Chapter 1

Peptidases and the Renin-Angiotensin System: The Alternative Angiotensin-(1-7) Cascade

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Abstract

The renin-angiotensin system (RAS) constitutes a key hormonal system in the physiological regulation of blood pressure via peripheral and central mechanisms. Dysregulation of the RAS is considered a major factor in the development of cardiovascular pathologies, and pharmacologic blockades of this system by the inhibition of angiotensin-converting enzyme (ACE) or antagonism of the angiotensin type 1 receptor (AT_1R) are effective therapeutic regimens. The RAS is now defined as a system composed of different angiotensin peptides with diverse biological actions mediated by distinct receptor subtypes. The classic RAS comprises the ACE-Ang II-AT_1R axis that promotes vasoconstriction, water intake, sodium retention and increased oxidative stress, fibrosis, cellular growth, and inflammation. The nonclassical or alternative RAS is composed primarily of the ACE2-Ang-(1-7)-AT_7R pathway that opposes the Ang II-AT_1R axis. In lieu of the complex aspects of this system, the current review assesses the enzymatic cascade of the alternative Ang-(1-7) axis of the RAS.

Keywords: angiotensin-(1-7), neprilysin, dipeptidyl peptidase 3, ACE, ACE2, renin

1. Introduction

The renin-angiotensin system (RAS) has been long defined as a circulating endocrine system composed of an enzymatic cascade that includes renin to initiate the RAS through the conversion of the large precursor protein angiotensinogen (>350 amino acids) to the inactive decapeptide Ang I and the subsequent generation of the bioactive octapeptide Ang II by

angiotensin-converting enzyme (ACE) (**Figure 1**). Ang II binds to the angiotensin type 1 receptor (AT_1R) to mediate both peripheral and central mechanisms in the regulation of blood pressure. Activation of the ACE-Ang II-AT_1R pathway is also associated with various pathological responses including fibrosis, inflammation, metabolic dysregulation, heart failure, cancer, aging, and diabetic injury [1–5]. Indeed, the targeting of this pathway by biochemical approaches that block the formation of Ang II through ACE inhibitors or binding of Ang II to the AT_1R by receptor antagonists is an effective therapy for the treatment of cardiovascular disease. It is now evident that these therapies have revealed alternative pathways within the RAS that may contribute to the beneficial actions of the RAS blockade. For example, the targeting of ACE reduces Ang II expression, but markedly enhances the circulating levels of Ang-(1-7), a ligand for the AT_7/MasR that generally opposes the actions of the Ang II-AT_1R pathway [6, 7]. The chronic treatment with AT_1R antagonists may also increase the formation of Ang-(1-7) through ACE2, as well as shunt Ang II to the AT_2R pathway that shares similar properties to the Ang-(1-7) system [8, 9]. Since the RAS is now viewed as a more complex array of components that can be functionally partitioned into distinct receptors and peptide ligands, we present an overview of the peptidases the may constitute the alternative Ang-(1-7) axis of the RAS. We include a discussion of renin, ACE, and chymase as they pertain to the formation of the substrates Ang I and Ang II for the direct processing to Ang-(1-7), as the well as the role of ACE to metabolize Ang-(1-7).

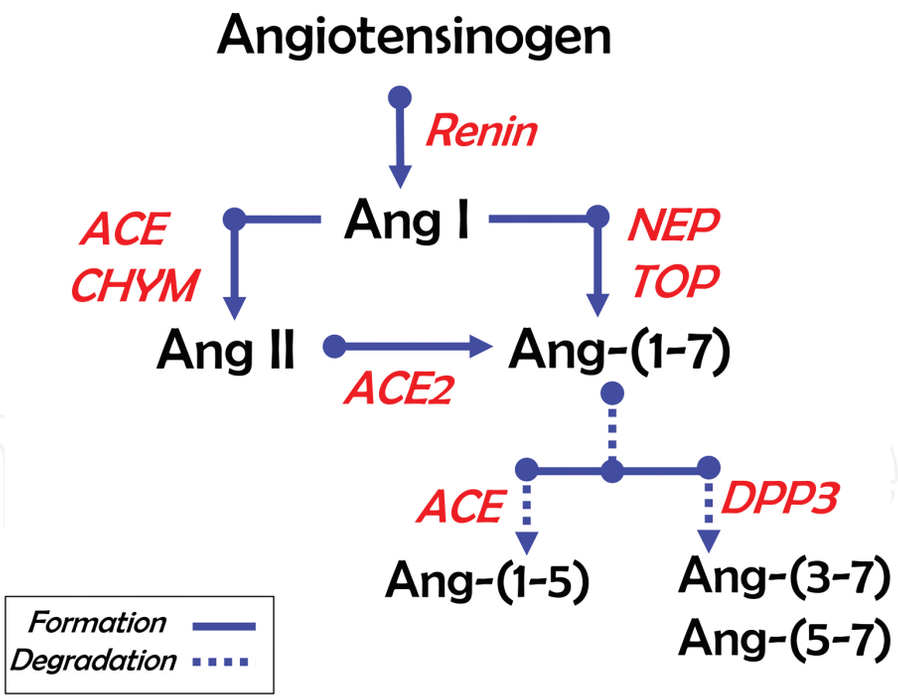

Figure 1. Processing cascade for angiotensin-(1-7). Renin cleaves angiotensinogen to Ang I which is further processed to the biologically active peptides Ang II by ACE or chymase (CHYM). Ang II undergoes further processing by the carboxypeptidase ACE2 to form Ang-(1-7). Ang-(1-7) is formed directly through non-Ang II pathways by the direct processing of Ang I by the endopeptidases neprilysin (NEP) and thimet oligopeptidase (TOP). Ang-(1-7) is subsequently metabolized by ACE to Ang-(1-5) and dipeptidyl peptidase 3 (DPP3) to Ang-(3-7) and Ang-(5-7). Adapted from Chappell [54].

