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DISSERTATION

Angiotensin-Converting Enzyme 2- and Prolyl Carboxypeptidase-Independent Conversion of Angiotensin II to Angiotensin-(1-7) in Circulation and Peripheral Tissues

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1. List of abbreviations

α-MSH	α-Melanocyte-stimulating hormone
ACE	Angiotensin-Converting Enzyme
ACE2	Angiotensin-Converting Enzyme 2
Ang-(1-7)	Angiotensin-(1-7)
Ang I	Angiotensin I
Ang II	Angiotensin II
AT_1R	Angiotensin II type 1 receptor
ELISA	Enzyme-linked Immunosorbent Assay
IC50	half maximal inhibitory concentration
i.p.	intraperitoneal
IP_3	inositol 1,4,5-triphosphate
mr	murine recombinant
PAL	Phenylalanine Ammonia Lyase
POP	Prolyl Oligopeptidase
PRCP	Prolyl Carboxypeptidase
PVDF	polyvinylidene fluoride
SARS	severe acute respiratory syndrome
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
XNT	1-[(2-dimethylamino) ethylamino]-4-(hydroxymethyl)-7-[(4-methylphenyl) sulfonyl oxy]-9H-xanthene-9-one
ZPP	Z-Pro-Prolinal

2. Abstract (Deutsch)

Das Renin-Angiotensin System (RAS) ist ein essentieller hormoneller Regelkreis, der an der Physiologie und Pathophysiologie der Blutdruck- und Elektrolytregulation beteiligt ist. Chronischer Überschuss an Angiotensin II (Ang II), dem zentralen Effektorpeptid des RAS, löst Vasokonstriktion, Natriumretention, Fibrose und Entzündung aus. Gegenseitige, kardiovaskulär protektive Effekte werden durch das neulich entdeckte Heptapeptid Angiotensin-(1-7) [Ang-(1-7)] und seinen Rezeptor Mas beobachtet. Drei Enzyme sind zur Zeit bekannt, die Ang II in Ang-(1-7) umwandeln können: Angiotensin-Converting Enzyme 2 (ACE2), Prolyl Carboxypeptidase (PRCP) und Prolyl Oligopeptidase (POP). Während ACE2 die größte Bedeutung als Ang-(1-7) bildendes Enzym zugeschrieben wird, ist die Rolle von PRCP und POP in diesem Vorgang zur Zeit unklar. In dieser Arbeit wurde die relative Beteiligung dieser drei Peptidasen an Ang-(1-7) Bildung in dem Kreislauf, Lungen und Nieren mittels *in vivo*, *ex vivo* und *in vivo* Methoden untersucht. Hierbei wurden sowohl genetisch modifizierte Mausmodelle als auch pharmakologische Inhibitoren der jeweiligen Enzyme eingesetzt. Nach einer intraperitonealen Ang II Injektion in Wildtyp Mäusen wurde ein deutlicher Anstieg des Peptids Ang-(1-7) in Plasma beobachtet. Ähnliche Zunahme der Ang-(1-7) Konzentration nach Ang II Injektion wurde auch in ACE2$^{-/-}$/PRCP$^{-/-}$ Mäusen festgestellt, die auf eine hauptsächlich ACE2- und PRCP-unabhängige Reaktion hinweist. Andererseits wurden in POP$^{-/-}$ Mäusen, denen in der gleichen Weise Ang II appliziert wurde, signifikant niedrigere Ang-(1-7) Konzentrationen gemessen als im Wildtyp. Pharmakologische Hemmung von POP mittels Z-Pro-Prolinal (ZPP) ließ ebenfalls reduzierte Ang-(1-7) Werte nachweisen, was nahe legt dass die Umwandlung von Ang II in Ang-(1-7) im Kreislauf vorwiegend POP-abhängig abläuft. Dementsprechend wurde in POP$^{-/-}$ Mäusen eine verspätete Erholungsphase nach Ang II-induzierter akuter Hypertonie festgestellt. In *ex vivo* Studien von Wildtyp Serum und Lungengewebe wurde Inkubation mit Ang II von einem Anstieg der Ang-(1-7) Konzentration gefolgt, der sich durch zusätzliche Gabe von ZPP inhibieren ließ. Dementsprechend wurden erniedrigte Ang-(1-7) Spiegel in POP$^{-/-}$ Serum und Lungenlysaten nach Exposition gegenüber Ang II nachgewiesen. Im Gegensatz dazu konnten Ang-(1-7) Werte in Nierenlysaten nicht signifikant von ZPP oder von genetischer POP Ablation beeinflusst werden. Nieren von Mäusen mit genetischer ACE2 Deletion zeigten deutlich verminderte Ang-(1-7) Bildung im Vergleich zu Wildtyp Nieren. Zusammenfassend zeigen die *in vivo* und *ex vivo* Studien, dass die Umwandlung von Ang II in Ang-(1-7) im Kreislauf und in den Lungen überwiegend POP vermittelt, während in den Nieren diese Reaktion hauptsächlich durch ACE2 katalysiert wird.

3. Abstract (English)

The Renin-Angiotensin system (RAS) is a regulatory hormonal network that plays a fundamental role in the physiology and pathophysiology of blood pressure regulation and electrolyte balance. Chronic overload of Angiotensin II (Ang II), the main RAS effector peptide, results in various effects such as vasoconstriction, sodium retention, fibrosis and inflammation. A protective axis of the RAS has recently been discovered encompassing Angiotensin-(1-7) [Ang-(1-7)] and its receptor Mas that counteract the deleterious effects of Ang II. There are three known enzymes that efficiently form Ang-(1-7) from Ang II: Angiotensin-Converting Enzyme 2 (ACE2), Prolyl Carboxypeptidase (PRCP) and Prolyl Oligopeptidase (POP). Although ACE2 has been recognized as the major Ang-(1-7) forming enzyme, the relevance of PRCP and POP for this conversion remains uncertain. This study focuses on the relative contribution of these three enzymes to circulatory, pulmonary and renal Ang-(1-7) formation *in vivo*, *ex vivo* and *in vitro* using mouse models of genetic ablation coupled with pharmacological inhibitors of the respective enzymes. Following intraperitoneal injection of Ang II into wild-type mice markedly elevated plasma Ang-(1-7) levels were observed. Similarly high levels of Ang-(1-7) were found in plasma of ACE2$^{-/-}$/PRCP$^{-/-}$ mice after Ang II injection indicating that this conversion is essentially independent of ACE2 and PRCP. In POP$^{-/-}$ mice infused with Ang II, however, Ang-(1-7) levels were significantly lower as compared to wild-type mice. Pharmacological inhibition of POP by Z-Pro-Prolinal (ZPP) also blunted the increase in plasma Ang-(1-7) levels suggesting that circulatory conversion of exogenous Ang II to Ang-(1-7) is largely POP-dependent. Moreover, recovery from Ang II-induced hypertension was delayed in POP$^{-/-}$ mice as compared to wild-type controls. In *ex vivo* studies incubation of wild-type mouse serum and lung lysates with Ang II resulted in a rapid increase in Ang-(1-7) which was markedly inhibitable by ZPP. Consistent with these findings with pharmacological inhibitors of POP, reduced Ang-(1-7) formation from Ang II in serum and lung lysates of POP$^{-/-}$ mice was also demonstrated. By contrast, Ang-(1-7) levels in kidneys after Ang II exposure were not significantly affected by either ZPP or genetic ablation of POP. Kidneys of mice with genetic deficiency of ACE2 showed reduced formation of Ang-(1-7) levels as compared to wild-type controls. In conclusion, this work demonstrates that POP is the major enzyme converting Ang II to Ang-(1-7) in the circulation and in lungs, whereas in kidneys this conversion is mainly ACE2-dependent.

