Ang II (Angiotensin II) Conversion to Angiotensin-(1-7) in the Circulation Is POP (Prolyloligopeptidase)-Dependent and ACE2 (Angiotensin-Converting Enzyme 2)-Independent

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Abstract—The Ang II (Angiotensin II)-Angiotensin-(1-7) axis of the Renin Angiotensin System encompasses 3 enzymes that form Angiotensin-(1-7) [Ang-(1-7)] directly from Ang II: ACE2 (angiotensin-converting enzyme 2), PRCP (prolylcarboxypeptidase), and POP (prolyloligopeptidase). We investigated their relative contribution to Ang-(1-7) formation in vivo and also ex vivo in serum, lungs, and kidneys using models of genetic ablation coupled with pharmacological inhibitors. In wild-type (WT) mice, infusion of Ang II resulted in a rapid increase of plasma Ang-(1-7). In *ACE2*$^{-/-}$/*PRCP*$^{-/-}$ mice, Ang II infusion resulted in a similar increase in Ang-(1-7) as in WT (563±48 versus 537±70 fmol/mL, respectively), showing that the bulk of Ang-(1-7) formation in circulation is essentially independent of ACE2 and PRCP. By contrast, a POP inhibitor, Z-Pro-Prolinal reduced the rise in plasma Ang-(1-7) after infusing Ang II to control WT mice. In POP$^{-/-}$ mice, the increase in Ang-(1-7) was also blunted as compared with WT mice (309±46 and 472±28 fmol/mL, respectively P=0.01), and moreover, the rate of recovery from acute Ang II-induced hypertension was delayed (P=0.016). In ex vivo studies, POP inhibition with ZZP reduced Ang-(1-7) formation from Ang II markedly in serum and in lung lysates. By contrast, in kidney lysates, the absence of ACE2, but not POP, obliterated Ang-(1-7) formation from added Ang II. We conclude that POP is the main enzyme responsible for Ang II conversion to Ang-(1-7) in the circulation and in the lungs, whereas Ang-(1-7) formation in the kidney is mainly ACE2-dependent. **(*Hypertension*. 2020;75:173-182. DOI: 10.1161/HYPERTENSIONAHA.119.14071.)** • **Online Data Supplement**

Key Words: angiotensins ■ hypertension ■ renin-angiotensin system

The knowledge of the renin angiotensin system (RAS) has expanded with the identification of enzymes and peptides downstream of Ang II (Angiotensin II), such as Ang-(1-7), Ang III, and others.[1–23] Ang-(1-7) forming enzymes do not only prevent and counteract Ang II overactivity but also foster Ang-(1-7) formation eliciting potentially beneficial actions of the latter peptide.[7,11,14,15,24,25] There are 3 known carboxypeptidases that form Ang-(1-7) by cleaving Ang II: ACE2 (angiotensin-converting enzyme 2),[1–5,15] PRCP (prolylcarboxypeptidase),[19,20,26–28] and prolyloligopeptidase (POP/PEP/PREP/PE).[10,23,29–34] The biological importance of conversion of Ang II to Ang-(1-7) is 2-fold: by lowering Ang II, its potentially detrimental actions may be prevented; in addition, Ang-(1-7) is being formed and this peptide has tissue-protective actions that are generally opposite to the unwanted chronic effects of excessive Ang II. This highlights the importance of enzymes that cleave Ang II to form Ang-(1-7).

It is also known from the work of Yamamoto et al[34] that Ang-(1-7) can be formed directly from Ang I. The authors showed that, combined ACE (angiotensin-converting enzyme)/Neprilysin inhibition decreased Ang-(1-7) formation from Ang I infusion suggesting a critical role of Neprilysin on the formation of Ang-(1-7) directly from Ang I.[34] In this study, however, Ang II was not infused. The goal of our study is to examine the relative contribution of enzymes that form Ang-(1-7) directly from Ang II. Of those, ACE2, a homolog of ACE with carboxypeptidase activity that is able to cleave both Ang I and Ang II is the best studied.[20,26,35] The enzyme embodies a metalloprotease active site and is widely distributed but principally expressed in the kidney, the heart, the intestine, and the testis.[36–38]

Two other enzymes are known to form Ang-(1-7) directly from cleavage of Ang II: PRCP and POP.[32–38] PRCP is a serine carboxyprotease that cleaves the C-terminal amino acid of various peptides like Ang II, where the penultimate amino acid is Proline.[26] The enzyme's activity is extremely pH dependent with an optimum in the acidic range and essentially no activity at the physiological pH of plasma.[20,26,35] POP is a serine protease that is involved in the enzymatic degradation of several biologically active peptides such as Ang I and Ang II.[36–38] Due to its molecular structure, POP hydrolyses peptide hormones and neuropeptides, excluding high molecular weight peptides

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and proteins.[36] POP cuts at the C-side of an internal proline and can cleave Ang I to form Ang-(1-7), and Ang II to form Ang-(1-7).[23,29,37] Ang II contains proline at the seventh amino acid position and Phenylalanine (Phe) at the eighth position. Therefore, the action of POP at the C-terminal end results in the formation of Ang-(1-7) and a release of Phe. POP is an intracellular enzyme localized in the cytoplasm[38,39] but a membrane-bound form isolated from bovine brain has also been reported.[40] The function of POP has mainly been studied in brain tissue, as it could be linked to some neurodegenerative diseases,[41] and in neutrophil inflammation.[42,43] Enzymatic activity for POP has been detected in lymphocytes, thrombocytes, renal cortex, epithelial cells, fibroblasts, and testis.[38] POP activity also was reported in plasma, but a physiological role of circulatory POP has not been established.[44,45] In this study, we examined formation of Ang-(1-7) following the administration of Ang II and the impact of POP deficiency on acute Ang II-induced hypertension. In addition, we examined the relative contribution to Ang-(1-7) formation from Ang II by POP and ACE2 ex vivo in serum, kidney, and lung tissues.

Methods

Methods used will be made available from the corresponding author on reasonable request (see online-only Data Supplement).

Ang II Injection to Evaluate Ang-(1-7) Formation

Male WT mice (C57Bl/6 background) at 10 to 20 weeks of age were intraperitoneally injected with Ang II (0.2 µg/g body weight). In some studies, 5 minutes after the Ang II bolus blood was drawn by cardiac puncture under euthasol anesthesia (see section Results) and the mice were euthanized by induction of double pneumothorax. In other studies following intraperitoneal Ang II, injection blood was collected from the tail tip snapped at 5 and 30 minutes post-infusion (this is specified under results). The same injection protocol was used for POP$^{-/-}$ and ACE2$^{-/-}$/PRCP$^{-/-}$ mice (males, 20–24 weeks old).

