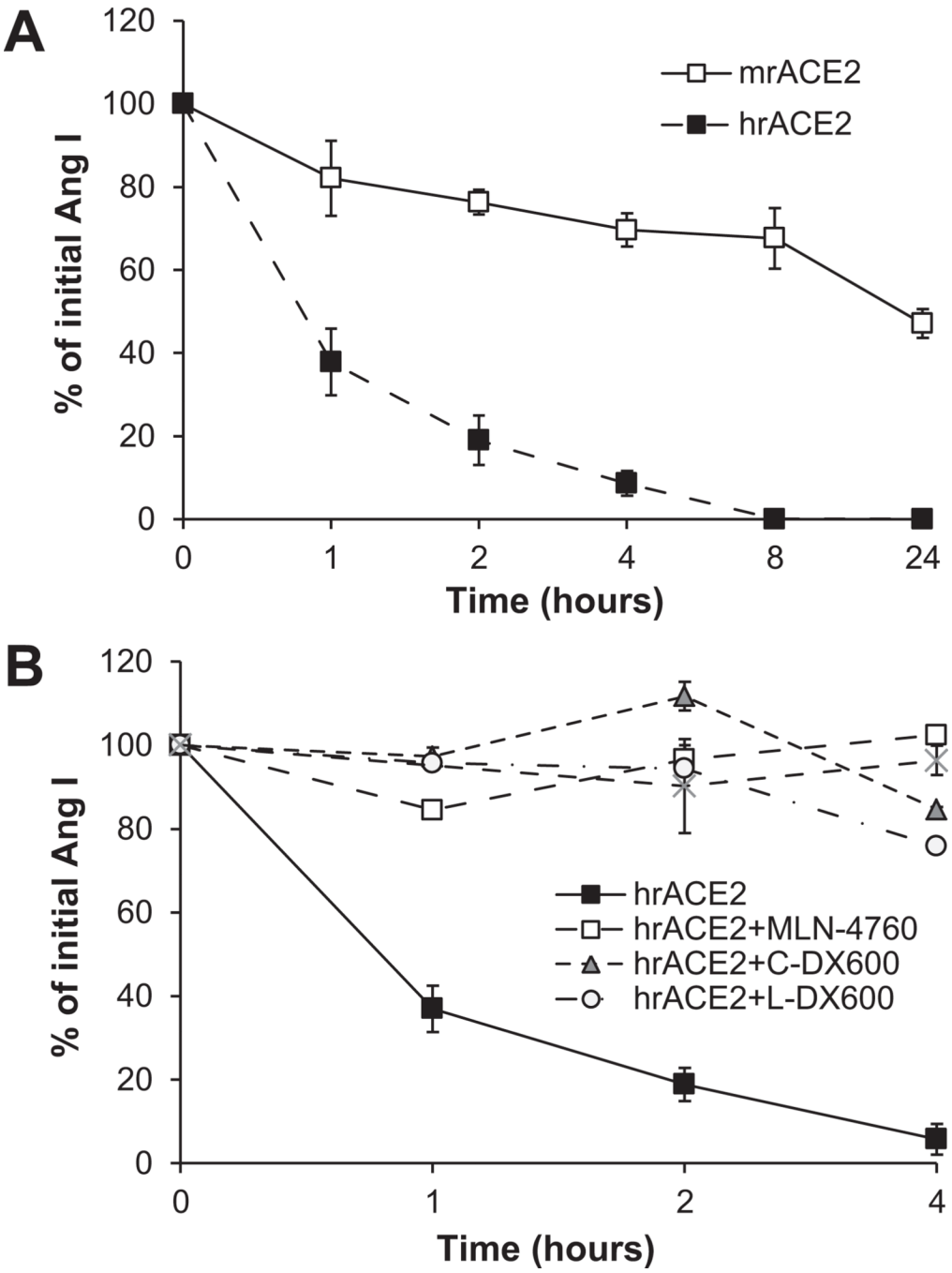

Figure 4.

Angiotensin I (10^{-8}M) was incubated with mouse rACE2 (Panel A) or human rACE2 (Panel A and B) and the disappearance of Ang I over time measured using an Ang I EIA for this peptide.