4. Synopsis

The following sections aim to elaborate on the findings of the scientific publication[1] by describing the current state of research, introducing the scientific problem, methodology, and presenting the most relevant results and discussion regarding the clinical implications of the work.

Introduction

The Renin-Angiotensin system (RAS) plays a central role in the regulation of blood pressure and hydroelectrolyte balance. Alterations, especially overactivity of this system have been linked to several cardiovascular disease states such as hypertension, diabetic and nondiabetic nephropathy, myocardial infarction and congestive heart failure[2-4]. After the discovery of renin in 1898[5], RAS was for about a century considered as an enzymatic cascade composed of Angiotensinogen, Angiotensin I (Ang I) and Angiotensin II (Ang II). The latter peptide was shown to act as the main effector molecule of the RAS by binding to Ang II type 1 receptor (AT_1R) exerting vasoconstriction, water intake and sodium retention[6]. Moreover, stimulation of AT_1R by Ang II has been reported to be associated with oxidative stress, hypertrophy, fibrosis and inflammation[7,8].

The discovery of Angiotensin-(1-7) [Ang-(1-7)] in 1988 by Santos et al. opened up new perspectives in the field of RAS[9]. In this study, Ang-(1-7) was described as a product from the degradation of Ang I by an Angiotensin-converting enzyme (ACE)-independent pathway in the dog brainstem. A year later the first study demonstrating the biological activity of the heptapeptide Ang-(1-7) was published[10,11]. Campagnole-Santos et al. showed that femtomole amounts of Ang-(1-7) injected into the Nucleus tractus solitarii in the brainstem resulted in significant blood pressure reduction in urethane-anesthetized rats. Further studies characterized the role of Ang-(1-7) in the central regulation of blood pressure including the modulation of the baroreceptor reflex[12]. Ang-(1-7) was later shown to counteract the effects of the ACE/Ang II/A/AT_1R axis by inducing vasodilation, antifibrosis, anti-inflammation and antiapoptosis[11,13-15]. In summary, Ang-(1-7) was found to play a protective role in the cardiovascular system[16-18]. Therefore, studies investigating *in vivo* function and metabolism of Ang-(1-7) have increasingly become important in understanding the role of RAS in cardiovascular diseases. Another milestone in characterizing the *in vivo* function of Ang-(1-7)

was the discovery of its receptor, Mas. Mas is a G protein-coupled receptor that was first identified as mas oncogene (named after the patient whose tumor cells were used for its discovery)[11,19]. Santos et al. were the first to demonstrate that Ang-(1-7) specifically binds to the Mas receptor[20]. Further evidence for the protective effects of the Ang-(1-7)/Mas receptor interaction was provided by numerous studies conducted with models of genetic deletion of Mas, which resulted in cardiac dysfunction, decreased baroreflex function and endothelial dysfunction[21-23]. Ang-(1-7) can be generated from Ang I or Ang II by endopeptidases or carboxypeptidases, respectively. The main endopeptidases involved in Ang-(1-7) formation from Ang I are Neprilysin (NEP; EC 3.4.24.11), Thimet oligopeptidase (THOP1; EC 3.4.24.15), and Prolyl Oligopeptidase (POP; EC 3.4.21.26) by releasing the last three amino acids of Ang I[24]. Carboxypeptidases, on the other hand, such as Angiotensin-Converting Enzyme 2 (ACE2; EC 3.4.17.23), Prolyl Carboxypeptidase (PRCP; EC 3.4.16.2) and POP (see above) cleave the C-terminal amino acid Phenylalanine and thereby form Ang-(1-7)[25-30]. This study[1] focuses on the conversion of Ang II to Ang-(1-7) as both the degradation of Ang II and the concurrent generation of Ang-(1-7) are beneficial and protective in terms of cardiovascular disease.

Since the discovery of ACE2 in 2000[25,26], this carboxypeptidase has been in the center of attention as the main enzyme converting Ang II to Ang-(1-7). ACE2 is a homologue of the human Angiotensin-Converting Enzyme (ACE) and as a type I transmembrane protein it consists of an extracellular domain with its catalytic site and an intracellular tail[7,25,26]. Several studies aimed to demonstrate the relevance of ACE2 in cardiovascular pathology. For instance, ACE2 deficient mice have been found to suffer from severe cardiac contractility defect, increased Ang II levels and upregulation of hypoxia-induced genes in the heart[31]. In another study, Kassiri et al. induced myocardial infarction by ligation of the left anterior descending artery in ACE2 knockout mice[32]. They found that ACE2 deficiency was associated with increased mortality, infarct size expansion and higher oxidative stress. Beyond its function as supposedly the main Ang-(1-7) forming enzyme, ACE2 can cleave Ang I and thereby form the nonapeptide Angiotensin-(1-9) which can further be degraded to Ang-(1-7) by ACE[16,25]. In addition, ACE2 has been reported to be involved in the degradation of various other peptides such as kinins, apelins and neurotensin[33]. ACE2 has also been found to participate in amino acid transport in the gut and kidney and to act as target of the severe acute respiratory syndrome (SARS) coronavirus[34]. In order to further comprehend the physiologic role of ACE2, several studies addressed the localization of *in vivo* expression of

this enzyme in rodents and humans. Tissue distribution of ACE2 has been investigated by Gembardt et al. by quantifying ACE2 mRNA and correlating peptidase activity in rodents[35]. This study demonstrated that ACE2 activity is highest in mouse ileum and kidney, whereas in lung tissue they found weak enzymatic activity. In a study published by Elased et al., ACE2 activity was assessed with a Mass spectrometry-based proteolytic assay[36]. They detected ACE2 activity in kidney and brain tissues but not in plasma of mice. Expression of ACE2 in humans was addressed by Hamming et al.[37]. They found relevant surface expression on lung alveolar epithelial cells and enterocytes of the small intestine as well as in vascular endothelial cells and arterial smooth muscle cells. In summary, these studies suggest that ACE2 expression can be detected in various tissues of the cardiovascular system and brain, while the distribution may vary depending on the species.