In all the samples, Ang-(1-7) was measured by ELISA (see online-only Data Supplement). In addition, in selected samples, the levels of plasma Ang-(1-7) together with Ang II and Ang-(1-5) were measured by LC/MS-MS (Attoquant Diagnostics) because our ELISA and RIA do not distinguish isoforms of those peptides, namely Ang III and Ang-(2-7). A radioimmunoassay (Hypertension Core Lab, Wake Forest University School of Medicine) was also used in the same selected samples. This RIA uses a C-terminal directed antibody and cross-reacts with Ang-(2–7), and Ang-(3–7) with little or no cross-reactivity with Ang I.[46]

ZPP, a POP inhibitor was given IP together with Ang II at a dose (1 µg/g BW) calculated to give a circulatory ZPP concentration in the 10 µmol/L range. At this concentration, this inhibition is selective for POP whereas at higher concentration (>100 µmol/L) also inhibits PRCP.[23,47] The dose of ZPP (1 µg/g BW) has shown in our preliminary studies a nearly complete inhibition of serum POP activity.

Blood Pressure Measurement After Ang II Injection

The effect of POP deficiency on systolic blood pressure after Ang II infusion was studied in male POP$^{-/-}$ and WT mice (*C57Bl/6* genetic background) at 20 to 24 weeks of age. Systolic blood pressure was measured noninvasively in anesthetized mice by determining tail blood volume with a volume-pressure recording sensor and an occlusion tail-cuff using a computerized system (CODA System, Kent Scientific, Torrington, CT) as previously described[14–16] (see also online-only Data Supplement).

Statistical Analyses

For comparison of 2 independent groups, a 2-tailed *t* test was used for normally distributed data. For not normally distributed data, a Mann-Whitney *U* test was used. For comparison of >2 independent groups, 1-way ANOVA was employed followed by Tukey multiple comparisons test. The significance over time between groups was evaluated by GLM model (SPSS, version 23). Results are presented in mean±SE and a $P<0.05$ was considered statistically significant.

Results

In Vivo Formation of Ang-(1-7) in Plasma After Infusion of Ang II

To examine Ang-(1-7) formation from Ang II, Ang II was injected intraperitoneally to wild-type mice. After an Ang II bolus, blood was collected and plasma levels of Ang-(1-7) 5 minutes later were taken as evidence of rapid formation of this peptide from infused Ang II.

In control animals (n=5) which did not receive an Ang II bolus, plasma Ang II levels measured by ELISA were low (8.8±1.8 fmol/mL), whereas in Ang II-infused animals (n=6) Ang II levels at 5 minutes were about 100-fold higher: (968±213 fmol/mL). The infusion of Ang II resulted in markedly elevated plasma Ang-(1-7) levels (1268±439 fmol/mL), measured by ELISA. To confirm the extremely high levels of plasma Ang-(1-7) after Ang II infusion, additional measurements of Ang-(1-7) were performed by RIA in the same plasma samples from Ang II-infused mice. The values of Ang-(1-7) obtained by RIA were also high and not significantly different from those obtained by ELISA (1702±268 and 1268±439, fmol/mL, respectively).

Plasma Ang II and Ang-(1-7) concentrations were additionally evaluated in the same Ang II postinfusion samples using liquid chromatography tandem mass spectrometry.[16] In addition, a major metabolite, the Ang-(1-5), formed from Ang-(1-7) was also measured by liquid chromatography tandem mass spectrometry (Figure 1). As previously reported,[16] noninfused animals had barely detectable levels of Ang II (7.5±1.9 fmol/mL), Ang-(1-7; 3.9±0 fmol/mL), and Ang-(1-5; 3.0±0 fmol/mL). In Ang II infused mice, Ang II and Ang-(1-7) were both markedly elevated (233±20 and 854±222 fmol/mL, respectively; Figure 1). This is consistent with the high levels of these peptides measured by ELISA and RIA following Ang II infusion. A high level of Ang-(1-5), moreover, was also found (233±24 fmol/mL; Figure 1). This shows that plasma Ang-(1-7) is formed at a high rate when there is a large supply of Ang II and that Ang-(1-7) is further degraded to Ang-(1-5).

In Vivo Formation of Ang-(1-7) After Ang II Infusion in a Model of Genetic Ablation of ACE2 and PRCP

We next examined Ang-(1-7) formation in a model of genetic ablation of 2 known Ang-(1-7) forming enzymes, ACE2 and PRCP. For this, we used a cross of ACE2 KO with PRCP KO that we have generated in our laboratory (see section Methods). Plasma levels of Ang II at 5 minutes post infusion were high and not significantly different between WT and ACE2$^{-/-}$/PRCP$^{-/-}$ mice (1229±307 and 783±166 fmol/mL, respectively $P=0.3$; Figure 2A). Plasma Ang-(1-7) levels also measured 5 minutes after Ang II infusion were markedly elevated and also not significantly different between WT and ACE2$^{-/-}$/PRCP$^{-/-}$ (563±51 and 537±70, fmol/mL,

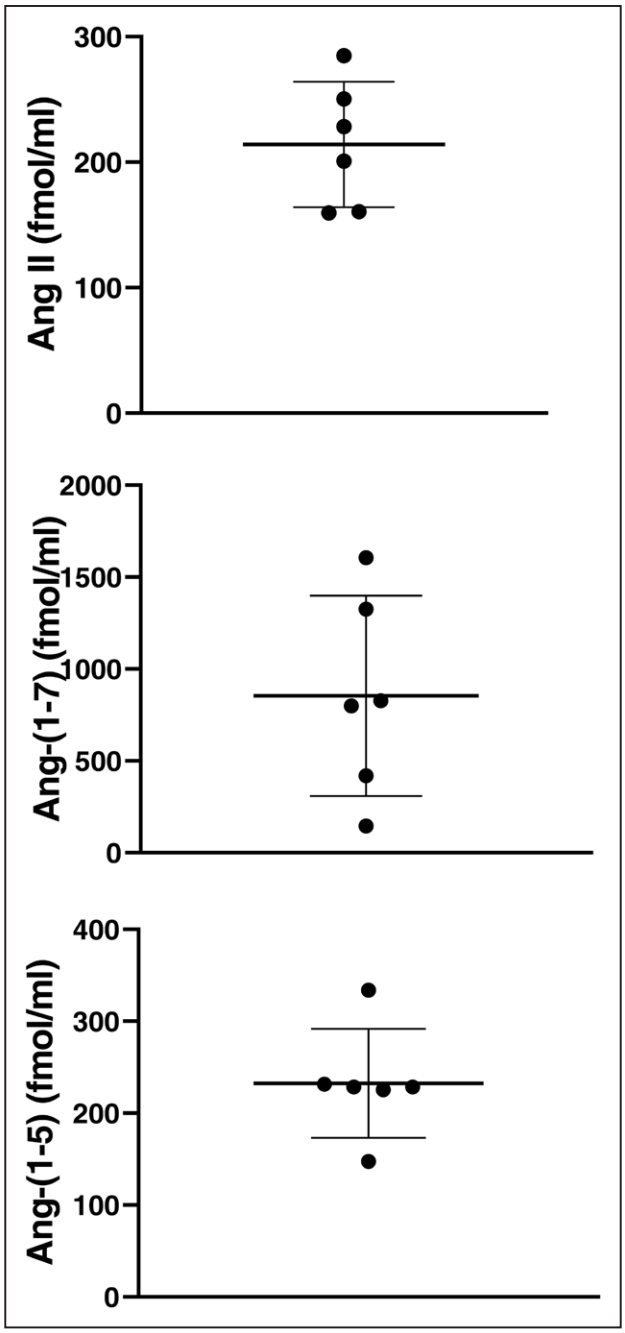

Figure 1. Angiotensin peptide levels in serum measured by liquid chromatography tandem mass spectrometry (LC/MS-MS) in Ang II (Angiotensin II)-infused mice. Plasma showed high levels of Ang II (upper), Ang-(1-7) (middle), and Ang-(1-5) (lower) measured by (LC/MS-MS). Blood was obtained by cardiac puncture from mice (n=6) at 5 min after Ang II i.p. infusion.