Mouse rACE2 had only a minimal effect on Ang I dissipation even at 24 hours (Panel A). In contrast, human rACE2 degraded Ang I effectively and almost completely by 4 hours and this was prevented by MLN-4760 and both variants of DX600 (Panel B). Control depicts Ang I incubated without rACE2.

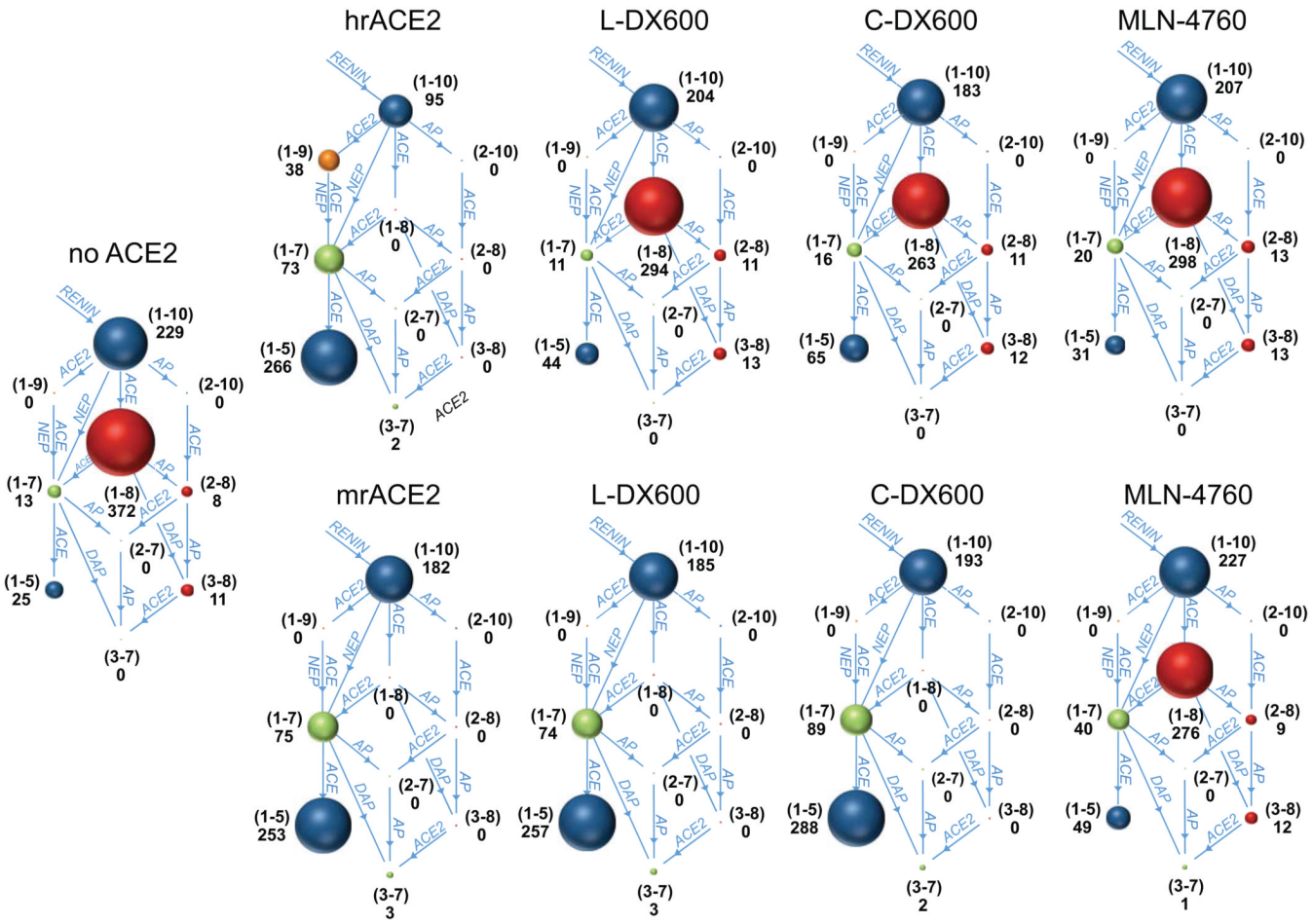

Figure 5.