PRCP is a serine carboxypeptidase that was first named angiotensinase C because of its ability to inactivate Ang II[38,39]. It was first purified from human kidney by Odya et al[40]. Here, the enzyme was found to cleave the C-terminal amino acid of peptides which carry Proline as the penultimate amino acid (Proline-X), such as Ang II. Enzymatic activity of PRCP was found to be pH-dependent favoring an acid pH optimum as stated by several authors [29,40,41]. Maier et al. investigated the phenotype of PRCP deficient mice to shed light on its potential role in cardiovascular pathology[29]. The mice were hypertensive, developed left ventricular hypertrophy and glomerular mesangial expansion. Of note, plasma Ang II and Ang-(1-7) levels were not significantly different between PRCP deficient mice and wild-type controls indicating that PRCP may not relevantly influence circulating Ang-(1-7) levels. Another biological function of PRCP is the regulation of food intake by metabolizing α-Melanocyte-stimulating hormone (α-MSH)[42]. PRCP has been reported to be expressed in several human and rodent tissues such as kidneys, liver, heart, spleen, the central nervous system, and in human urine but not in erythrocytes or plasma[39,42,43]. Miller et al. even proposed the evaluation of urinary PRCP activity to assess renal hypertension and diabetic nephropathy[44]. Cellular and subcellular expression of PRCP has been assessed in various studies. Kumamoto et al. observed PRCP activity in polymorphonuclear neutrophil granulocytes, macrophages, and lymphocytes isolated from human blood[45]. From various cultured human tissues, fibroblasts showed the highest PRCP activity. The same study concluded that the subcellular activity of PRCP was localized in the lysosomal compartment as supported by Skidgel et al[46].

POP is a serine peptidase that can hydrolyse several biologically active peptides shorter than 30-mer such as substance P, neurotensin, bradykinin, Ang I and Ang II, however, its *in vivo* function has not yet been fully elucidated [30,47,48]. In the RAS, POP can degrade both Ang I and Ang II to form Ang-(1-7) by hydrolyzing the Proline7-Phenylalanine8 bond and hereby releasing the final three, or the ultimate C-terminal amino acid, respectively[24,30,49]. Beyond its function as an angiotensinase, POP also exerts central nervous system effects by metabolizing neuropeptides. Neurodegenerative disorders such as Alzheimer's disease, Lewy body dementia and Parkinson's disease have been linked to reduced POP activity in certain areas of the brain[50]. Although the molecular mechanisms of these effects are not yet conclusive, some studies anticipate that POP regulates intracellular inositol 1,4,5-triphosphate (IP_3) signaling[51,52]. The emerging evidence that POP may be involved in several central nervous system pathologies has led to the development of pharmacological POP inhibitors. These inhibitors have been found to reverse amnesia in rodents[53-55]. Some of them were assessed in preclinical trials to treat age-related memory deficits and memory loss related to Alzheimer's disease[56]. Tissue distribution of POP has been evaluated by several authors. Goossens et al. found that POP is highly present in human renal cortex, epithelial cells, fibroblasts, testis, lymphocytes and thrombocytes whereas in body fluids the activity is low[48]. Irazusta et al. showed that POP activity in the central nervous system is higher than in any other peripheral tissues[57]. POP was purified from bovine serum by Cunningham et al.[58] and, although the physiologic function of circulating POP is still not widely understood, some authors suggest that it may be involved in systemic and/or neuroinflammation[59]. Interestingly, some authors reported increased POP activity in prostate, lung, ovarian and colorectal cancer as compared to healthy tissues[48,60]. Regarding subcellular localization, POP is a cytoplasmic enzyme but a membrane-bound form in the Golgi apparatus and rough endoplasmic reticulum have also been described[61,62].

As described above, all three enzymes, ACE2, PRCP and POP are widely expressed in tissues of the cardiovascular system and exert various effects in the organism. Although there are numerous studies evaluating their *in vivo* function, there is limited previous work addressing the relative contribution of these peptidases to circulating Ang-(1-7) levels. ACE2 has been in the center of attention as the main representative of the Ang-(1-7) forming enzymes but evidence regarding the role of PRCP and POP in circulatory Ang II metabolism is lacking. This study[1] highlights the importance of POP in Ang-(1-7) formation from exogenous Ang II in serum and peripheral tissues using genetic mouse models of ACE2/PRCP and POP

deficiency and pharmacological inhibitors of these enzymes. Additionally, response to Ang II-induced acute hypertension was examined in POP deficient mice to further assess the *in vivo* function of POP.

Methods

In these studies *in vivo*, *ex vivo* and *in vitro* methods were applied to characterize the enzymatic contribution of ACE2, PRCP and POP to Ang-(1-7) formation from Ang II in circulation and peripheral tissues[1]. All studies were conducted with the review and approval of the Institutional Animal Care and Use Committee. The experiments were conducted in Prof. Daniel Batlle's laboratory in the Division of Nephrology and Hypertension at Northwestern University, Feinberg School of Medicine.

Mouse models: Wild-type, ACE2$^{-/-}$/PRCP$^{-/-}$ and POP$^{-/-}$ mice

For the *in vivo* Ang II injection studies male wild-type mice on C57Bl/6 background were used as controls. ACE2 deficient mice (ACE2$^{-/-}$) on C57Bl/6 genetic background were initially provided by Dr. S. Gurley from Duke University School of Medicine. A genetic mouse model (KST302 derived in C57Bl/6 background) of PRCP deficiency (PRCP$^{-/-}$) generated by Baygenomics using gene trap technology was provided by Dr. A. Schmaier from Case Western Reserve University[29]. A cross of these two lines (ACE2$^{-/-}$ and PRCP$^{-/-}$) was performed to generate ACE2$^{-/-}$/PRCP$^{-/-}$ mice at Northwestern University. POP deficient mice (POP$^{-/-}$) were generated by Deltagen (San Carlos, CA) and fully backcrossed and maintained on C57Bl/6 background at the University of Helsinki. For the Ang II injection protocols age-matched male ACE2$^{-/-}$/PRCP$^{-/-}$ and POP$^{-/-}$ mice were used (see below). To verify the knockout models, activity levels of the respective enzymes (ACE2, PRCP and POP) were measured in plasma and kidney lysates. In plasma and kidney lysates of ACE2$^{-/-}$/PRCP$^{-/-}$ mice activity of these enzymes were barely detectable as opposed to their wild-type counterparts. POP deficient mice showed similarly negligible POP activity levels in plasma and kidney samples. These data confirmed the absence of effective enzyme activity of ACE2, PRCP and POP in their respective knockout models.

In vivo Ang-(1-7) formation from exogenous Ang II

The aim of the *in vivo* studies in wild-type, ACE2$^{-/-}$/PRCP$^{-/-}$ and POP$^{-/-}$ mice was to determine the contribution of these carboxypeptidases to circulating Ang-(1-7) levels after an i.p. bolus of Ang II. In the initial *in vivo* studies a group of male wild-type mice (C57Bl/6 background) at 10-20 weeks of age were injected with an i.p. bolus of Ang II (0.2 µg/g body weight). Five minutes post-injection blood sampling via cardiac puncture was performed under euthasol (Pentobarbital sodium and Phenytoin sodium) anesthesia. Next, the animals were euthanized by induction of double pneumothorax. In another set of experiments consecutive blood sampling was performed by tail tip snapping at 5 and 30 minutes post-injection. The same Ang II injection protocol was applied in the ACE2$^{-/-}$/PRCP$^{-/-}$ and POP$^{-/-}$ groups (males, 20-24 weeks old).