2. Renin

Renin [EC 3.4.23.15] belongs to the family of aspartyl-type acid proteases (molecular size: 30–40 kDa); however, the protease exhibits a more neutral pH optima of 6.5–8.0. The only known substrate for renin is angiotensinogen, and renin cleaves off the N-terminal portion of angiotensinogen to form the inactive peptide Ang I. The formation of Ang I is considered the enzymatic event that initiates the RAS cascade (**Figure 1**). The enzyme is synthesized predominantly in the juxtaglomerular (JG) cells of the kidney and is stored in both inactive (prorenin) and active forms for subsequent release into the afferent arteriole to reach the circulation. The collecting duct (CD) cells are another source of renin within the kidney that primarily secretes the active form of renin from the apical aspect of the principal cells into the tubular fluid [10, 11]. The distal secretion of renin into the tubular fluid would presumably contribute to the processing of angiotensinogen that may arise from either proximal tubule synthesis or apical release or the glomerular filtration of the protein. Interestingly, the regulation of JG renin appears to be distinct from CD renin as Ang II reduces JG renin release and synthesis constituting the classic short-loop negative feedback system for RAS activation, whereas the peptide stimulates CD renin release suggesting a positive feedback system for renin in the distal nephron [12, 13].

There is evidence for alternative gene products of renin expressed in the kidney, brain, heart, and adrenal gland [14–17]. These products arise from an alternative start site for renin transcription that is situated downstream from the typical start site and yields a shorter, but active form of the enzyme. The truncated form of renin lacks the secretory signal of the protein and the enzyme is not secreted, but resides within the cell. Peters and colleagues find that truncated renin specifically localized to the mitochondria and that the active form of renin, but not prorenin, was internalized by mitochondria [14, 18, 19]. The overexpression of the active renin isoform protected the cells under high glucose conditions and this effect was not reversed by the renin inhibitor aliskerin or an AT_1R antagonist suggesting that the beneficial effects may not reflect activation of the Ang II-AT_1R axis [20]. In support of these findings, we recently reported the presence of active renin in isolated mitochondria from the sheep renal cortex [21]. In this case, we also quantified the mitochondrial levels of both Ang II and Ang-(1-7) (50–60 fmol/mg protein), which may indicate that the intracellular form of renin contributes to the cellular expression of angiotensin peptides within the kidney [21]. In the renal NRK-52 epithelial cell line, immunocytochemical staining for renin was evident in the nucleus of these cells [22]. Renin activity as measured by aliskerin-sensitive inhibition of Ang I formation from angiotensinogen was also evident in isolated nuclei in the renal epithelial NRK-52 cells, as well as quantifiable levels of Ang I, Ang II, and Ang-(1-7) [22]. In confirmation of intracellular renin within the kidney, Ishigami and colleagues report a truncated renin transcript expressed in the proximal tubules of the mouse kidney [16]. Overexpression of the renin isoform within the proximal tubules was associated with a sustained elevation in blood pressure and no change in the circulating levels of renin [16]. Although the intracellular distribution of the renin isoform was not ascertained, the fact that overexpression of tubular renin augmented blood pressure is consistent with previous studies demonstrating that the increased expression of

proximal tubule angiotensinogen, the AT_1R, or intracellular Ang II also resulted in a sustained increase in blood pressure [23–28].

3. Angiotensin-converting enzyme

The predominant pathway of the classical RAS for the conversion of Ang I to the bioactive peptide Ang II is catalyzed by the metallopeptidase ACE [EC 3.4.15.1], a dipeptidyl carboxypeptidase that cleaves two residues from the carboxy terminus of Ang I (**Figure 1)**. The peptidase is a membrane-bound, glycosylated protein (120–180 kDa) ubiquitously expressed in multiple tissues [29]. Soluble forms of the enzyme are present in the circulation, cerebrospinal fluid (CSF), lymph fluid, and urine that fully retain peptidase activity [29]. The soluble form of ACE arises from the hydrolysis of the membrane-anchoring or stalk region of the protein that may reflect the processing by A Disintegrin and Metalloproteinase (ADAM) family of metalloenzymes, although the precise role of ACE shedding in cardiovascular disease is presently unclear. Somatic ACE is characterized by two active sites termed N and C terminal domains that likely arose from the gene duplication of germinal or testicular ACE that contains only the single C terminal-active site. Importantly, in addition to forming Ang II, ACE degrades a number of other peptides that exhibit cardiovascular actions including bradykinin, substance P, acetyl-SDKP, and Ang-(1-7) [29]. Indeed, the cardioprotective effects of ACE inhibitors may reflect the protection of these peptides from metabolism in addition to the blockade of Ang II formation. We showed that ACE hydrolyzes Ang-(1-7) to the pentapeptide Ang-(1-5) and that ACE inhibition markedly reduced the clearance of the peptide [30, 31]. The reduced metabolism of Ang-(1-7) likely contributes to the elevation in circulating levels of Ang-(1-7) following the chronic treatment with ACE inhibitors in experimental animals and in humans [32]. Moreover, treatment with an ACE inhibitor was required to clearly demonstrate the accumulation of Ang-(1-7) derived from either Ang II or Ang I in isolated proximal tubules [33]. These data suggest a pivotal role for ACE to regulate the balance of Ang II and Ang-(1-7), two peptides within the RAS that exhibit strikingly different actions from one another [7].

4. Chymase

Chymases [EC 3. 4.21.39] comprise a family of serine peptidases that may generate Ang II by hydrolysis of the Phe^8-His^9 bond of Ang I and other peptide precursors [Ang-(1-12), Ang-(1-25)] (α-chymases) or metabolize Ang II at Tyr^4-Ile^5 to form Ang-(1-4) and Ang-(5-8) (β-chymases) [34–39]. Humans express α-chymase while rodents express primarily β-chymases, as well as other isoforms (mouse MCP-4 and rat MCP-5) that more closely resemble α-chymase in regard to the processing of Ang I to Ang II [35]. The human and mouse enzymes may also play a role in the conversion of the endothelin precursor to the active peptide, as well as the activation of various inflammatory cytokines [40]. Chymases (35 kDa) are synthesized and stored in an inactive proform within mast cells and neutrophils that are released with other proteases (cathepsin G, tryptases, and renin) upon degranulation following injury or inflam-

mation [41]. Although chymases are soluble enzymes, they associate with the cell membrane and may locate to the extracellular surface of tissues following release. The serine protease inhibitor chymostatin is typically used to demonstrate specificity; however, chymostatin inhibits other Ang II-generating enzymes (cathepsin G and elastase-2). Thus, chymostatin sensitivity for Ang II generation does not necessarily demonstrate the involvement of chymase and more selective approaches should be considered [42–45]. The extent that chymase or other peptidases participate in the formation of circulating or tissue Ang II through an ACE-independent pathway remains equivocal [46].