respectively; *P*=0.8; Figure 2B). These in vivo findings show that Ang-(1-7) formation from Ang II in plasma is essentially ACE2- and PRCP-independent.

In Vivo Formation of Ang-(1-7) After Ang II Infusion in the Face of Pharmacological POP Inhibition

In these studies, we used ZPP, an inhibitor of POP, given to WT and ACE2$^{-/-}$/PRCP$^{-/-}$ mice infused with Ang II Plasma levels of Ang II at 5 minutes post infusion were markedly elevated and not significantly different from each other (1651±379 and 1004±169 fmol/mL respectively; *P*=0.3; Figure 2A). The administration of ZPP largely prevented the elevated Ang-(1-7) levels seen at 5 minutes as compared with WT not infused with this inhibitor (186±60 and 563±51 fmol/mL, respectively; *P*=0.001; Figure 2B). Similarly, in ACE2$^{-/-}$/PRCP$^{-/-}$ mice, ZPP administration also resulted in markedly lower Ang-(1-7) plasma levels as compared with mice without ZPP infusion (50±30 and 537±70 fmol/fmol/mL, respectively; *P*=0.0002; Figure 2B). In WT and ACE2$^{-/-}$/PRCP$^{-/-}$ mice treated with ZPP, levels of Ang-(1-7) were reduced and not significantly different from each other (*P*=0.08).

By 30 minutes post-infusion (Figure S3A in the online-only Data Supplement), the levels of plasma Ang II had markedly decreased in all 4 groups studied. Likewise, the levels of plasma Ang-(1-7) had decreased markedly in WT and WT treated with ZPP, as well as in ACE2$^{-/-}$/PRCP$^{-/-}$ The latest group had levels of Ang-(1-7) which were barely detectable (see Figure S3B).

Plasma Ang II and Ang-(1-7) Levels in WT and POP$^{-/-}$ Mice

Baseline Ang II levels in POP$^{-/-}$ mice were higher but not significantly different from WT mice (57.8±14 and 31.1±4.4 fmol/mL, respectively; *P*=ns; Figure 3A). Plasma Ang-(1-7) levels in WT mice were significantly higher than in the POP$^{-/-}$ where the levels were not detectable (25.9±11.3 and 0.0 fmol/mL; *P*=0.032; Figure 3B**).**

In Vivo Formation of Ang-(1-7) After Ang II Infusion in Mice With Genetic POP Ablation

To confirm the effects of ZPP, we next examined Ang-(1-7) formation in POP$^{-/-}$ mice after Ang II infusion using the same protocol as in the sections above. In both WT and POP$^{-/-}$ mice, plasma Ang II 5 minutes after its infusion was elevated and significantly higher in the POP$^{-/-}$ than in the WT mice (1120±93 versus 596±234 fmol/mL, respectively *P*=0.027; Figure 3C). This suggests a major role of POP in the degradation of Ang II as in previous protocols the levels of Ang II after its infusion were not significantly different than those observed in the ACE2$^{-/-}$/PRCP$^{-/-}$ mice. After Ang II infusion, POP$^{-/-}$ mice exhibited lower plasma Ang-(1-7) levels than WT mice (309±46 and 472±28 fmol/mL, respectively; *P*<0.01; Figure 3D). Plasma Ang-(1-7) to Ang II ratio after Ang II infusion was also markedly lower in the POP$^{-/-}$ as compared with the WT (0.23±0.02 vs 1.53±0.48, respectively; *P*=0.017). These findings show that genetic POP deficiency is associated with a significantly reduced ability to convert Ang II to Ang-(1-7) in plasma in vivo. This results in higher levels of Ang II and lower levels of Ang-(1-7) as compared with WT mice.

Blood Pressure Response to Ang II Infusion in POP$^{-/-}$ Mice

The effect of Ang II bolus injection (0.2 µg/g BW) on systolic blood pressure was examined in POP$^{-/-}$ and WT mice (n=10 in each group) under light anesthesia. Systolic BP peaked in the first minute after Ang II infusion and was not significantly different between POP$^{-/-}$ and WT mice (189±5 versus 186±8 mm Hg; Figure 3E). The recovery from Ang

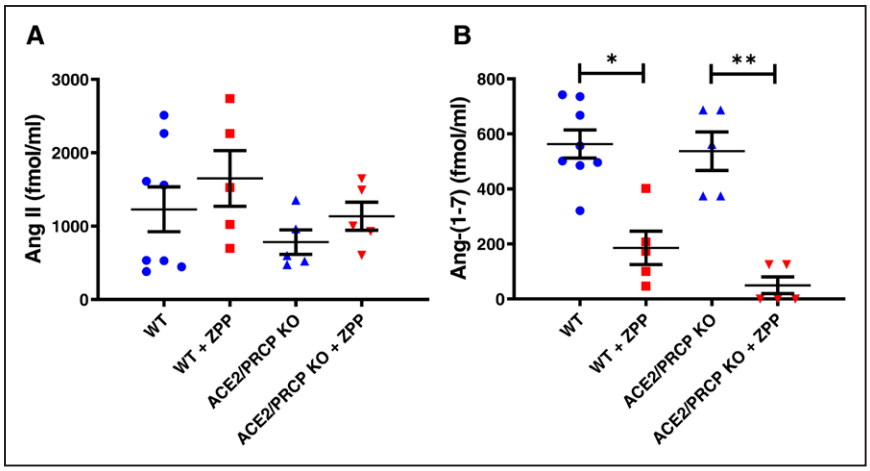

Figure 2. Ang II (Angiotensin II) and Ang-(1-7) levels in plasma measured 5 min post-infusion of Ang II. **A**, Plasma Ang II levels in wild-type (WT) and ACE2$^{-/-}$/PRCP$^{-/-}$ mice were not significantly different from each other in the absence or with administration of Z-Pro-Prolinal (ZPP). **B**, Ang-(1-7) levels in WT mice with ZPP administration (WT+ZPP) were markedly reduced as compared with WT without ZPP (WT). In the ACE2$^{-/-}$/PRCP$^{-/-}$ mice, ZPP administration also led to much lower Ang 1-7 levels as compared with ACE2$^{-/-}$/PRCP$^{-/-}$ mice without ZPP (*P<0.05; **P<0.01). In these studies, blood was obtained from tail vein at 5 min and also at 30 min (see online-only Data Supplement) after Ang II.