The blood collected by cardiac puncture or tail tip snapping was placed on a peptidase inhibitor cocktail[63] and plasma separated by centrifugation at 3000 rpm for 10 minutes at 4°C. A solid phase immobilized epitope immunoassay kit (Cayman Chemical, Ann Arbor, MI) was used to measure Ang II concentration in the samples. This EIA method cross-reacts with Ang-(1-7) less than 0.1% whereas there is a more prominent cross-reactivity with the peptides Angiotensin III (36%), Angiotensin IV (33%) and Angiotensin-(4-8) (41%). Ang-(1-7) was measured by commercial Ang-(1-7) ELISA (Peninsula Laboratories) with a detection range of 0.01-10 ng/ml. According to the producer, this EIA has no relevant cross-reactivity with Ang I, Ang II, Angiotensin-(1-9), Angiotensin-(1-5), or Angiotensin III. For confirmatory purposes, in selected samples Ang II, Ang-(1-7) as well as Angiotensin-(1-5) levels were additionally measured by LC/MS-MS (Attoquant Diagnostics) and by a radioimmunassay (Hypertension Core Lab, Wake Forest University School of Medicine). The latter uses a C-terminal directed antibody, which has been reported to cross-react with Angiotensin-(2-7) and Angiotensin-(3-7).[64] In the *in vivo* studies, aiming to determine the contribution of POP, ZPP was co-injected (i.p.) with Ang II at a dose of 1 µg/g body weight.

Blood pressure measurement in wild-type and POP$^{-/-}$ mice during Ang II-induced hypertension

Wild-type (C57Bl/6 genetic background) and POP$^{-/-}$ mice at 20-24 weeks of age were put under light ketamine anesthesia (160 mg/kg body weight i.p.) and were thereafter placed on a

heating platform for 10 minutes. Ang II was then injected i.p. at a dose of 0.2 mg/kg and systolic blood pressure was measured every 30 seconds for 15 minutes. The blood pressure was assessed non-invasively by determining tail blood volume using a volume-pressure recording sensor and an occlusion tail-cuff (CODA System, Kent Scientific, Torrington, CT). Measurements that did not meet the qualitative criteria pre-determined in the manufacturer's algorithm (e.g. volume <5ml, wrong shape, etc.) were automatically excluded by the computer program. The recovery of acute Ang II-induced hypertension was assessed via slope analysis of the systolic blood pressure decline over time. For the slope analysis, single missing data points due to the exclusion were imputed by the Last Observation Carried Forward (LOCF) approach.

Ex vivo Ang-(1-7) formation from Ang II in peripheral tissue lysates and serum

The *ex vivo* studies addressed the contribution of ACE2, PRCP and POP in peripheral tissues to Ang-(1-7) formation from exogenous Ang II. For these studies kidney and lung lysates, as well as sera of wild-type C57Bl/6, ACE2$^{-/-}$/PRCP$^{-/-}$ and POP$^{-/-}$ mice were incubated with Ang II. First, Ang II was titrated in the lysates and serum to achieve detectable Ang-(1-7) levels using commercial Ang-(1-7) ELISA (see above). Concurrently, the degradation of the exogenously administered Ang II was monitored using the solid phase immobilized epitope immunoassay kit described above. After determining the optimal substrate concentration (Ang II 10^{-5} M), the tissue load of the kidney- and lung lysates was calculated. A protocol loading 2 mg of kidney/lung tissue or 100 µl of serum was implemented for the following experiments. Furthermore, in selected samples the POP inhibitor Z-Pro-Prolinal (ZPP, from Enzo) or the specific ACE2 inhibitor MLN-4769 (gift from Millenium Pharmaceuticals, Cambridge, MA) was added. This protocol was then used to quantify the efficacy of the carboxypeptidases ACE2, PRCP and POP in peripheral tissue lysates and serum.

Enzymatic activities of ACE2 and POP

The purpose of these studies was to support our *in vivo* and *ex vivo* findings by determining enzyme activities of ACE2 and POP in peripheral tissues and serum samples. For the ACE2 activity measurements an ACE2-specific intramolecularly quenched fluorogenic substrate, Mca-APK-Dnp (Anaspec), was used as previously described by Ye et al.[65] Another, POP-specific fluorogenic substrate (Z-Gly-Pro-AMC) was used to measure POP activity in serum

and the tissue lysates. For this, 1 µg of total protein of the tissue sample or 1 µl serum were diluted in POP act assay buffer (TRIS buffered saline, pH 7.4 consisting of 19.98 mM Tris, 136 mM NaCl) in a 96 well black polystyrene microtiter plate. 10 µl of 10^{-4}M Z-Gly-Pro-AMC substrate working solution (Bachem, Cat# I-1145.0050) was added and incubated for 60 minutes. Fluorescence measurements were then conducted at excitation wavelength 380 nm and emission wavelength 460 nm using an FLX800 microplate fluorescence reader. Each sample was measured as duplicate whereby one of the samples was replaced by a blank well consisting of the same components as mentioned above but using 10 µl of 10^{-4}M POP inhibitor S-17092 (Sigma-Aldrich) instead of the POP act assay buffer. Total fluorescence was then obtained by subtracting blank values from values of the respective wells without inhibitor. Enzyme activity (relative fluorescence units, RFU) was then converted to a concentration of enzyme by reference to a standard curve of recombinant ACE2 and POP (R&D Systems) assayed under the same conditions.

Protein abundance by Western Blot

The Western Blot studies provide another line of evidence of the enzymatic predominance of ACE2 in kidneys and POP in lungs and serum. Protein extraction was performed in kidney cortex and lung lysates using the protocol previously described by the research group[66]. Equal amounts of total protein (50 µg/well) of kidney and lung lysates were loaded onto the gel. To estimate protein abundance in these tissues, mrACE2 and mrPOP (R&D Systems) were loaded as standards (0.2-25 ng/well). Proteins were separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to PVDF (polyvinylidene fluoride) membrane. The PVDF membrane was incubated with primary anti-ACE2 antibody (R&D Systems) or anti-POP antibody (Novus) and followed by exposure to horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology). The specificity of the ACE2 antibody was confirmed by absence of any bands in Western blot using ACE2KO samples. Specificity of POP antibody was verified by the producer (Novus) using a Protein Array containing target protein plus 383 other non-specific proteins.

In vitro studies with murine recombinant enzymes

Enzymatic cleavage of the octapeptide Ang II by carboxypeptidases, like ACE2 and POP, results in release of Ang-(1-7) and the C-terminal amino acid Phenylalanine. In these studies

Ang II was incubated with equivalent amounts of murine recombinant (mr) ACE2 or mrPOP and the formation of Ang-(1-7) and Phenylalanine was measured with commercial ELISA (see above) or a fluorometric method, respectively[67]. The latter assay has recently been developed at Northwestern University and is used for measuring carboxypeptidase activities that release a C-terminal Phenylalanine based on a reaction with the yeast enzyme of Phenylalanine Ammonia Lyase (PAL)[67].