5. Neprilysin

Neprilysin [EC 3.4.24.11; ~95 kDa] is a metalloendopeptidase that is predominantly anchored to the plasma membrane. The peptidase was initially characterized in brain to hydrolyze the opiate peptide enkephalin, hence the original terminology of the enzyme as "enkephalinase." Neprilysin was subsequently found in a number of peripheral tissues with particularly high expression within the brush border on the apical region of the renal proximal tubules. Interest in the renal expression of neprilysin reflected the potential role of the peptidase to metabolize natriuretic peptides, and selective inhibitors alone or combined with an ACE inhibitor ("vasopeptidase inhibitor") were developed as cardiovascular therapies to prolong the renal actions of these peptides [47–52]. Although the vasopeptidase inhibitor omapatrilat was clinically withdrawn regarding a greater incidence of angioedema that may reflect increased levels of bradykinin, a new generation of agents that combine a neprilysin inhibitor and an AT_1R antagonist may be a promising therapeutic approach for the treatment of heart failure [53]. One potential caveat to this approach would be that the neprilysin inhibitor component may reduce circulating levels of Ang-(1-7) and potentially attenuate the cardioprotective effects of the combined antagonist/inhibitor.

Vascular neprilysin is responsible for the extracellular conversion of Ang I to Ang-(1-7) in the circulation, particularly under conditions of chronic ACE inhibition (**Figure 1**) [54]. Neprilysin hydrolyzes the Pro^7-Phe^8 bond of Ang I to form Ang-(1-7), as well as the Tyr^4-Ile^5 bond to generate Ang-(1-4) that reflects the enzyme's preference for aromatic and hydrophobic residues. We further showed that renal neprilysin on the proximal tubules and the renal cortex readily converted both Ang I and Ang-(1-12) to Ang-(1-7), as well as that a neprilysin inhibitor attenuated the blood pressure-lowering effects of the ACE inhibitor lisinopril in the spontaneously hypertensive rat [33, 55, 56]. Apart from the conversion of Ang I to Ang-(1-7), neprilysin is capable of metabolizing a number of other peptides including adrenomedullin, Ang II [to Ang-(1-4)], endothelin, bradykinin, and the natriuretic peptides ANP and BNP.

6. Thimet oligopeptidase

Thimet oligopeptidase [EC 3.4.24.15, 80 kDa] is primarily a soluble metalloendopeptidase that resides within the cell. Similar to neprilysin, thimet oligopeptidase prefers aromatic and

hydrophobic residues and cleaves multiple peptide substrates. In regard to the RAS, thimet oligopeptidase hydrolyzes Ang I exclusivity at the Pro-Phe bond to form Ang-(1-7) (**Figure 1**). We recently showed that both neprilysin and thimet oligopeptidase contributed to the processing of Ang I to Ang-(1-7) within isolated mitochondria [21]. In the human proximal tubule HK-2 cell line, cytosolic thimet oligopeptidase was the sole activity responsible for the generation of Ang-(1-7) from exogenous Ang I [57]. Moreover, thimet oligopeptidase activity in isolated nuclei processed Ang I exclusively to Ang-(1-7) and may be a potential candidate that contributes to the nuclear levels of Ang-(1-7) within the cell [22]. The RAS was originally characterized as a classic endocrine or circulating system, but there is very compelling evidence for the intracellular expression of the RAS peptides, receptors, and synthetic components [7, 54, 58–60]. It is presently unclear the cellular mechanisms for the intracellular expression of Ang II or Ang-(1-7), although the intracellular peptidase thimet oligopeptidase may play a role in the cellular Ang-(1-7) axis.

7. Angiotensin-converting enzyme 2

ACE2 is a membrane-bound monocarboxypeptidase [EC 3.4.17.23; 120 kDa] that converts Ang II directly to Ang-(1-7) (**Figure 1**). ACE2 was initially characterized as a homolog to ACE and exhibits approximately ~40% homology with ACE [60–62]. ACE2 was initially reported to cleave Ang I not to Ang II, but to the nonapeptide Ang-(1-9) [63]. Subsequent studies found that Ang II exhibits far better kinetic values for ACE2 that would argue that the endogenous substrate is Ang II, not Ang I [64]. Among a number of peptide substrates (>100) that were screened for ACE2, Vickers et al. reported that only apelin 13 exhibited comparable kinetic values to that of Ang II [65]. In the murine heart, Ang II was primarily converted to Ang-(1-7) by ACE2 and that in the presence of the ACE2 inhibitor MLN-4760 or in ACE2 null mice there was essentially no metabolism of Ang II in the heart [66]. By contrast, under identical kinetic conditions, Ang I was primarily converted to Ang-(1-9) by carboxypeptidase A and not ACE2 in both the wild-type and ACE2 knockout mice.

In comparison to ACE, the circulating levels of ACE2 are typically quite low. Whether this reflects a reduced degree of shedding, lower vascular expression or both is not presently clear. Rice et al. reported that the molar concentration of ACE in human serum averaged 7 nM in over 500 subjects while ACE2 content was *200-fold lower* (33 pM) and was detectable in <10% of the patient population [67]. Circulating NEP content (290 pM) was also lower than ACE and evident in <30% of these patients [67]. Serum and urinary ACE2 activities are elevated in diabetes, heart failure, and hypertension [68–70]. Circulating ACE2 activity increased approximately threefold in the diabetic hypertensive mRen2.Lewis rat; however, serum ACE activity also increased in the diabetic rats. As measured under identical kinetic conditions, serum ACE was far higher than ACE2 suggesting that the capacity to generate Ang II (or metabolize Ang-(1-7) and bradykinin) remains greater than the capability to form Ang-(1-7) from Ang II [68].