II-induced acute hypertension was blunted in POP$^{-/-}$ as compared with WT mice (slope 3.06±0.48 versus 4.81±0.45 mm Hg/minute, respectively; P<0.05). This is consistent with higher levels of Ang II and lower levels of Ang-(1-7) found in POP$^{-/-}$ mice as compared with WT mice shown in (Figure 3C and 3D).

Ex Vivo Formation of Ang-(1-7) From Ang II in Kidney and Lung Lysates

Since enzymes residing in highly perfused organs, such as kidneys and lungs, might also contribute to circulating Ang-(1-7) levels, ex vivo studies on Ang-(1-7) formation from exogenous Ang II were performed in lysates from those 2 organs. In WT,

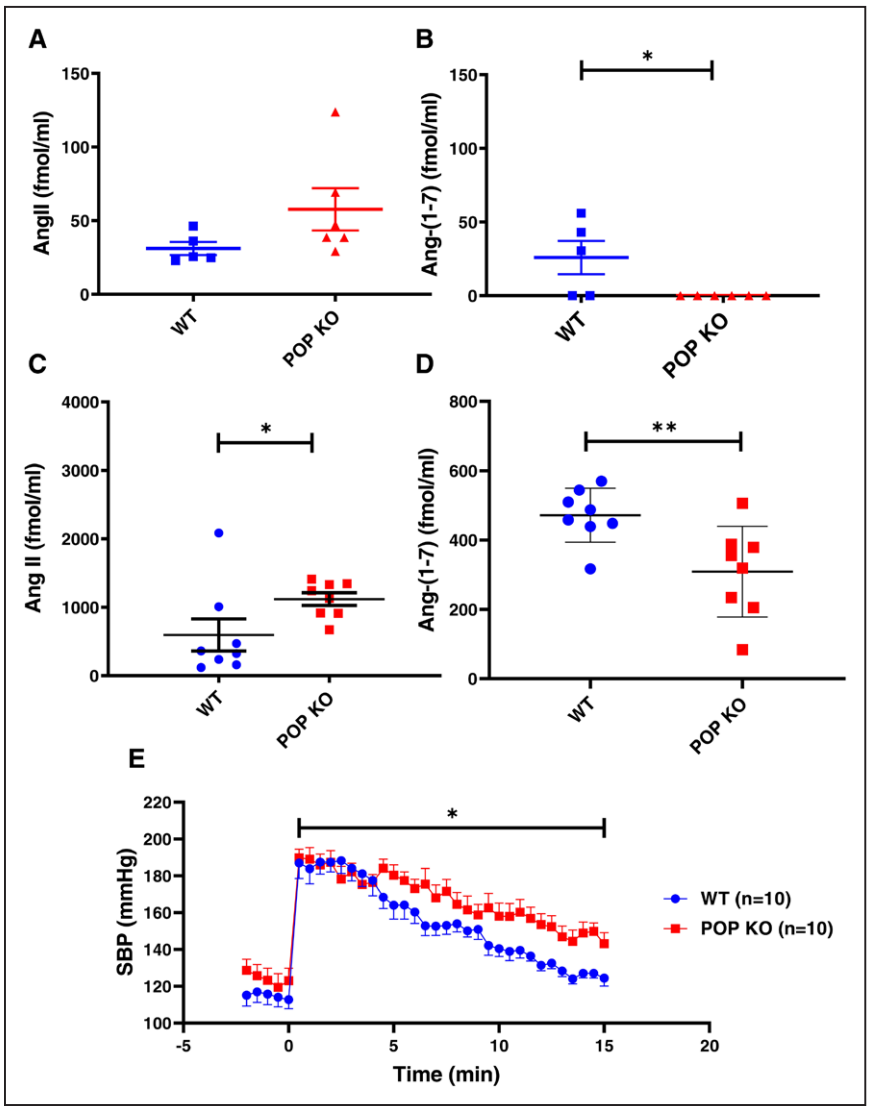

Figure 3. Studies in POPKO mice. **A**, Endogenous Ang II (Angiotensin II) levels in plasma of POP$^{-/-}$ mice were higher but not significantly different from wild-type (WT) controls. **B**, Endogenous plasma Ang-(1-7) levels in POP$^{-/-}$ mice were not detectable whereas in WT mice the levels were low, but detectable (P=0.032). **C**, Ang II levels in plasma measured 5 min after i.p. injection of Ang II were significantly higher in POP$^{-/-}$ mice (n=8) than in WT mice (*P<0.05). **D**, Ang-(1-7) levels in plasma also measured 5 min after i.p. injection of Ang II were lower in POP$^{-/-}$ mice than in WT mice (**P<0.01). Baseline levels were measured in blood obtained by cardiac puncture while post-Ang II infusion blood was obtained by tail snip. **E**, Systolic blood pressure (SBP) after single i.p. bolus injection of Ang II (time point 0) under light ketamine anesthesia WT and POP$^{-/-}$ mice (n=10 in each group). The SBP was recorded every 30 s for 15 min after Ang II injection. The slope of SBP decline was significantly slower in POP$^{-/-}$ than in WT (3.06±0.48 vs 4.81±0.45 mm Hg/min, respectively, *P<0.05.)

kidneys (n=10) spiked with Ang II, Ang-(1-7) levels peaked at 15 minutes (Figure 4) As depicted in one example under methods (Figure S2) by 15 minutes, there is a rapid decline in Ang II such that by 60 minutes essentially all of the added Ang II has dissipated. ZPP had a minimal and not significant effect on Ang-(1-7) formation at 15 minutes as compared with WT kidneys not incubated with this inhibitor (417±32 versus 352±75, pmol/mL, respectively; *P*=0.3). To confirm the lack of effect of ZPP in the kidney tissue, we used another inhibitor of POP, S-17092.[48,49] In WT kidney lysates, the latter inhibitor also did not reduce Ang-(1-7) formation significantly (peak at 15 minutes: 417±32 versus 385±35 pmol/mL; *P*=ns).

In kidney lysates from ACE2$^{-/-}$/PRCP$^{-/-}$ mice, the peak Ang-(1-7) levels were much lower than in WT (94±18 versus 417±32 pmol/mL; Figure 4A). Addition of a POP inhibitor, ZPP (n=7), reduced Ang-(1-7) to undetectable levels at all time points assessed (Figure 4A). In the aggregate, the data show that the formation of Ang-(1-7) in mouse kidneys is highly dependent on ACE2 and minimally dependent on POP.