Statistical analyses

For normally distributed data, a two-tailed T-test was applied for comparison of two independent groups. For not normally distributed data, a Mann-Whitney test was used. For comparison of more than two independent groups, one-way ANOVA was employed followed by Tukey's multiple comparison tests. The significance over time between groups was evaluated by GLM model (SPSS, version 23). Results are expressed as means ± SE and a p-value <0.05 was considered statistically significant.

Results

In vivo Ang-(1-7) formation from Ang II in wild-type, $ACE2^{-/-}$/$PRCP^{-/-}$ and $POP^{-/-}$ mice

The initial approach to assess *in vivo* Ang-(1-7) formation from Ang II was the i.p. injection of a bolus of Ang II to C57BL/6 wild-type mice. A rapid increase in plasma Ang II and, unexpectedly, Ang-(1-7) levels was observed, which led to the hypothesis that circulatory Ang II cleavage to Ang-(1-7) occurs massively. To demonstrate the ACE2- and PRCP-independent conversion of circulatory Ang II to Ang-(1-7) a genetic model of ACE2 and PRCP deficiency ($ACE2^{-/-}$/$PRCP^{-/-}$) was used. Analogously to the Ang II injection protocol in wild-type mice, in a group of $ACE2^{-/-}$/$PRCP^{-/-}$ rapid formation of Ang-(1-7) was found. These results support that Ang-(1-7) conversion from Ang II in the circulation is mainly ACE2- and PRCP-independent.

Next, the contribution of POP to Ang-(1-7) formation from exogenous Ang II was examined in wild-type and $ACE2^{-/-}$/$PRCP^{-/-}$ mice using the pharmacological POP inhibitor ZPP. Simultaneous injection of ZPP and Ang II resulted in significantly lower Ang-(1-7) formation in both wild-type and $ACE2^{-/-}$/$PRCP^{-/-}$ groups as compared to the corresponding groups

without ZPP. For confirmatory purposes another set of experiments was conducted using mice with genetic ablation of POP ($POP^{-/-}$). Using the same *in vivo* protocol as above, $POP^{-/-}$ mice formed significantly less Ang-(1-7) from Ang II than the wild-type group. These findings suggest that POP plays a crucial role in the circulatory conversion of Ang II to Ang-(1-7).

Blood pressure recovery in $POP^{-/-}$ mice after Ang II injection

To further characterize the role of POP in circulatory Ang II metabolism an *in vivo* blood pressure study was conducted. Response to acute Ang II-induced hypertension was studied in wild-type and $POP^{-/-}$ mice. Although the peak blood pressure after 1 minute was not significantly different in the two groups, recovery in the $POP^{-/-}$ group was found to be delayed as compared to wild-type mice. These data suggest that altered Ang II degradation due to POP deficiency results in delayed recovery from Ang II-induced hypertension.

***Ex vivo* conversion of Ang II to Ang-(1-7) in kidney and lung lysates**

Endothelial enzymes in highly perfused organs, such as kidneys and lungs, might influence the degradation of Ang II and thus contribute to plasma Ang-(1-7) levels. To quantify the contribution of these organs an *ex vivo* protocol was implemented, in which kidney and lung lysates were incubated with Ang II followed by consecutive Ang-(1-7) measurements.

Wild-type kidney lysates showed Ang-(1-7) formation with a peak at 15 minutes followed by a rapid decline. ZPP did not significantly influence the Ang-(1-7) levels. On the other hand, in kidneys of $ACE2^{-/-}$/$PRCP^{-/-}$ Ang-(1-7) formation from Ang II was markedly lower indicating that Ang-(1-7) formation from Ang II in kidneys is essentially ACE2-dependent. Similarly to kidneys, in wild-type lung lysates the conversion of Ang II to Ang-(1-7) peaked at 15 minutes, however, reaching lower peak Ang-(1-7) concentrations while exposed to the same amount of Ang II. As opposed to kidneys, ZPP completely prevented Ang-(1-7) formation in wild-type lung lysates. Lungs of $ACE2^{-/-}$/$PRCP^{-/-}$ mice generated similarly high amounts of Ang-(1-7) as wild-types. These findings show that in lungs formation of Ang-(1-7) from exogenously added Ang II is mainly attributable to POP and is not significantly influenced by ACE2 or PRCP.

To further elaborate on the role of POP in Ang-(1-7) formation in kidneys and lungs the same *ex vivo* protocol was applied for organs of $POP^{-/-}$ mice. Formation of Ang-(1-7) in kidney lysates of $POP^{-/-}$ mice was not significantly different from that in wild-type mice. The additional specific ACE2 inhibitor MLN-4760 completely prevented Ang-(1-7) formation in kidneys of the $POP^{-/-}$ group. In lung lysates of $POP^{-/-}$ mice, however, the levels of Ang-(1-7) after incubation with Ang II were barely detectable, which is consistent with the *ex vivo* findings using ZPP. MLN-4760 had no significant effect in $POP^{-/-}$ lung lysates.

In summary, the *ex vivo* studies clearly show the essential contribution of ACE2 to Ang-(1-7) formation from Ang II in kidneys and the predominance of POP in lungs.

***Ex vivo* conversion of Ang II to Ang-(1-7) in mouse serum**

To further examine the rapid formation of Ang-(1-7) from Ang II observed *in vivo* a set of experiments was performed using serum of wild-type, $ACE2^{-/-}/PRCP^{-/-}$ and $POP^{-/-}$ mice. Similarly to the *ex vivo* protocol in kidney and lung lysates, sera were incubated with Ang II followed by consecutive Ang-(1-7) measurements. Firstly, the effect of exogenous ZPP in wild-type mice was studied. Wild-type sera incubated with Ang II in the presence of ZPP formed significantly less Ang-(1-7) over time than sera without this inhibitor.

The next step was to evaluate the contribution of ACE2, PRCP and POP to Ang-(1-7) formation in serum using the respective genetic knockout models. No significant difference in peak Ang-(1-7) levels was found between the $ACE2^{-/-}/PRCP^{-/-}$ and wild-type groups. As opposed to the $ACE2^{-/-}/PRCP^{-/-}$ model, sera of $POP^{-/-}$ mice showed significantly reduced Ang-(1-7) formation as compared to both wild-type and $ACE2^{-/-}/PRCP^{-/-}$ mice. These findings are in line with the previous *in vivo* and *ex vivo* results and confirm that POP is the major Ang-(1-7) forming enzyme from Ang II in the circulation.

Protein abundance of ACE2 and POP in serum, lungs and kidneys

As another line of evidence, protein abundance of ACE2 and POP was assessed using Western blot in serum, lung and kidney lysates. ACE2 was present in kidneys but could not be detected in either serum or lung tissue, which is consistent with our *ex vivo* data in these organs. POP, on the other hand, was detected in serum, lungs and kidney lysates.