Similar to ACE, ACE2 has a potentially significant role in the RAS pathway as a single catalytic step metabolizes Ang II to attenuate the Ang II-AT1 receptor pathway, and generate Ang-(1-7)

that would stimulate the Ang-(1-7)-AT7/Mas receptor axis (**Figure 1**) [71, 72]. Indeed, ACE2-deficient animals exhibit exaggerated responses to Ang II or under chronic conditions of an activated RAS [73–76]. In turn, overexpression of ACE2 or administration of the soluble form of the peptidase, which retains full enzymatic activity, attenuates the Ang II-dependent increase in blood pressure and indices of target organ injury [77–85]. In regard to the benefits of an activated ACE2 pathway, several compounds have been identified that may act as allosteric activators of ACE2 including xanthenone (XNT) and diminazene aceturate (DIZE) to promote a higher ratio of Ang-(1-7) to Ang II [86]. Chronic treatment with DIZE ameliorated the extent of pulmonary hypertensin and fibrosis, renal tissue injury, and myocardial infarction consistent with enhanced levels of Ang-(1-7) and a reduction in Ang II [84, 87–90]. Interestingly, DIZE treatment was also associated with increased mRNA levels of ACE2 suggesting that DIZE may exhibit actions apart from the direct activation of the peptidase [87, 91]. However, it should be noted that the effects of DIZE on ACE2 activity or expression have not been confirmed by others. Haber et al. [92] found no effect of DIZE on soluble ACE2 activity or an influence on Ang II-dependent hypertension using similar doses of DIZE as previously reported. Velkosa et al. [93] also found no direct effect of various concentrations of DIZE on renal ACE2 activity and slightly increased renal ACE2 activity in the 5/6 nephrectomized rat following a 2-week administration. Of particular interest, the Velkosa study reported that DIZE normalized the marked increase in renal ACE activity suggesting that ACE may be a more relevant target than ACE2 [93]. DIZE also failed to increase ACE2 activity or enhance the local vascular actions of Ang-(1-7) in the isolated pig coronary arteries [94].

Conceptually, the use of ACE2 as a therapeutic agent to chronically alter the balance of Ang II and Ang-(1-7) is challenging. ACE activity in the circulation and the vasculature surface is significant with a very high capacity to generate Ang II. This reflects not simply the abundance of ACE but the significant capability of angiotensinogen and renin to generate the ACE substrate Ang I. Moreover, reduced Ang II levels by exogenous ACE2 should stimulate the generation of Ang II that reflects the activation of negative feedback mechanisms on renin. Therefore, it is difficult to conceive sufficiently high levels of ACE2 that can be achieved to chronically reduce Ang II *and* increase Ang-(1-7) except with the possible addition of an ACE inhibitor. In this case, supplementation of ACE2 may degrade residual levels of Ang II and the circulating levels of Ang-(1-7) may be augmented, particularly as the Ang-(1-7)-degrading pathway in the circulation is attenuated by the ACE inhibitor.

8. Dipeptidyl peptidase 3

We demonstrated a role for ACE in the metabolism of Ang-(1-7), but there are other potential pathways that may regulate endogenous levels of the peptide [95]. Marshall and colleagues reported that ACE and a second peptidase activity in the sheep cerebrospinal fluid degraded Ang-(1-7) [95–97]. Interestingly, the non-ACE-degrading activity accounted for a greater contribution of metabolism than ACE [96]. Moreover, this activity was inversely correlated to CSF levels of Ang-(1-7) in control and betamethasone-exposed sheep, a model of fetal programming that exhibits elevated blood pressure and an attenuated baroreflex [7]. Subsequent

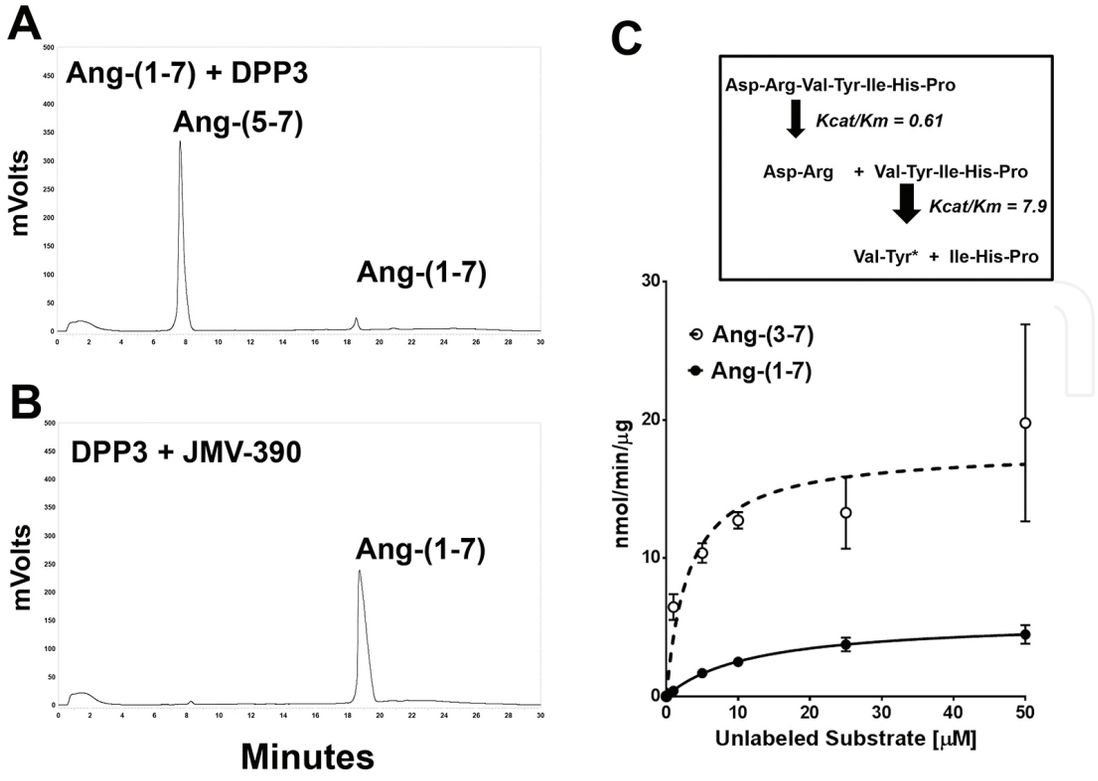

Figure 2. Kinetics of human DPP 3 hydrolysis of Ang-(1-7) and Ang-(3-7). (A) Chromatographs reveal that human DPP 3 cleaves Ang-(1-7) to the tripeptide Ang-(5-7). (B) Chromatograph reveals that the JMV-390 inhibitor attenuates Ang-(1-7) metabolism by human DPP 3. (C) Kinetic analysis of the hydrolysis of Ang-(1-7) and Ang-(3-7) by human DPP 3. Data adapted from Cruz-Diaz et al. [99].