In lung lysates from WT mice, the Ang II decline was slower than with kidney lysates when equivalent amounts of both tissues were exposed to the peptide (Figure S2). The levels of Ang-(1-7) after 15 minutes of incubation with Ang II declined at 30 minutes and reached almost undetectable levels at 60 minutes of incubation (Figure 4B). Generation of Ang-(1-7) in lung lysates from ACE2$^{-/-}$/PRCP$^{-/-}$ mice was not significantly different from that of WT lung lysates (peak values at 15 minutes, 313±31 versus 230±33 pmol/mL, respectively; *P*=0.09; Figure 4B). Addition of ZPP completely prevented Ang-(1-7) formation from Ang II in lung lysates from both WT and ACE2$^{-/-}$/PRCP$^{-/-}$ mice (Figure 4B). Using another POP-inhibitor, S-17092, the peak Ang-(1-7) formation at 15 minutes was also prevented entirely (3±3 versus 182.8±23 pmol/mL, respectively; Figure S4A). These findings show that in mouse lungs, Ang-(1-7) formation from Ang II is essentially ACE2 independent and entirely prevented by POP inhibitors. We also measured for comparison the formation of Ang-(1-7) from exogenous Ang I in lung tissue (Figure S4B). Inhibition of POP with S-17092 significantly, but incompletely reduced the peak formation of Ang-(1-7). An inhibitor of Neprilysin (Thiorphan) also reduced the peak level of Ang-(1-7) from Ang I to an extent similar to S-17092. The combination of both inhibitors markedly reduced Ang-(1-7) formation (Figure S4B).

To further determine the contribution of POP in the formation of Ang-(1-7) from Ang II, we used kidney and lung tissues from POP$^{-/-}$ mice and their respective WT counterparts (Figure 4C and 4D). In kidneys from WT and POP$^{-/-}$ mice, Ang-(1-7) formation from Ang II over time was almost identical (Figure 4C). The specific ACE2 inhibitor, MLN-4760 (10^{-5} M) completely obliterated generation of Ang-(1-7) from Ang II (Figure 4C). In contrast to kidney lysates, lung lysates from POP$^{-/-}$ mice showed almost undetectable Ang-(1-7) formation from Ang II (Figure 4D). Addition of MLN had no effect in the POP$^{-/-}$ lungs. In summary, in kidneys lysates, Ang-(1-7) formation is predominantly ACE2-dependent whereas in lung lysates the Ang-(1-7) formation from Ang II is essentially ACE2-independent and entirely POP dependent.

Ex Vivo Formation of Ang-(1-7) From Ang II in Mouse Serum

To determine the contribution of POP to Ang-(1-7) generation in serum, Ang-(1-7) formation from added Ang II was studied ex vivo with (n=6) and without (n=6) addition of ZPP (10^{-5} M; Figure 5A). Incubation of the WT sera with Ang II resulted in formation of Ang-(1-7) that peaked at 15 minutes and was no longer detectable at 120 minutes of incubation (Figure 5A). At 15 minutes, ZPP lowered Ang-(1-7) formation by 85.8% (4.5±4.5 versus 31.8±6.3 pmol/mL; *P*=0.01), and at 30 minutes by 72% (8.9±4.5 versus 31.8±6.7 pmol/mL; *P*=0.01), respectively.

We next evaluated the contribution of ACE2, PRCP, and POP to ex vivo conversion of Ang II to Ang-(1-7) in sera obtained from mice deficient in those enzymes. For this, Ang

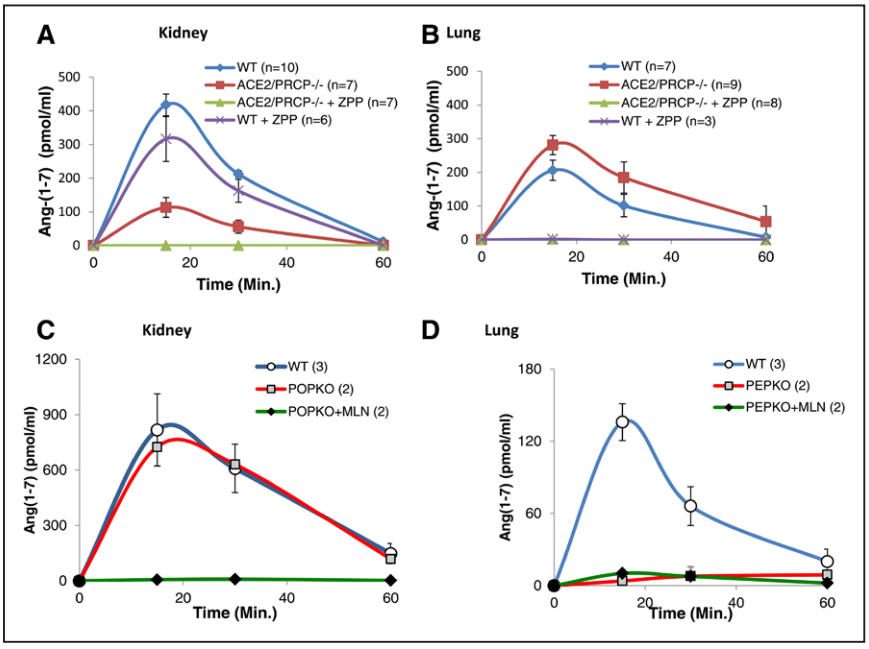

Figure 4. Ex vivo Ang-(1-7) formation from Ang II (Angiotensin II) in kidney and lung lysates as assessed by the peak levels of Ang-(1-7) at 15 min (see section Methods). Experiments with each lysate were performed on 2 different occasions in duplicate. **A**, In wild-type (WT) kidney lysates, the POP inhibitor, Z-Pro-Prolinal (ZPP), did not affect Ang-(1-7) formation from Ang II significantly as compared with WT lysates without ZPP. In ACE2$^{-/-}$/PRCP$^{-/-}$ kidney lysates, Ang-(1-7) was markedly decreased. In ACE2$^{-/-}$/PRCP$^{-/-}$ kidney lysates, ZPP addition decreased Ang-(1-7) to essentially nondetectable levels. **B**, In WT lung lysates, ZPP completely prevented Ang-(1-7) formation from Ang II (compare WT and WT+ZPP). In ACE2$^{-/-}$/PRCP$^{-/-}$ lung lysates, Ang-(1-7) levels were not reduced (actually, they were higher) and ZPP resulted in undetectable Ang-(1-7) levels. **C**, In POP$^{-/-}$ kidney lysates, Ang-(1-7) formation from Ang II was not different from that of WT lysates. In contrast, in POP$^{-/-}$ kidney lysates, an addition of MLN-4760, a specific inhibitor of ACE2, was associated with no detectable Ang-(1-7) formation from Ang II. **D**, In POP$^{-/-}$ lung lysates, Ang-(1-7) formation was completely prevented as compared with WT lung lysates and addition of MLN-4760 had no further effect.