The effect of human rACE2 (upper panels) and mouse rACE2 (lower panels) on Ang peptides *ex vivo* in plasma. Control (no recombinant ACE2) is shown in the middle of the graph as this provides a baseline control for comparisons with samples to which either human (upper panels) or mouse rACE2 (lower panels) was added without and with ACE2 inhibitors. Human or mouse rACE2 were added at a final plasma concentration of 5μg/ml. Angiotensin peptides were measured using LC-MS/MS as described in the Methods (see concentrations of all measured Ang peptides in Table S2). In this figure, the diameter of the spheres reflects the concentration of the respective peptide metabolite, which is also given in pg/mL next to each individual sphere. 0 pg/mL indicates concentrations below quantification limits, which are defined by a signal-to-noise ratio below 10. The amino acid sequence of each angiotensin metabolite is schematically given in brackets near the corresponding sphere. The sequence annotation is based on the decapeptide Angiotensin I (1–10) which is N- or C-terminally cleaved by the indicated peptidases. Specific peptidases are shown in the blue by the arrows connecting their substrate and product. AP: aminopeptidases; NEP: neutral endopeptidase; DAP: di-aminopeptidase; ACE: angiotensin-converting enzyme, ACE2: angiotensin converting enzyme 2.

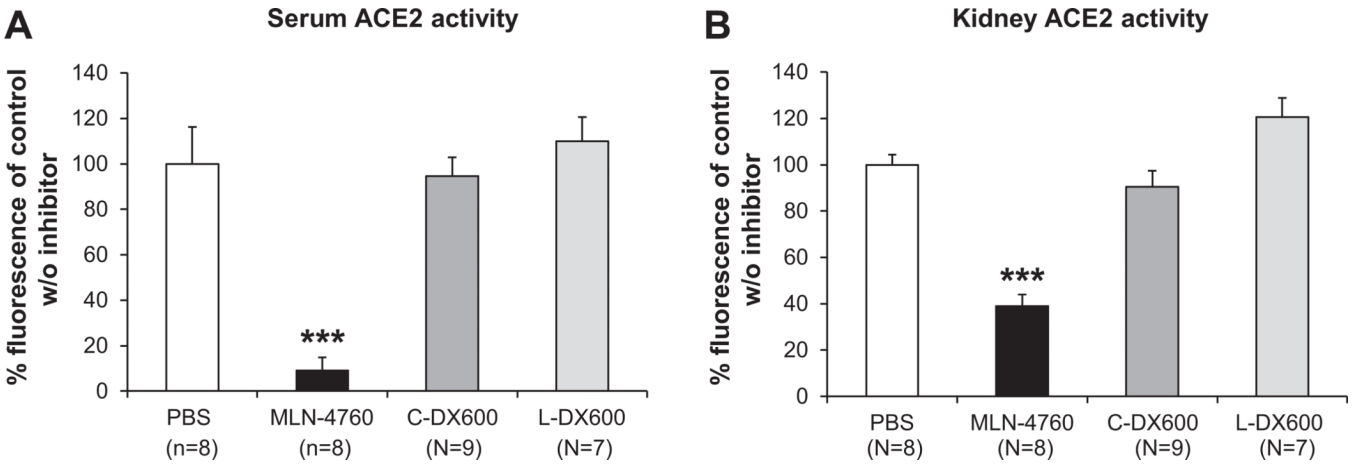

Figure 6.

ACE2 activity measured in sera (Panel A) and kidneys (Panel B) from mice that received, MLN-4760, C-DX600 or L-DX600 (all at the dose of 1mg/kg BW) or PBS (Vehicle) in a single i.p. bolus injection. ACE2 activity in sera and kidneys from mice that received MLN-4760 (n=8) were significantly lower ($p<0.001$) than in vehicle control mice (n=8). Mice receiving C-DX600 (n=9) or L-DX600 (n=7) had ACE2 activity levels not different from vehicle control (PBS) mice in sera (Panel A) and in the kidneys (Panel B); *** $p<0.001$ vs. PBS, vs. C-DX600 and vs. L-DX600.