studies found the Ang-(1-7)-degrading activity in sheep brain and kidney cortex, as well as in the human proximal tubule HK-2 cell line [57, 98]. The enzyme activity exhibited unusual characteristics as Ang I and other peptides equal to or greater than 10 residues were not substrates for the peptidase [57, 98]. In addition, the peptidase was sensitive to both chelating agents such as o-phenanthroline and EDTA, and the sulfhydryl inhibitors APMA and PCMB [57, 98]. Additional studies identified the inhibitor JMV-390, originally developed to block the metalloendopeptidases neprilysin, thimet oligopeptidase, and neurolysin, that potently inhibited the Ang-(1-7)-degrading activity in the brain and kidney [IC_{50} <1 nM] [57, 98]. Conversely, specific inhibitors against these endopeptidases did not attenuate the Ang-(1-7)-degrading activity [98]. Interestingly, the peptidase activity accounted for the sole degradative pathway in the cytosolic fraction and in the media of the HK-2 cells [57]. Utilizing the HK-2 cells as the source of the Ang-(1-7)-degrading activity, we recently purified the peptidase from the cell cytosol by ion exchange and hydrophobic interaction chromatography and identified the enzyme as dipeptidyl peptidase 3 [EC 3.4. 14.4, DPP 3] [99].

DPP 3 belongs to a family of metalloaminopeptidases that sequentially cleave two residues from the N-terminus of peptides no more than eight residues in length and this likely explains our previous results that Ang I, apelin-13, and neurotensin were not substrates for the Ang-(1-7)-degrading activity in the CSF and brain [98]. We obtained a human recombinant form of

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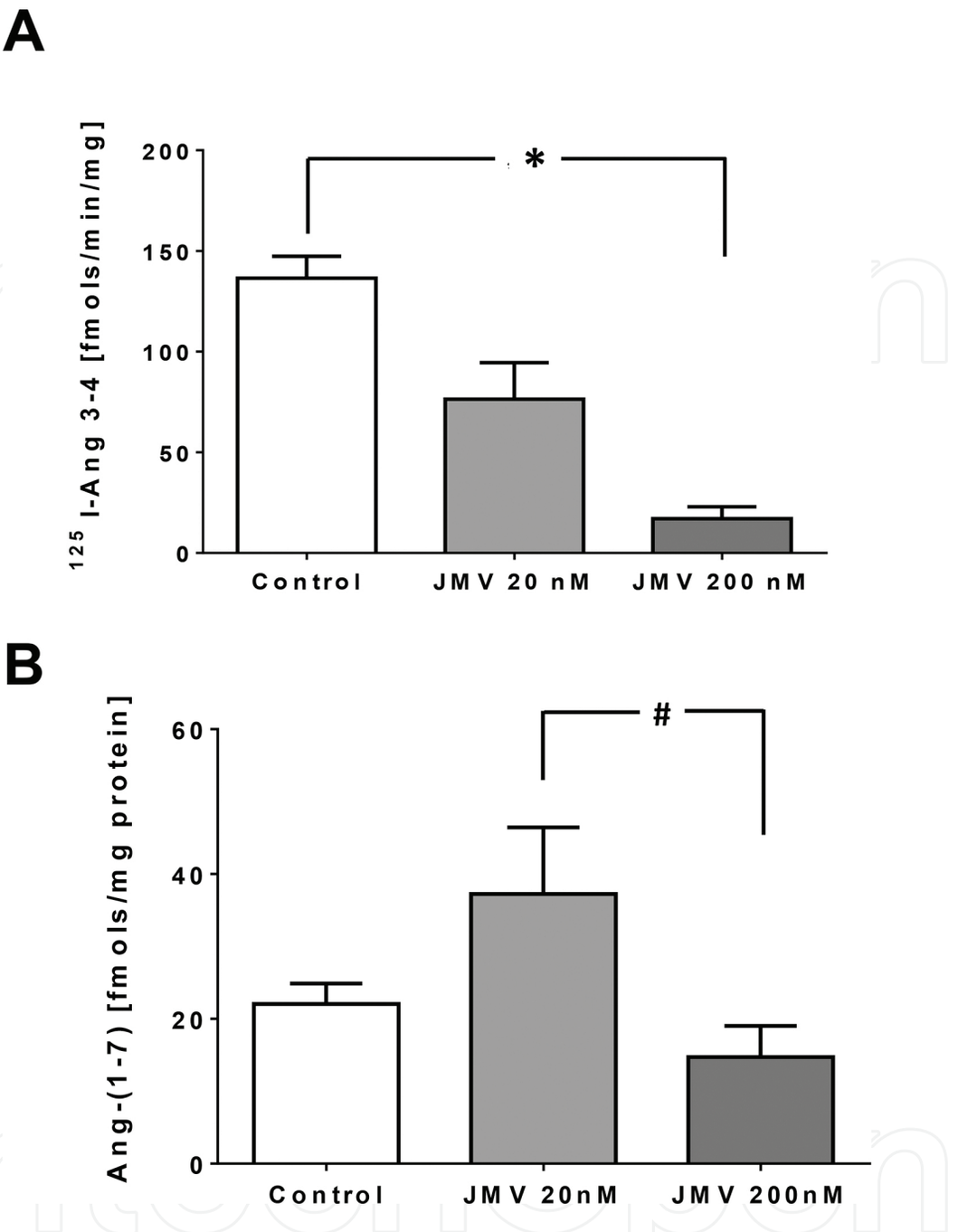

Figure 3. The JMV-390 inhibitor reduces DPP 3 activity and alters Ang-(1-7) cellular content of HK-2 cells. (A) Increasing doses of the JMV-390 inhibitor reduced DPP 3 activity (^{125}I-Ang-(3-4) generation—fmol/min/mg protein) in HK-2 cells. (B) Low-dose JMV-390 (20 nM) tended to increase the cellular content of Ang-(1-7) (fmols/mg protein), but high dose (200 nM) significantly reduced Ang-(1-7). $^{*}P < 0.05$ versus control; $^{\#}P < 0.05$ versus 20 nM JMV. Data adapted from Cruz-Diaz et al. [99].