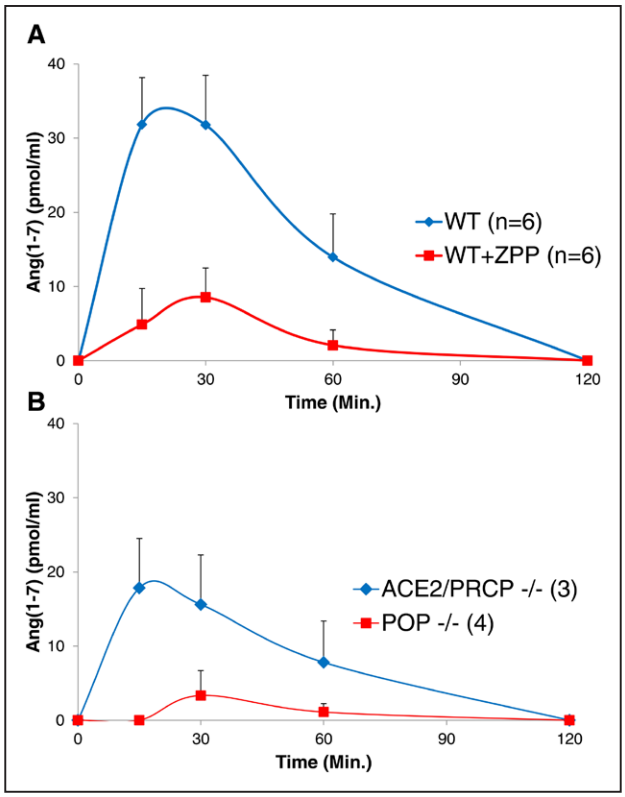

Figure 5. Ex vivo formation of Ang-(1-7) from Ang II (Angiotensin II) in serum. Ex vivo Ang-(1-7) formation after incubation with Ang II in sera from (**A**) wild-type (WT) mice, without Z-Pro-Prolinal (ZPP) and with ZPP and (**B**) serum from from ACE2$^{-/-}$/PRCP$^{-/-}$ mice and POP$^{-/-}$ mice. **A**, In wild-type (WT) sera (n=6) in the presence of the dual POP (prolyloligopeptidase)/PRCP (prolylcarboxypeptidase) inhibitor, ZPP (10^{-5} M), Ang-(1-7) formation from Ang II is markedly lower than in the absence of ZPP (P<0.01 by GLM statistics over time). **B**, In sera from POP$^{-/-}$ mice (n=4), Ang-(1-7) formation from Ang II is markedly lower than in the sera from ACE2$^{-/-}$/PRCP$^{-/-}$ mice (n=3; P<0.01 by GLM statistics over time).

II was incubated with sera from the various KO models and the resultant formation of Ang-(1-7) was compared. Incubation of the sera from ACE2$^{-/-}$/PRCP$^{-/-}$ mice with Ang II resulted in essentially the same formation of Ang-(1-7) as in WT mice (Figure 5B). There was no significant difference between WT mice (n=6) and ACE2$^{-/-}$/PRCP$^{-/-}$ (n=3) at 30 minutes (32±6 versus 17±7 pmol/mL, respectively; P=ns) or at 15 minutes (31.8±6.7 versus 17.8±6 pmol/mL, respectively; P=ns), which is the peak of Ang 1-7 formation.

In contrast, the amount of Ang-(1-7) formed from Ang II by the sera from POP$^{-/-}$ mice was markedly reduced compared with both ACE2$^{-/-}$/PRCP$^{-/-}$ mice and WT mice (P<0.01; Figure 5B).

ACE2 and POP Proteins in WT Sera, Lung, and Kidneys

By Western blot, ACE2 protein was not detectable either in serum or in the lungs but was abundant in kidneys of WT mice (Figure 6A). In contrast, POP protein was present in serum, lungs, and kidney lysates (Figure 6A).

In Vitro Ang-(1-7) and Phe Formation From Ang II

Conversion of Ang II to Ang-(1-7) is associated with a release of its carboxyterminal amino acid, Phe, that is directly proportional to the amount of the generated Ang-(1-7).[50] We assessed the reaction by both the formation of Ang-(1-7; Figure 6B) and the Phe formation (Figure 6C) after incubating Ang II with equivalent amounts of murine recombinant (mr) POP and mrACE2 in vitro.

Murine rACE2 formed more Ang-(1-7) than mrPOP (5.3±0.4 versus 1.3±0.6 pmol/mL; P<0.001; Figure 6B). In agreement with this finding, Phe generation by mrACE2 was much higher than that formed by equivalent amount of mrPOP (789±29 RFU/ng mrACE2 versus 127±12 RFU/ng mrPOP; P<0.001; Figure 6C). These data show that rACE2 is intrinsically considerably more effective in catalyzing the conversion of Ang II to Ang-(1-7) than rPOP.

Discussion

The ACE2/Ang-(1-7) axis of the RAS has received increased attention and ACE2 is generally considered the main Ang-(1-7) forming enzyme from Ang II. There are 2 other known angiotensinases, PRCP (prolylcarboxypeptidase) and (POP) prolyl endopeptidase, which have not been as extensively studied but could be as important as ACE2 in the processing of Ang II to Ang-(1-7) in body fluids, certain organs, and cell types.[10,19,22,23,29–33] In the current study, the conversion of Ang II to Ang-(1-7) by these enzymes was examined in vivo and ex vivo.

We found similarly high levels of Ang-(1-7) in plasma being generated following acute Ang II injections to WT mice as in mice with combined genetic ACE2 and PRCP deficiency. By contrast, reduced plasma Ang-(1-7) levels were found in mice with genetic POP deficiency infused with Ang II. Furthermore, the administration of ZPP, an inhibitor of PEP, markedly decreased plasma Ang-(1-7) formation from infused Ang II in WT and ACE2$^{-/-}$/PRCP$^{-/-}$ mice. Our study, therefore, has demonstrated that the levels of Ang-(1-7) found in the circulation immediately after Ang II infusion are largely POP dependent and ACE2 and PRCP independent. It should be noted that Ang-(1-7) formation in vivo can originate from Ang I cleavage.[34] As demonstrated in a study by Yamamoto et al,[34] the formation of circulatory Ang-(1-7) from infused Ang I was entirely Neprilysin dependent. While it is important to note the there is direct formation of Ang-(1-7) from Ang I, in the present study, we are mainly concerned with the relative importance of enzymes that cleave Ang II to form Ang-(1-7).