In vitro Ang-(1-7) and Phenylalanine formation from Ang II using mouse recombinant ACE2 and POP

Ang-(1-7) levels in the mrACE2 group were significantly higher than those in the mrPOP group. Consistently, Phenylalanine formation in the mrACE2 was found to be markedly higher than in the Ang II samples incubated with mrPOP. These results indicate that mrACE2 is more potent in the *in vitro* conversion of Ang II to Ang-(1-7) than mrPOP.

Discussion

The classical RAS has recently been expanded by the discovery of a protective axis with the biologically active heptapeptide Ang-(1-7) and its receptor Mas[9,11,19]. While ACE2 is currently recognized as the main Ang-(1-7) forming enzyme, no previous research has addressed the contribution of other peptidases such as PRCP and POP to circulating and tissue Ang-(1-7) levels[7,16]. The aim of this study[1] was to further extend current knowledge of ACE2, PRCP and POP and to characterize their role in Ang-(1-7) formation in the circulation, kidneys and lungs using *in vivo* and *ex vivo* approaches.

The principal observation was that an acute i.p. injection of Ang II into wild-type mice resulted in markedly increased plasma Ang-(1-7) levels 5 minutes post-injection. A potential explanation of this phenomenon is that the rapid increase in plasma Ang-(1-7) levels are due to enzymatic cleavage by circulatory peptidases. Considering the fact that there are three known peptidases, ACE2, PRCP and POP that efficiently convert Ang II to Ang-(1-7), their contribution to this conversion was examined. Prior research suggests that ACE2 has low serum activity[63,65] and that PRCP mainly exerts its effects at acidic pH[29,40], favoring POP as the main circulatory Ang-(1-7) generating enzyme. In line with this hypothesis, in a model of combined genetic ablation of ACE2 and PRCP similarly high plasma levels of Ang-(1-7) were observed after acute Ang II injection as in wild-type mice. Pharmacological POP inhibition, on the other hand, resulted in markedly reduced Ang-(1-7) formation in both wild-type and $ACE2^{-/-}/PRCP^{-/-}$ mice. Consistent with these findings, in a model of genetic POP ablation, Ang-(1-7) levels following Ang II injection were found to be decreased as compared to wild-type controls. These *in vivo* findings show that conversion of exogenous Ang II to Ang-(1-7) is largely POP-dependent and ACE2- and PRCP-independent. Yamamoto et al.

intravenously administered Ang I to rats and examined the formation of Ang II and Ang-(1-7) under ACE/Neprilysin/POP inhibition[68]. They concluded that Neprylisin plays a crucial role in circulatory Ang I metabolism. They did not study, however, the effect of direct Ang II infusions. This is the first study[1] to investigate the degradation of exogenously administered Ang II in the face of pharmacological or genetic inhibition of ACE2, PRCP and POP.

Another line of evidence to demonstrate the relevance of POP in circulatory Ang II metabolism is the experiment investigating blood pressure response in $POP^{-/-}$ mice following exposure to Ang II. In this model of Ang II-induced acute hypertension, the $POP^{-/-}$ group showed delayed recovery in systolic blood pressure. Both insufficient degradation of Ang II and/or reduced formation of Ang-(1-7) could account for this finding, although according to previous work Ang-(1-7) barely influences blood pressure[63]. Thus, the most likely explanation for the blunted blood pressure recovery in POP deficient mice is the altered plasma Ang II metabolism. It should be pointed out that POP is involved in the degradation of other vasoactive peptides such as bradykinin[47]. Therefore, changes in blood pressure response in the $POP^{-/-}$ model may not only reflect changes in RAS metabolism.

As an *ex vivo* correlate of the findings stated above, sera of wild-type, $ACE2^{-/-}$/$PRCP^{-/-}$ and $POP^{-/-}$ mice were incubated with Ang II. Consistently with the *in vivo* results, $POP^{-/-}$ mice formed significantly lower amounts of Ang-(1-7) than wild-types indicating that POP is the main circulatory enzyme responsible for Ang-(1-7) formation from Ang II. Incubation of $ACE2^{-/-}$/$PRCP^{-/-}$ sera, on the other hand, showed essentially identical Ang-(1-7) levels as in wild-type mice. Protein abundance of POP was accordingly high in serum as opposed to that of ACE2. Low plasma ACE2 activity was reported by several authors[63,65,69], which is consistent with these findings. There is some controversy in the literature surrounding circulatory POP activity. Although Goossens et al. reported low POP activity in body fluids including plasma[48], Tenorio-Laranga et al. found significant levels of POP activity in serum, cerebrospinal fluid, seminal fluid, and urine[70]. The data obtained by Western blot in this study[1] supports the latter finding. The *ex vivo* protocol does not perfectly depict the *in vivo* situation as POP has been shown to be present on endothelial cells which may therefore additionally influence Ang II metabolism[71]. It should be noted that circulating cells such as lymphocytes, thrombocytes and macrophages have been reported to highly express POP and therefore also may contribute to *in vivo* Ang-(1-7) generation from exogenous Ang II[48,72].

To support the *in vivo* findings and localize the site of Ang II-Ang-(1-7) conversion, *ex vivo* studies were conducted in kidney and lung tissue homogenates. These organs receive a substantial fraction of the cardiac output and could therefore contribute to circulating Ang-(1-7) levels. ACE2$^{-/-}$/PRCP$^{-/-}$ kidneys showed markedly reduced Ang-(1-7) formation from Ang II, whereas lung lysates of the same model showed no significant difference as compared to wild-type lungs. These results indicate that the kidneys largely rely on ACE2 to form Ang-(1-7) from Ang II, whereas the lungs do not. The effect of PRCP is negligible in this constellation as the *ex vivo* studies were conducted at physiologic pH where this enzyme is inactive[29,40,41]. One could anticipate, however, that PRCP may contribute to Ang-(1-7) conversion from Ang II *in vivo* at sites of lower pH in the kidney, such as the lumen of the collecting duct. As reported by Maier et al. PRCP is highly present in this area of the kidney[29]. ZPP, a specific POP inhibitor, had marginal effects in kidney lysates while it completely prevented Ang-(1-7) formation in lungs suggesting a pivotal role of POP in pulmonary Ang-(1-7) formation. This was further supported by studies with organs of POP$^{-/-}$ mice. Correspondingly, by Western blot, POP was abundantly present in lungs and kidneys, while ACE2 protein was not detectable in lungs but highly present in kidney lysates. This is in line with previous findings[35,36,48,73]. According to Myöhänen et al. POP protein is highly present in human alveolar macrophages and pneumocytes and moderately present in epithelial cells of tubules and glomeruli of the kidney[60]. It should be noted, however, that in humans ACE2 has been reported to be expressed on lung alveolar epithelial cells[37]. As demonstrated by Western blot, POP is present in kidneys, however, its contribution to Ang-(1-7) formation from Ang II is low as shown by the studies with ZPP and the POP$^{-/-}$ mouse model. This slight discrepancy is still not completely understood but might result from the 10- to 100-fold higher efficiency constant of ACE2 than that of POP for the conversion of Ang II to Ang-(1-7) as reported by Chappell et al[74].