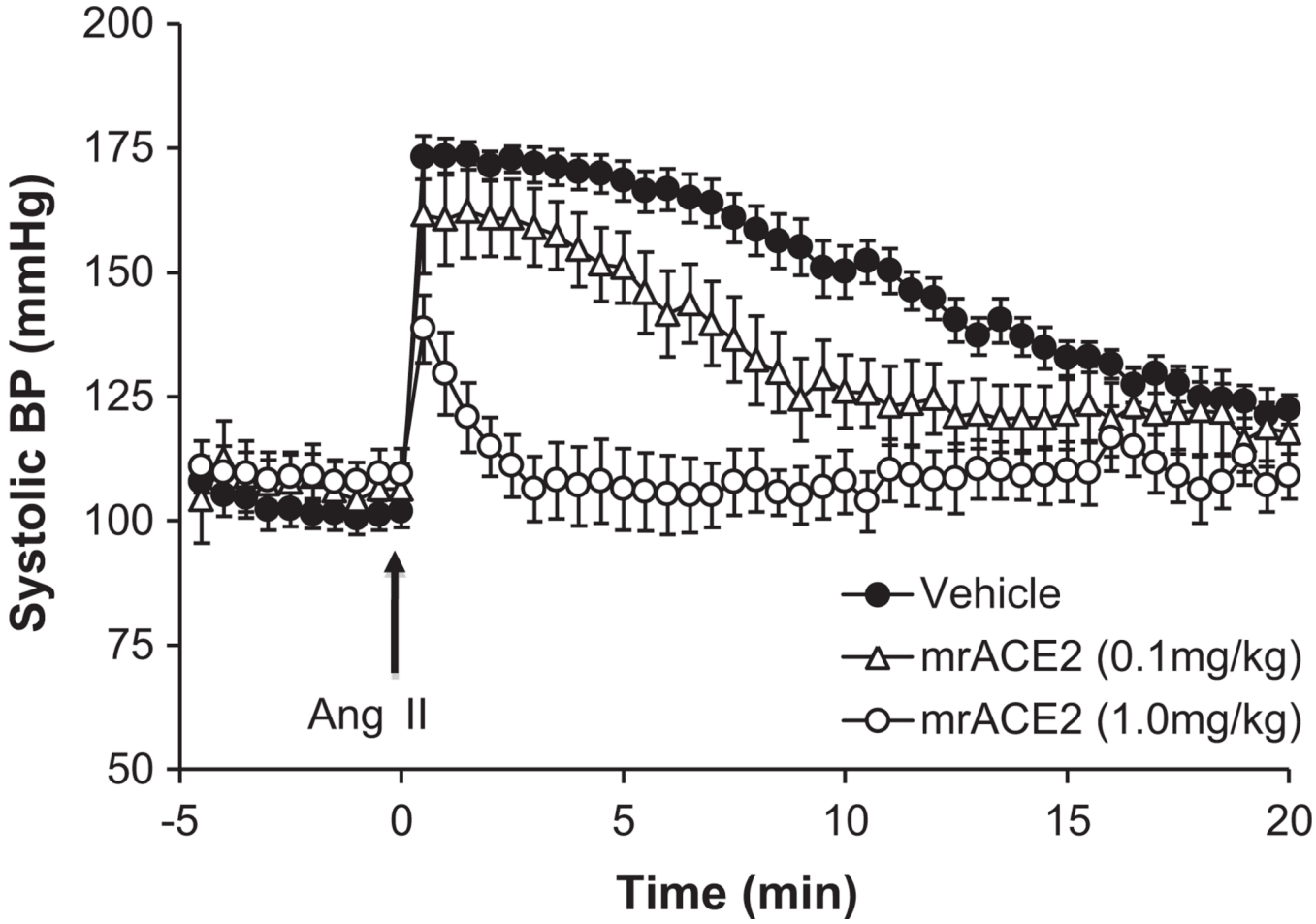

Figure 7. Continuous SBP measurement in anesthetized mice

Mice received PBS (Vehicle) or mrACE2 (0.1 mg/kg or 1 mg/kg) in a single IP injection 2 hours before blood pressure measurement. SBP was recorded at 30-second intervals 5 minutes before an IP bolus of Ang II (0.2 mg/kg, arrow, time point 0 minutes) and 20 minutes thereafter. There was no difference in baseline SBP between mrACE2 and PBS-infused animals before Ang II bolus. A bolus of Ang II to mice pretreated with vehicle (PBS, n=8) was associated with a rapid increase in SBP. In mice administered with mrACE2 before Ang II injection (1 mg/kg, n=7), the SBP increase was blunted and normalized within the first 5 minutes after Ang II injection. The lower dose mrACE2 (0.1 mg/kg, n=7) had an intermediate effect on blood pressure recovery as compared to vehicle treated mice and mice pre-treated with the highest dose of mrACE2.