DPP 3 to show that the enzyme metabolized Ang-(1-7) in two cleavage steps [99]. DPP 3 initially hydrolyzes Ang-(1-7) at the Arg^2-Val^3 bond to form Ang-(3-7) and the dipeptide Arg^1-Asp^2. Ang-(3-7) is then very rapidly cleaved at Tyr^4-Ile^5 to form Ang-(5-7) and Val^3-Tyr^4. The kinetic analysis of DPP 3 hydrolysis revealed a higher efficiency constant (kcat/km) for Ang-(3-7) than

Ang-(1-7) (**Figure 2**) [99]. The preferred hydrolysis of Ang-(3-7) by DPP 3 would account for our inability to demonstrate the accumulation of Ang-(3-7) following the initial metabolism of Ang-(1-7). In regard to an *in vivo* role for DPP 3 to modulate Ang-(1-7), human HK-2 cells were treated with varying doses of the JMV-390 inhibitor and we assessed both the endogenous content of Ang-(1-7) and the intracellular DPP-3/Ang-(1-7)-degrading activity in the cells. As shown in **Figure 3**, 20- and 200-nM JMV-390 reduced DPP3 activity by >30 and >80%, respectively, as compared to control suggesting that the inhibitor effectively penetrates the cells [99]. The lower dose of JMV increased the cellular content Ang-(1-7) approximately twofold, although this did not reach statistical significance. The higher dose of JMV, however, significantly reduced the intracellular levels of the peptide [99]. We interpret the latter results that the high dose may spill over to block other peptidases including thimet oligopeptidase that may be involved in the generation of Ang-(1-7) in the renal cells [57]. Thus, the blockade of Ang-(1-7)-forming enzymes by the high-dose JMV may override any protective effects of DPP 3 inhibition. We are currently assessing alternative approaches to block DPP 3 within the HK-2 cells and assess both secreted and intracellular levels of Ang-(1-7), as well as Ang-(1-7)-dependent actions on these cells.

9. AGE-induced peptidase

Our recent studies assessed the potential role of Ang-(1-7) in the progression of epithelial-to-mesenchymal transition (EMT) in the renal epithelial NRK-52 cell line by either advanced glycation end products (AGEs) or the cytokine TGF-β [100]. Ang-(1-7) essentially abolished the cellular indices for EMT in the NRK-52 cells likely through the inhibition of the noncanonical ERK 1/2-signaling pathway stimulated by AGE (100). Interestingly, AGE exposure reduced the intracellular levels of Ang-(1-7) but did not alter the cellular content of Ang II [100]. Processing of Ang I to Ang-(1-7) by thimet oligopeptidase tended to be reduced by AGE; however, the intracellular metabolism of Ang-(1-7) to Ang-(1-4) was significantly increased by AGE exposure [100]. These data suggest that AGE-induced EMT may reflect lower Ang-(1-7) tone in the renal epithelial cells that may be permissive for the progression of EMT and the increase in tissue fibrosis. Our preliminary data suggest that DPP 3 is not responsible for the AGE-induced metabolism of Ang-(1-7) in these cells and that another peptidase activity may participate in the cellular metabolism of the peptide. As shown in **Figure 4A**, the NRK-52 cytosolic fraction readily hydrolyzed the quenched Ang-(1-7) fluorescent substrate Abz^{1}-Ang-(1-7)-$Tyr^{7}(NO_2)$ to Abz-Ang-(1-4). By contrast, DPP 3 in the HK-2 renal cells did not cleave the Ang-(1-7) fluorescent peptide (**Figure 4B**). The failure of DPP 3 to cleave the fluorescent Ang-(1-7) substrate is consistent with the inability of DPP 3 to hydrolyze blocked N-terminal residues of peptides such as the Abz moiety on the Ang-(1-7) substrate. Moreover, hydrolysis of the N-terminally blocked Ang-(1-7) fluorescent substrate by the cytosolic fraction of the NRK-52 cells suggests the peptidase is likely an endopeptidase. Apart from the identification of the Ang-(1-7)-degrading activity in the NRK-52E cells, it remains to be determined whether intracellular levels of Ang-(1-7) influence the EMT process.

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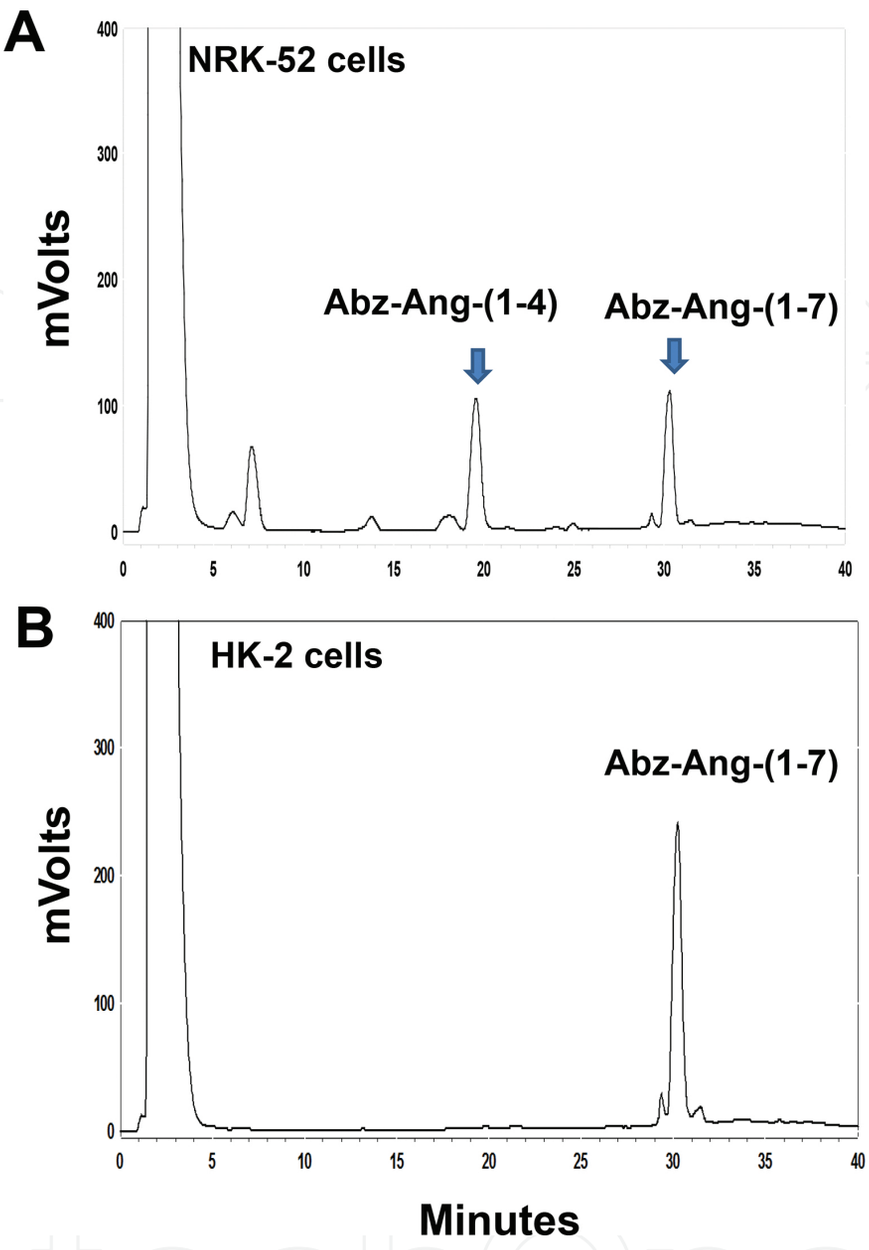