The physiological importance of POP in terms of systemic blood pressure was examined in a model of acute hypertension induced by Ang II infusion using the same dosing and timing as in the studies examining the conversion of Ang II to Ang-(1-7). In this model, the blood pressure (BP) response was altered in mice with the genetic absence of POP as shown by a blunted recovery from Ang II-induced hypertension This can be attributed to the diminished Ang II degradation and Ang-(1-7) formation in the absence of POP. It is not possible to precisely discern the contribution of Ang II (pro-hypertensive) or Ang-(1-7; antihypertensive) or both on the BP response in this model. Of note, in the POP$^{-/-}$ model, the peak level of Ang II after its infusion was higher than in WT. By contrast, it was not significantly different between WT and ACE2$^{-/-}$/PRCP$^{-/-}$ mice. As previous studies have shown no effect or only a marginal effect of Ang-(1-7) on BP,[15,51] we surmise that

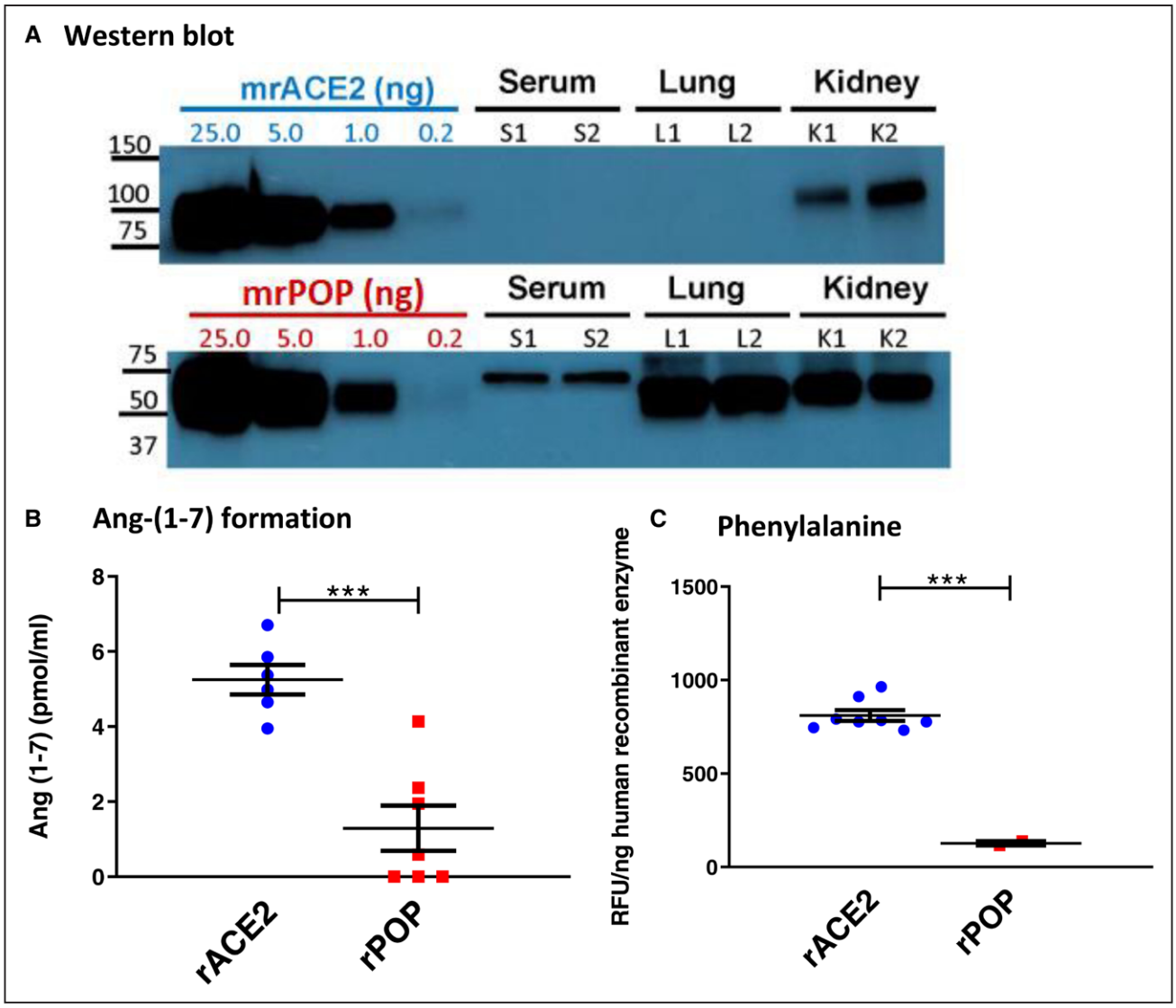

Figure 6. ACE2 (angiotensin-converting enzyme 2) and POP proteins in wild-type (WT) sera, lung, and kidneys and the effect of rACE2 and rPOP on Ang-(1-7) and Phenylalanine formation. **A**, ACE2 and POP proteins in WT sera, lung, and kidneys by Western blot. ACE2 protein is abundant in kidneys, but not in serum or lungs. POP protein is present in serum, lungs, and kidneys. **B**, In vitro Ang II (Angiotensin II) to Ang-(1-7) conversion assessed by equivalent amounts of recombinant (r) ACE2 and rPOP is higher with rACE2 than with rPOP (***P<0.001). **C**, generation of free Phenylalanine (Phe) from Ang II as a substrate by equivalent amounts of recombinant (r) ACE2 and rPOP is higher with rACE2 than with rPOP (***P<0.001). For Western blot, different amounts of mouse recombinant protein standards were loaded to estimate ACE2 and POP protein expression levels in sera (1 µL), lung, and kidney lysates (50 µg total protein) from 2 WT mice.

in this model of acute Ang II-induced hypertension, the sluggish recovery observed in POP$^{-/-}$ mice was largely attributable to decreased rate of Ang II degradation as compared with the WT after Ang II infusion. It should be noted that POP is also involved in the metabolism of other vasoactive peptides such as bradykinin. Therefore, in our POP$^{-/-}$ model, the changes observed in blood pressure response might be influenced by RAS independent mechanisms. It should also be acknowledged that the i.p. route gives more time for extravascular processing of Ang II to Ang-(1-7) outside the circulation as compared with the i.v. route.

To gain further insight into where in the body the conversion of Ang II to Ang-(1-7) may take place after Ang II infusion, we performed ex vivo studies in plasma, lung, and kidney lysates. We found that POP protein was present in substantial amounts in mouse serum by western blot. ACE2, by contrast, was scarcely present in serum, which is consistent with previous reports.[14,15,52,53] In sera from WT mice with the POP inhibitor, ZPP, formation of Ang-(1-7) from added Ang II, moreover, was markedly diminished. Consistent with this finding, a marked attenuation of Ang-(1-7) formation was also observed in sera from mice with genetic POP deficiency. The presence of POP in plasma and its ability to form Ang-(1-7) demonstrated in these studies supports the role of this enzyme as a contributor to Ang-(1-7) formation in vivo. This does not mean, however, that POP resident in plasma accounts predominantly for the rapid conversion of Ang II to Ang-(1-7). POP could originate from the vascular endothelium and there is evidence that endothelial cells in culture have POP.[54] In addition, circulating cells such as macrophages that are rich in this enzyme[55] could contribute to the conversion of Ang II to Ang-(1-7) after Ang II infusion.