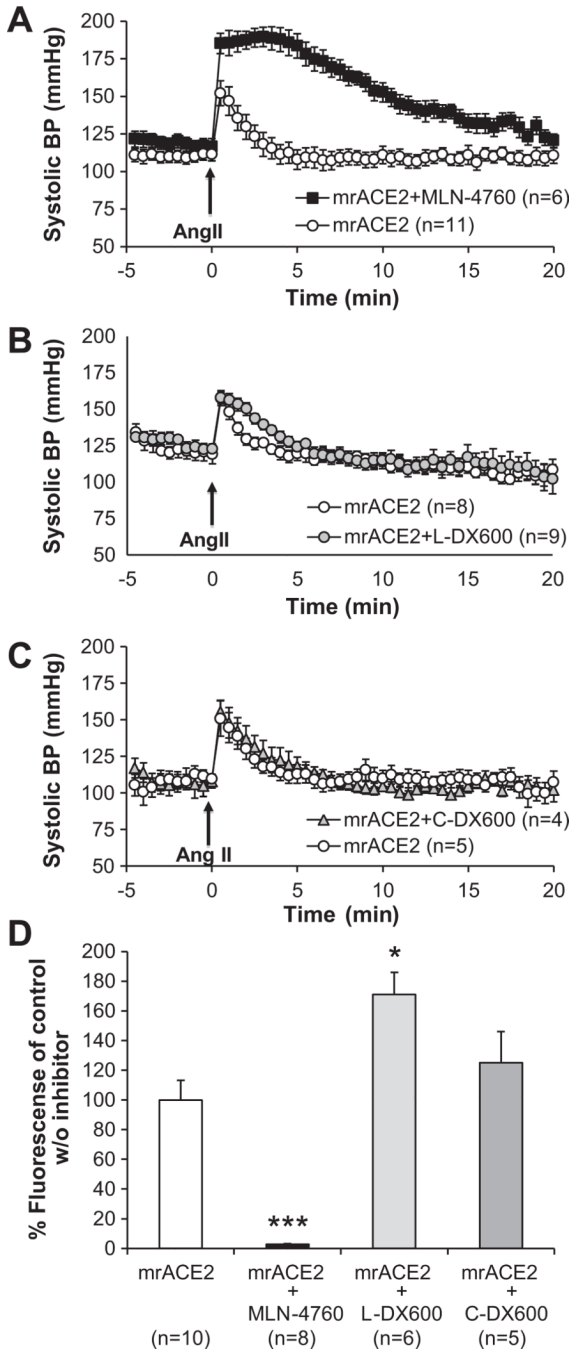

Figure 8.

The effect of MLN-4760 (panel A), linear DX600 (panel B) and cyclic DX600 (panel C) on mrACE2-mediated SBP decrease in mice infused with Ang II. Mice received mrACE2 (1.0 mg/kg) in a single IP injection 2 hours before blood pressure measurement. An IP bolus of Ang II (0.2 mg/kg, n=11) or Ang II (0.2 mg/kg) along with MLN-4760 or DX600 (1 mg/kg; arrow, time point 0 minutes). It is shown that MLN-4760 completely blocks the effect of mrACE2 on blood pressure recovery (Panel A). In contrast, DX600 has only marginal (linear DX600, panel B) or no effect (cyclic DX600, panel C) on SBP which recover in a manner similar to mice that received mrACE2 alone.

In a separate set of mice equally infused with Ang II and mrACE2, serum ACE2 activity was measured 5 min. after IP bolus of Ang II with or without MLN-4760 or DX600 (Panel D). MLN-4760 markedly reduced serum ACE2 activity ($p<0.001$) while both forms of DX600 had no significant inhibitory effect on serum ACE2 activity; *** $p<0.001$ vs. mrACE2 alone, vs. mrACE2+C-DX600 and vs. mrACE2+L-DX600; * $p<0.05$ vs. mrACE2 alone.