Figure 4. Metabolism of Abz[1]-Ang-(1-7)-Tyr$_7$ (NO_2). (A) HPLC chromatograph reveals that the cytosolic fraction of the NRK-52E cells cleaves the Ang-(1-7)-fluorescent substrate Abz-Ang-(1-7) to Abz-Ang-(1-4). (B) By contrast, HK-2 cytosolic fraction does not hydrolyze the Ang-(1-7)-fluorescent substrate suggesting an endopeptidase activity distinct from DPP 3. The large void peak is absorbance from the DMSO solvent to dissolve the fluorescent substrate. Substrate concentration and incubation conditions are identical for both cell types. The HK-2 cell data are adapted from Wilson et al. [57].

10. Characterization of peptidase pathways

In the review of the peptidases of the Ang-(1-7) axis, a brief discussion of the various biochemical approaches to characterize these enzymatic components is warranted. Ideally, utilization of the endogenous peptide substrates including Ang I, Ang II, and Ang-(1-7) should

be assessed to identify the peptidase activities involved in the RAS [101]. One advantage is that the contribution of various peptidases for a given peptide is directly comparable to determine the predominant pathway in a particular tissue or treatment condition. Peptidase activities derived by different synthetic substrates are not comparable unless standardized to the enzyme concentration. Moreover, the use of endogenous peptide substrates may reveal novel peptidase activities involved in angiotensin processing [102, 103]. Peptidase assays developed in our laboratory typically utilize ^{125}I-radiolabled peptides coupled to high-performance liquid chromatography (HPLC)-based separation and automated in-line γ-detection. Advantages of this are that only microliter amounts of serum or microgram quantities of tissue are normally required that reflects detection sensitivity in the fmol range and the lack of detector interference or quenching [33].

More recent studies have incorporated mass spectroscopy (MS) detection of angiotensin metabolism in tissues, cells, and plasma, as well as the derivation of processing networks [104]. Velez and colleagues applied HPLC-MS analysis of Ang I processing in rat glomeruli to reveal the predominant processing of Ang I to Ang-(1-7) catalyzed by neprilysin [104]. Interestingly, the authors could not demonstrate an Ang I to Ang II pathway even following the blockade of the Ang-(1-7) pathway with a neprilysin inhibitor. Hildesbrand et al. [103] utilized a HPLC-tandem quadrupole system (HPLC-MS/MS) to reveal multiple metabolism pathways from Ang I to its N-terminal metabolites Ang-(5-10) and Ang-(4-10), as well as Ang II and Ang-(1-7) in immobilized proteins from human plasma. Suski et al. [105] reported that Ang I was primarily converted to Ang-(1-7) in vascular smooth muscle cells (VSMCs) as characterized by HPLC-MS/MS and confirms our earlier study that thimet oligopeptidase directly processed Ang I to Ang-(1-7) in rat VSMC [106]. Grobe and colleagues have applied "in situ" MALDI to characterize both renal and cardiac metabolism of exogenous Ang II [107, 108]. Ang-(1-7) was the primary product from Ang II in the renal cortex while Ang III was the major metabolite in the medulla [108]. In the heart, Ang III and Ang-(1-7) were products of Ang II metabolism catalyzed by APA and ACE2, respectively [107]. These data confirm earlier HPLC-based studies on the contribution of ACE2 to Ang-(1-7) formation in the mouse and human heart [66, 109]. Although this approach cannot distinguish intracellular versus membrane or extracellular processing and requires relatively high-substrate concentrations, it is likely that these systems will develop the required sensitivity and resolution to detect peptides *in situ*, as well as characterize the extent of enzymatic processing.

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References

[1] Aroor, A. R.; Demarco, V. G.; Jia, G.; Sun, Z.; Nistala, R.; Meininger, G. A.; Sowers, J. R. The role of tissue renin-angiotensin-aldosterone system in the development of endothelial dysfunction and arterial stiffness. Front Endocrinol (Lausanne) 2013;4:161.

[2] Ferrario, C. M.; Strawn, W. B. Role of the renin-angiotensin-aldosterone system and proinflammatory mediators in cardiovascular disease. Am J Cardiol 2006;98:121–128.

[3] Te, R. L.; van Esch, J. H.; Roks, A. J.; van den Meiracker, A. H.; Danser, A. H. Hypertension: renin-angiotensin-aldosterone system alterations. Circ Res 2015;116(6):960–975.

[4] Kobori, H.; Nangaku, M.; Navar, L. G.; Nishiyama, A. The intrarenal renin-angiotensin system: from physiology to the pathobiology of hypertension and kidney disease. Pharmacol Rev 2007;59(3):251–287.