The *in vivo* and *ex vivo* data stated above support that circulatory and pulmonary Ang-(1-7) formation from Ang II is essentially catalyzed by POP while kidneys mainly rely on ACE2. This, however, does not sufficiently elucidate the potency of these enzymes under *in vitro* circumstances. Conversion of Ang II to Ang-(1-7) concurrently results in the release of its C-terminal amino acid Phenylalanine. Using a fluorometric assay we recently developed[67], the amount of generated Phenylalanine can be determined after Ang II incubation. This is directly proportional to the amount of formed Ang-(1-7). This assay, coupled with commercial Ang-(1-7) ELISA, was used to assess the potency of ACE2 and POP *in vitro.* mrACE2 was found

to form considerably more Ang-(1-7) and Phenylalanine than mrPOP which is consistent with Chappell's et al. findings[74]. Even though mrACE2 was shown to be more potent than mrPOP *in vitro*, several other factors, for instance tissue distribution, subcellular localization and local pH, have to be taken into consideration while evaluating *in vivo* function of these enzymes.

A number of potential limitations of the study[1] need to be considered. To begin with, the *in vivo* Ang II injection protocols do not distinguish between intra- or extravascular processing of Ang II and do not consider the subcellular localization of the participating enzymes either. Nevertheless, they allow an estimation of the contribution of ACE2, PRCP and POP to circulatory Ang-(1-7) formation. The dose of injected Ang II was remarkably high as compared to physiologic plasma Ang II levels[75]. Ang II at this concentration may serve as substrate for peptidases with physiologically low affinity for this peptide and therefore influence its degradation. This dose (0.2 µg/g body weight) was chosen to accentuate differences between wild-type and the knockout models and to appropriately detect Ang-(1-7) by ELISA. It should be noted that the inhibitor ZPP was used to quantify the contribution of POP but it also inhibits PRCP[30,76]. The *in vivo* studies were conducted with a ZPP concentration of 1µg/g, thus the estimated circulatory ZPP concentration is below 10 µM range. As reported by Velez et al.[30], 100 nM already provides sufficient inhibition of POP whereas PRCP has been reported to have a half maximal inhibitory concentration (IC50) of several hundred µM to mM[76]. In studies conducted with $ACE2^{-/-}/PRCP^{-/-}$ mice PRCP inhibition by ZPP is negligible. The *in vivo* studies also lack advanced cardiovascular monitoring such as continuous arterial blood pressure or cardiac output measurements which could further elucidate the function of the mentioned angiotensinases. It also needs to be considered that other cells or tissues such as the vascular endothelium or the brain might also contribute to circulating Ang-(1-7) levels, which, however, were not investigated in this paper. The study[1] did not address either the possible function of POP in kidneys and lungs or its cellular and subcellular localization which could further elucidate its role in RAS-related and -unrelated peptide metabolism. Further data collection such as mRNA expression and immunohistochemistry is required to determine the tissue expression and exact localization of POP in these organs.

The results have several implications for research into a more detailed understanding of circulatory, pulmonary and renal Ang II degradation and Ang-(1-7) formation. Further work needs to be performed to establish the exact role of POP, especially in the circulation. A

comprehensive cardiovascular, renal and pulmonary phenotyping of the $POP^{-/-}$ model could shed light on its function in RAS, particularly in terms of hypertension and cardiovascular disease. Chronic Ang II infusion studies in ACE2/PRCP/POP knockout models coupled with long term blood pressure measurements using radiotelemetry devices and circulatory Angiotensin peptide measurements are needed to estimate the role of these enzymes in Ang II-induced hypertension. This work[1] has revealed that POP deficiency alters circulatory Ang II metabolism and blood pressure response to Ang II-induced hypertension, of which both could have possible clinical implications. Ongoing preclinical and clinical studies target the Ang-(1-7)/Mas axis as a therapeutic strategy to foster this protective pathway of the RAS[16,77]. These studies test recombinant human ACE2, ACE2 activating substances such as 1-[(2-dimethylamino) ethylamino]-4-(hydroxymethyl)-7-[(4-methylphenyl) sulfonyl oxy]-9H-xanthene-9-one (XNT) and Mas agonists such as pharmacologically stabilized cyclic Ang-(1-7)[16,78,79]. POP, despite being a potent Ang-(1-7) forming enzyme that might play a protective role in cardiovascular disease, has not been evaluated in these studies. Myöhänen et al. reported relevant expression of POP in several human tissues such as kidneys and lungs[60], thus one could anticipate that pharmacological activation of the POP/Ang-(1-7) axis may be beneficial in these organs. In particular, in lungs where POP is abundant, preclinical studies suggest that Ang-(1-7) attenuates pathophysiological processes such as pulmonary hypertension and pulmonary fibrosis[80] although there is some controversy surrounding these results[81].

In conclusion, this is the first study to demonstrate that formation of Ang-(1-7) from exogenous Ang II in the circulation and lungs is essentially POP-dependent. In contrast, this conversion in kidneys is mainly mediated by ACE2. Genetic deficiency of POP resulted in delayed recovery from Ang II-induced hypertension. If these findings are confirmed by others, they will provide new insights into the physiology and pathophysiology of the RAS. Hopefully this work will serve as a base for future studies on the role of POP in the RAS and may be the first step to consider POP as a novel therapeutic target in cardiovascular disease.

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5. Statutory Declaration

“I, Peter Daniel Serfözö, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic „Angiotensin-Converting Enzyme 2- and Prolyl Carboxypeptidase-Independent Conversion of Angiotensin II to Angiotensin-(1-7) in Circulation and Peripheral Tissues“, in German: „Angiotensin-Converting Enzyme 2- und Prolyl Carboxypeptidase-unabhängige Umwandlung von Angiotensin II in Angiotensin-(1-7) in dem Kreislauf und in Peripheren Geweben“, independently and without the support of third parties, and that I used no other sources and aids than those stated.
All parts which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (in particular regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts and tables) are exclusively my responsibility.

Furthermore, I declare that I have correctly marked all of the data, the analyses, and the conclusions generated from data obtained in collaboration with other persons, and that I have correctly marked my own contribution and the contributions of other persons (cf. declaration of contribution). I have correctly marked all texts or parts of texts that were generated in collaboration with other persons. My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; www.icmje.org) on authorship. In addition, I declare that I shall comply with the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice. I declare that I have not yet submitted this dissertation in identical or similar form to another Faculty.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me.”