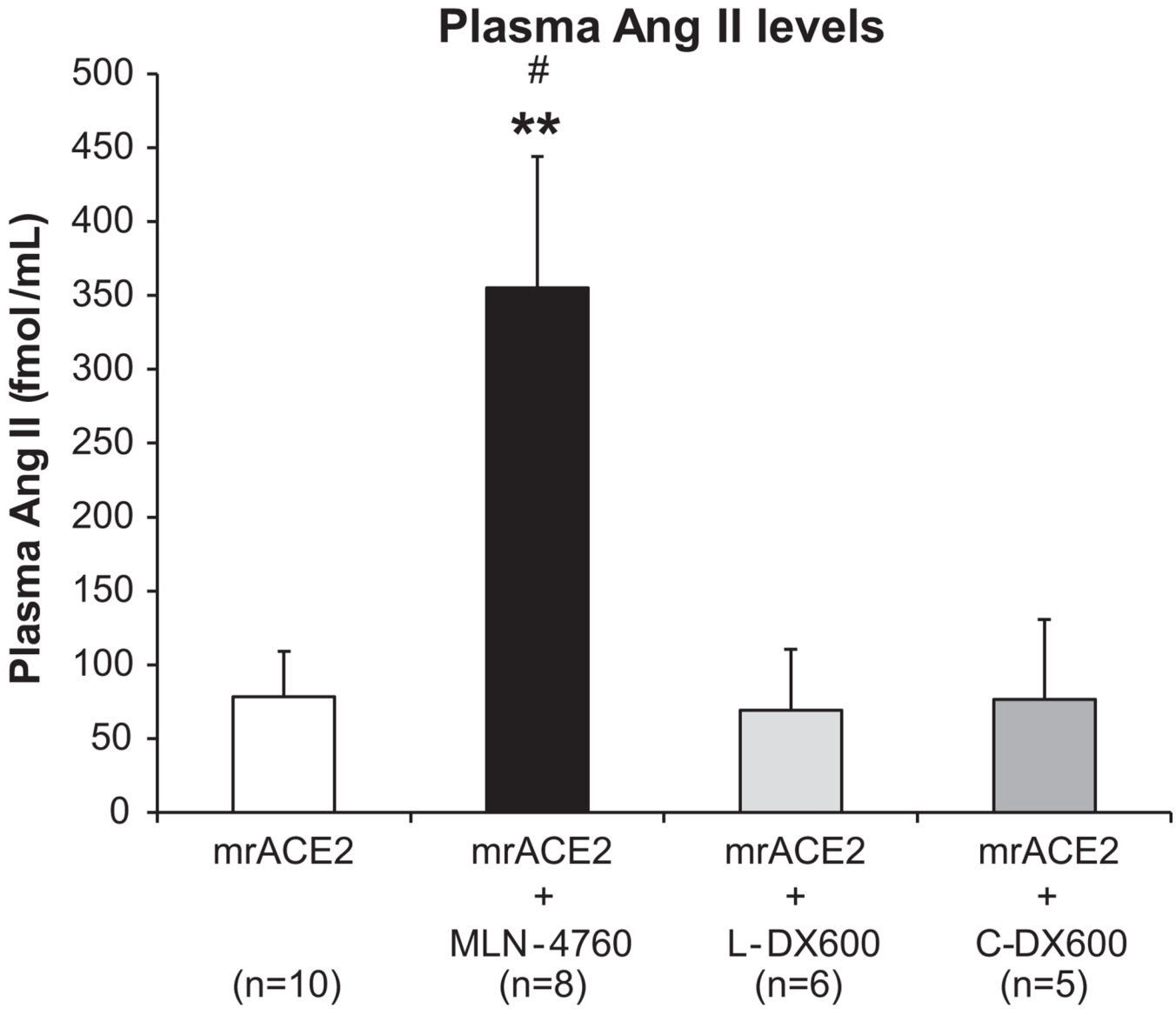

Figure 9.

Plasma Ang II measured 5 min. after IP bolus of Ang II with or without MLN-4760 or DX600 in the same mice as in Figure 8D. Plasma Ang II in mice that received mrACE2 and MLN-4760 was significantly higher than in mice that received mrACE2 alone. Mice receiving linear or cyclic DX600 had plasma Ang II levels similar to those of mrACE2 alone; **p<0.01 vs. mrACE2 alone; # p<0.05 vs. mrACE2+C-DX600 and vs. mrACE2+L-DX600.