Another possibility that we think most likely is that POP in plasma comes from the lung circulation, similar to circulating plasma ACE.[56] This issue was partly addressed by examining Ang-(1-7) formation from exogenous Ang II in ex vivo studies using lung lysates. Our ex vivo studies provide compelling evidence for POP as the main Ang-(1-7) forming enzyme from Ang II in the lungs whereas ACE2 and PRCP have only a marginal if any effect in this organ. According to the Human Protein Atlas, ACE2 protein expression was not detectable in human pneumocytes or in alveolar macrophages (https://www.proteinatlas.org/ENSG00000130234-ACE2/tissue). ACE2 mRNA expression was also low and mainly localized in pneumocytes and endothelial cells. Other studies show also low ACE2 mRNA and protein expression in rodent lungs.[57,58] Our results are therefore consistent with these previous studies showing low lung ACE2 activity.

In sharp contrast to the lungs, in mouse kidneys, the conversion of Ang II to Ang-(1-7) was almost entirely ACE2-dependent. Kidney lysates from ACE2$^{-/-}$/PRCP$^{-/-}$ showed markedly lower Ang-(1-7) generation from Ang II than those of WT kidneys. In POP$^{-/-}$ kidney lysates, which exhibited almost identical generation of Ang-(1-7) from Ang II as the WT kidneys, a specific ACE2 inhibitor (MLN-4760) completely abrogated the formation of Ang-(1-7). These ex vivo studies overall show that ACE2 is responsible for the formation of Ang-(1-7) from Ang II in mouse kidney, which is consistent with findings reported for human kidneys.[59] In addition, in our study, ZPP did not reduce Ang-(1-7) formation from Ang II in kidney lysates from WT mice suggesting minimal, if any, effect of PRCP on Ang-(1-7) formation from Ang II by the kidney at a physiological pH. One cannot exclude, however, the possibility that PRCP could play an important role in Ang II to Ang-(1-7) conversion in certain regions of the kidney where acidic conditions prevail, such as the lumen of the collecting duct. In fact, PRCP is abundant in the kidney collecting tubules, where the prevailing pH is low.[20]

The relative potency of each of the Ang-(1-7) forming enzymes and their kinetic properties need to be taken into consideration together with the relative site abundance of each enzyme. About 10× higher catalytic constant for the conversion of Ang II to Ang-(1-7) for ACE2 than POP was previously reported by Chappell et al.[60] Earlier studies by Ward et al documented the kinetic parameters of POP-related Ang II conversion. In these studies, the calculated K_m (5.7±0.9 µmol/L) and V_{max} (1.70±0.09 µmol×minute^{-1}×mg^{-1}) values for POP with Ang II as a substrate[61] allowed us an approximation of POP catalytic efficiency (kcat/Km) to be ≈5× to 10× lower than those reported for human ACE2.[4,5] Using 2 independent methods: Ang-(1-7) formation and Phe generation[50] (which is a cleavage by-product of Ang II to Ang-(1-7) conversion)[50] we found, moreover, that ACE2 is much more efficient than POP which is consistent with findings of Chappell et al.[60] We show that both enzymes are present in the kidney by western blot but the higher potency of ACE2 confers much greater capacity for Ang-(1-7) formation from Ang II in this organ. Moreover, one has to take into account the subcellular localization of the enzymes. POP is mainly a cytosolic enzyme but also has been reported to have membrane-bound isoforms.[62,63] As a limitation of this study, we wish to point out that the doses of Ang II used to demonstrate Ang-(1-7) generation are high relative to the levels of Ang II normally present in plasma. Peptidases, however, are most efficient at their Km values and the dose of ANG II used in our studies was only twice the Km for POP and ACE2. While the balance between these 2 peptidases in the metabolism of Ang II that we have demonstrated under conditions of high Ang II levels, both in vivo and ex vivo, may not mimic the physiological levels of Ang II, the doses of this peptide used are appropriate to demonstrate Ang (1-7) formation. The fact that in the POP KO the levels of Ang II were higher than in the corresponding WT controls supports, moreover, the importance of POP in the metabolism of this peptide in vivo. In contrast, the baseline levels of Ang II were not higher in ACE2 KO mice as compared with wild type.[64]

Our study did not address the possible function of POP within the kidney or the lungs. As the conversion of Ang II to Ang-(1-7) within the kidney is largely POP-independent, it is possible that this enzyme exerts kidney actions unrelated to this conversion. For instance, POP has been reported to foster antifibrotic processes in kidney tissue by releasing N-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP) from thymosin-β4.[65] By contrast, POP is the main Ang-(1-7) forming enzyme in the lungs and moreover the lung circulation is possibly a major source of POP protein for conversion of Ang II to Ang-(1-7) in the circulation. Myöhänen et al[66] reported that POP can be localized in several human peripheral tissues including kidneys and lungs. Further studies ideally using human tissues and human plasma are needed to elucidate the physiological function of the POP/Ang-(1-7) axis. Our findings show that conversion of Ang II to Ang-(1-7) in the circulation and lungs is essentially POP-dependent and ACE2-independent. In contrast, this reaction in the kidney is predominantly mediated by ACE2. These findings may have important implications for therapeutic purposes when targeting Ang-(1-7) formation from Ang II in the circulation and specific organs. Future studies are also needed to fully evaluate the kidney and cardiovascular phenotype of POP$^{-/-}$ mice.

Perspectives

There are at least 3 enzymes known to form Ang-(1-7) directly from AngII, but their relative importance in the circulation and various organs is not known.This is the first study to demonstrate that conversion of Ang II to Ang-(1-7) in the circulation and lungs is essentially POP-dependent and ACE2-independent. In contrast, this conversion in the kidney is predominantly mediated by ACE2. Deficiency of POP by slowing down the dissipation of Ang II in the circulation may predispose to hypertension. Our findings therefore may have important implications for therapeutic purposes when targeting Ang-(1-7) formation from Ang II in the circulation and specific organs.

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Disclosures

D. Batlle and J. Wysocki are co-inventors of patent 10,443,049. D. Batlle is founder/owner of Angiotensin Therapeutics, Inc.

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Novelty and Significance

What Is New?

- The first study to characterize ACE2 (angiotensin-converting enzyme 2)- and PRCP (prolylcarboxypeptidase)-independent Ang-(1-7) formation from Ang II (Angiotensin II) in circulation and peripheral tissues using ACE2$^{-/-}$/PRCP$^{-/-}$ and POP$^{-/-}$ genetic mouse models and pharmacological inhibitors.

What Is Relevant?

- Circulatory and pulmonary Ang-(1-7) formation from exogenous Ang II is essentially ACE2 independent and POP dependent while in kidneys the conversion is mainly dependent on ACE2.
- Blood pressure recovery from acute Ang II-induced hypertension is delayed in POP-deficient mice.

Summary

This study demonstrates the relevance of the POP/Ang-(1-7) axis in the circulation and in the lungs while confirming the importance of ACE2 in kidney Ang-(1-7) formation from Ang II.