Date Signature

6. Declaration of contribution

Peter Daniel Serfözö contributed the following to the below listed publication:

Publication:

Serfozo P, Wysocki J, Gulua G, Schulze A, Ye M, Liu P, Jin J, Bader M, Myohanen T, Garcia-Horsman JA, Batlle D. Ang II (Angiotensin II) Conversion to Angiotensin-(1-7) in the Circulation Is POP (Prolyloligopeptidase)-Dependent and ACE2 (Angiotensin-Converting Enzyme 2)-Independent. Hypertension 2019:Hypertensionaha11914071

Contribution:

- Planning and conducting the *in vivo* Ang II injection studies in wild-type C57Bl/6 and $ACE2^{-/-}$/$PRCP^{-/-}$ mice: intraperitoneal Ang II injection, induction of anesthesia, followed by euthanasia, blood sampling, plasma isolation, peptide extraction and performing Ang II and Ang-(1-7) ELISA under supervision of Jan Wysocki MD PhD. LC/MS-MS measurements were performed by the company Attoquant Diagnostics, while Radioimmunoassay measurements in the Hypertension Core Lab, Wake Forest University School of Medicine. Studies with $POP^{-/-}$, and additional wild-type mice were conducted by the co-authors Jan Wysocki MD PhD, Minghao Ye MD, Gvantca Gulua and Arndt Schulze.
- Developing the *ex vivo* protocols for mouse lung- and kidney lysates: organ extraction, preparing the tissue lysates, titrating the substrate (Ang II) concentration and determining the optimal tissue load, followed by Ang II- and Ang-(1-7) ELISA.
- Planning and conducting *ex vivo* experiments in wild-type- and $ACE2^{-/-}$/$PRCP^{-/-}$ mouse kidney- and lung lysates applying the protocol mentioned above. Performing studies with- or without the enzyme inhibitors MLN-4769 and ZPP. Determining Ang II and Ang-(1-7) levels by ELISA. Analogous studies were additionally conducted by Gvantca Gulua (increasing total number of observations "n"). The experiments with $POP^{-/-}$ organs as well as with serum samples were performed by Jan Wysocki MD PhD, Gvantca Gulua and Arndt Schulze.
- $POP^{-/-}$ mice were provided by Timo Myöhänen PhD and J. Arturo Garcia Horsman from the University of Helsinki.

- Co-development of the fluorometric Phenylalanine assay which was applied for the *in vitro* studies (see the publication Liu P, Wysocki J, Serfozo P, Ye M, Souma T, Batlle D, Jin J. A Fluorometric Method of Measuring Carboxypeptidase Activities for Angiotensin II and Apelin-13. Scientific Reports 2017;7:45473). The assay was essentially developed by Pan Liu, PhD and Jing Jin MD PhD who were consulted about its application and technical details. The *in vitro* studies with recombinant enzymes were conducted by Jan Wysocki MD PhD.
- Interpretation of the results, writing and editing the manuscript and its revisions. Additional revisions were carried out by the co-authors Prof. Dr. Daniel Batlle, Jan Wysocki MD PhD, Gvantca Gulua, Jing Jin MD PhD, Pan Liu, PhD, Timo Myöhänen PhD, J. Arturo Garcia Horsman PhD and Prof. Dr. Michael Bader.
- Designing and creating the Figures 2, 4 and supplemental Figure S2. These were partially modified by Jan Wysocki MD PhD and Gvantca Gulua. Creating and formulating all of the Figure legends. The remaining figures were created and edited by the co-authors Jan Wysocki MD PhD, Gvantca Gulua and Arndt Schulze.
- Performing statistical analysis with T-Test. The methods Mann-Whitney, ANOVA and Tukey's multiple comparison test were applied by Jan Wysocki MD PhD.

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Signature of doctoral candidate

7. Extract from the Journal Summary List

Journal Data Filtered By: **Selected JCR Year: 2018** Selected Editions: SCIE,SSCI Selected Categories: **"PERIPHERAL VASCULAR DISEASE"** Selected Category Scheme: WoS

Gesamtanzahl: 29 Journale

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	CIRCULATION	166,484	23.054	0.211290
2	CIRCULATION RESEARCH	52,988	15.862	0.072290
3	HYPERTENSION	36,482	7.017	0.046530
4	ARTERIOSCLEROSIS THROMBOSIS AND VASCULAR BIOLOGY	33,223	6.618	0.036000
5	STROKE	64,814	6.046	0.082630
6	ANGIOGENESIS	2,956	5.759	0.004410
7	Journal of Stroke	925	5.571	0.003580
8	CURRENT OPINION IN LIPIDOLOGY	4,140	4.844	0.006320
9	THROMBOSIS AND HAEMOSTASIS	16,590	4.733	0.022810
10	JOURNAL OF THROMBOSIS AND HAEMOSTASIS	18,886	4.662	0.028230
11	International Journal of Stroke	4,172	4.466	0.015210
12	ATHEROSCLEROSIS	23,442	4.255	0.033500
13	JOURNAL OF HYPERTENSION	17,501	4.209	0.026380
14	AMERICAN JOURNAL OF PHYSIOLOGY-HEART AND CIRCULATORY PHYSIOLOGY	27,828	4.048	0.022820
15	Current Atherosclerosis Reports	2,392	3.769	0.004770
16	EUROPEAN JOURNAL OF VASCULAR AND ENDOVASCULAR SURGERY	9,293	3.642	0.012760
17	CURRENT HYPERTENSION REPORTS	2,900	3.606	0.006430
18	Journal of Atherosclerosis and Thrombosis	3,249	3.478	0.004800

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Selected JCR Year: 2018; Selected Categories: "PERIPHERAL VASCULAR DISEASE"

8. Publication

https://doi.org/10.1161/HYPERTENSIONAHA.119.14071

9. Curriculum vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

10. Complete list of publications

- **Serfozo P**, Wysocki J, Gulua G, Schulze A, Ye M, Liu P, Jin J, Bader M, Myohanen T, Garcia-Horsman JA, Batlle D. Ang II (Angiotensin II) Conversion to Angiotensin-(1-7) in the Circulation Is POP (Prolyloligopeptidase)-Dependent and ACE2 (Angiotensin-Converting Enzyme 2)-Independent. Hypertension 2019:Hypertensionaha11914071.
- Liu P, Wysocki J, **Serfozo P**, Ye M, Souma T, Batlle D, Jin J. A Fluorometric Method of Measuring Carboxypeptidase Activities for Angiotensin II and Apelin-13. Scientific Reports 2017;7:45473.

Abstracts:

- Gulua G, **Serfozo P**, Wysocki J, Ye M, Liu P, Jin J, Bader M, Myöhänen T, Garcia-Horsman JA, Batlle D. Abstract P2014: Prolyl Endopeptidase (PEP) is the Main Angiotensin (1-7) Forming Enzyme From Angiotensin II (1-8) in the Circulation: Implications for Hypertension. Hypertension 2019;74:AP2014-AP
- **Serfözö PD**, Wysocki J, Liu P, Ye M, Müller T, Jin J, Batlle D. MP490FORMATION OF ANG(1−7) FROM ANGII(1−8) IS LARGELY INDEPENDENT OF ACE2 AND PRCP. Nephrology Dialysis Transplantation 2017;32:iii608-iii9.

11. Acknowledgements

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