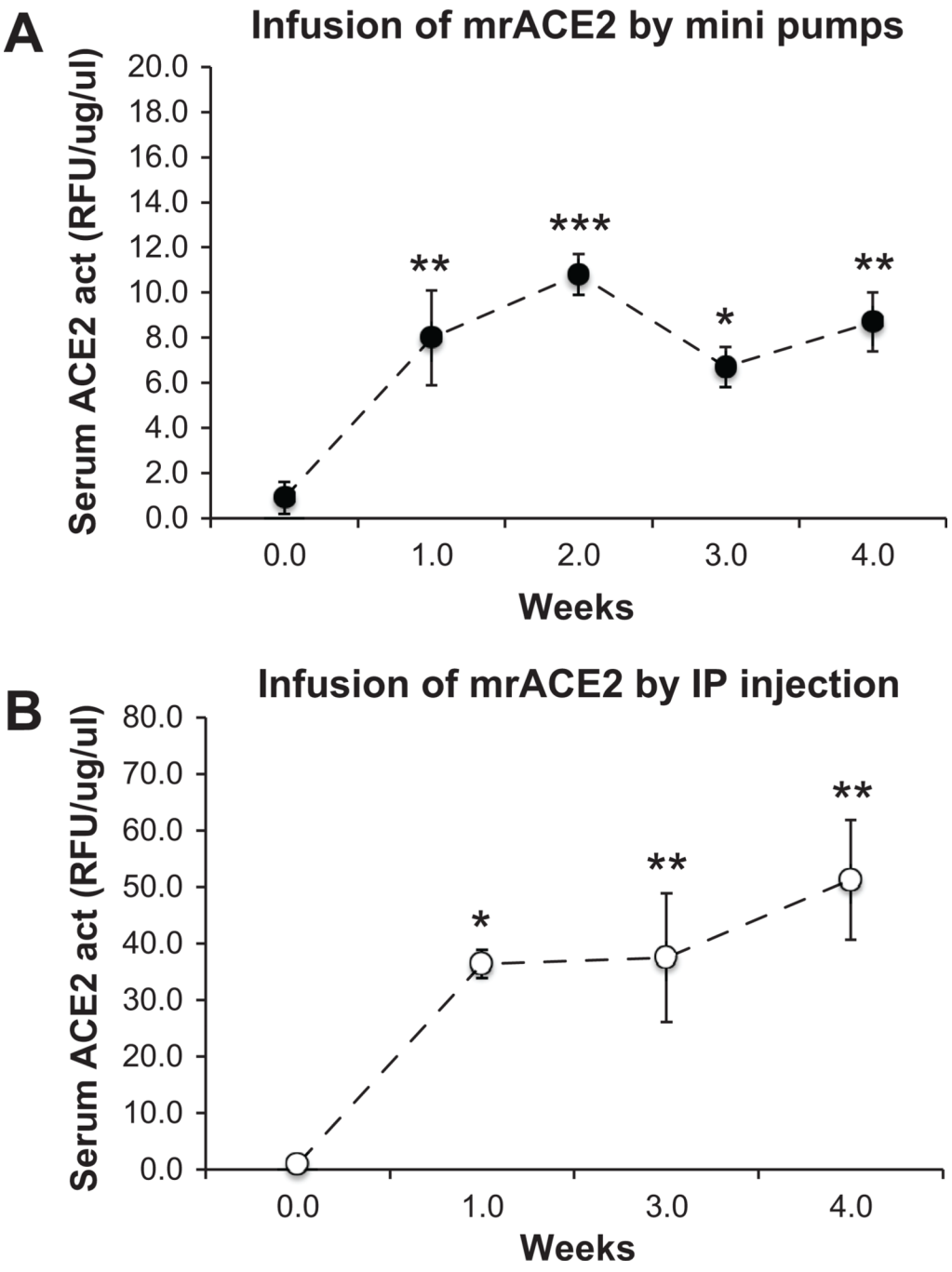

Figure 10.

Weekly measurements of serum ACE2 activity in blood collected from tail vein in mice that received murine rACE2 using osmotic minipumps (0.5 mg/kg/d) (n=5) (Panel A) or using daily IP bolus injections (0.2 mg/kg/d) (n=5) (Panel B). Measurements were taken 2 hours after injection when the level ACE2 activity was the highest. It shows a sustained increase over time with either form of administration; *p<0.05, **p<0.01, ***p<0.001 vs. time point '0' (prior to mrACE2 infusion).