[5] Navar, L. G.; Prieto, M. C.; Satou, R.; Kobori, H. Intrarenal angiotensin II and its contribution to the genesis of chronic hypertension. Curr Opin Pharmacol 2011;11(2): 180–186.

[6] Santos, R. A. Angiotensin-(1-7). Hypertension 2014;63(6):1138–1147.

[7] Chappell, M. C.; Marshall, A. C.; Alzayadneh, E. M.; Shaltout, H. A.; Diz, D. I. Update on the Angiotensin converting enzyme 2-Angiotensin (1-7)-MAS receptor axis: fetal programming, sex differences, and intracellular pathways. Front Endocrinol (Lausanne) 2014;4:201.

[8] Carey, R. M.; Padia, S. H. Role of angiotensin AT(2) receptors in natriuresis: Intrarenal mechanisms and therapeutic potential. Clin Exp Pharmacol Physiol 2013;40(8):527–534.

[9] Sumners, C.; de Kloet, A. D.; Krause, E. G.; Unger, T.; Steckelings, U. M. Angiotensin type 2 receptors: blood pressure regulation and end organ damage. Curr Opin Pharmacol 2015;21:115–121.

[10] Gonzalez, A. A.; Lara, L. S.; Luffman, C.; Seth, D. M.; Prieto, M. C. Soluble form of the (pro)renin receptor is augmented in the collecting duct and urine of chronic angiotensin II-dependent hypertensive rats. Hypertension 2011;57(4):859–864.

[11] Navar, L. G.; Kobori, H.; Prieto, M. C.; Gonzalez-Villalobos, R. A. Intratubular renin-angiotensin system in hypertension. Hypertension 2011;57(3):355–362.

[12] Gonzalez, A. A.; Liu, L.; Lara, L. S.; Seth, D. M.; Navar, L. G.; Prieto, M. C. Angiotensin II stimulates renin in inner medullary collecting duct cells via protein kinase C and independent of epithelial sodium channel and mineralocorticoid receptor activity. Hypertension 2011;57(3):594–599.

[13] Prieto-Carrasquero, M. C.; Harrison-Bernard, L. M.; Kobori, H.; Ozawa, Y.; Hering-Smith, K. S.; Hamm, L. L.; Navar, L. G. Enhancement of collecting duct renin in angiotensin II-dependent hypertensive rats. Hypertension 2004;44(2):223–229.

[14] Peters, J.; Clausmeyer, S. Intracellular sorting of renin: cell type specific differences and their consequences. J Mol Cell Cardiol 2002;34(12):1561–1568.

[15] Lavoie, J. L.; Liu, X.; Bianco, R. A.; Beltz, T. G.; Johnson, A. K.; Sigmund, C. D. Evidence supporting a functional role for intracellular renin in the brain. Hypertension 2006;47(3):461–466.

[16] Ishigami, T.; Kino, T.; Chen, L.; Minegishi, S.; Araki, N.; Umemura, M.; Abe, K.; Sasaki, R.; Yamana, H.; Umemura, S. Identification of bona fide alternative renin transcripts expressed along cortical tubules and potential roles in promoting insulin resistance in vivo without significant plasma renin activity elevation. Hypertension 2014;64(1):125–133.

[17] Peters, J.; Wanka, H.; Peters, B.; Hoffmann, S. A renin transcript lacking exon 1 encodes for a non-secretory intracellular renin that increases aldosterone production in transgenic rats. J Cell Mol Med 2008;12(4):1229–1237.

[18] Clausmeyer, S.; Sturzebecher, R.; Peters, J. An alternative transcript of the rat renin gene can result in a truncated prorenin that is transported into adrenal mitochondria. Circ Res 1999;84(3):337–344.

[19] Wanka, H.; Kessler, N.; Ellmer, J.; Endlich, N.; Peters, B. S.; Clausmeyer, S.; Peters, J. Cytosolic renin is targeted to mitochondria and induces apoptosis in H9c2 rat cardiomyoblasts. J Cell Mol Med 2009;13(9A):2926–2937.

[20] Wanka, H.; Staar, D.; Lutze, P.; Peters, B.; Hildebrandt, J.; Beck, T.; Baumgen, I.; Albers, A.; Krieg, T.; Zimmermann, K.; Sczodrok, J.; Schafer, S.; Hoffmann, S.; Peters, J. Anti-necrotic and cardioprotective effects of a cytosolic renin isoform under ischemia-related conditions. J Mol Med (Berl) 2016;94(1):61–69.

[21] Wilson, B. A.; Nautiyal, M.; Gwathmey, T. M.; Rose, J. C.; Chappell, M. C. Evidence for a Mitochondrial Angiotensin-(1-7) System in the kidney. Am J Physiol Renal Physiol 2016; 308(6):F594-F601.

[22] Alzayadneh, E. M.; Chappell, M. C. Nuclear expression of renin-angiotensin system components in NRK-52E renal epithelial cells. J Renin Angiotensin Aldosterone Syst 2014;307(5):R487-R489.

[23] Lavoie, J. L.; Lake-Bruse, K. D.; Sigmund, C. D. Increased blood pressure in transgenic mice expressing both human renin and angiotensinogen in the renal proximal tubule. Am J Physiol Renal Fluid Electrolyte Physiol 2004;286(5):F965–F971.

[24] Crowley, S. D.; Gurley, S. B.; Herrera, M. J.; Ruiz, P.; Griffiths, R.; Kumar, A. P.; Kim, H. S.; Smithies, O.; Le, T. H.; Coffman, T. M. Angiotensin II causes hypertension and cardiac hypertrophy through its receptors in the kidney. Proc Natl Acad Sci U S A 2006;103(47): 17985–17990.

[25] Li, X. C.; Cook, J. L.; Rubera, I.; Tauc, M.; Zhang, F.; Zhuo, J. L. Intrarenal transfer of an intracellular fluorescent fusion of angiotensin II selectively in proximal tubules increases blood pressure in rats and mice. Am J Physiol Renal Physiol 2011;300(5):F1076–F1088.

[26] Li, X. C.; Hopfer, U.; Zhuo, J. L. Novel signaling mechanisms of intracellular angiotensin II-induced NHE3 expression and activation in mouse proximal tubule cells. Am J Physiol Renal Physiol 2012;303(12):F1617–F1628.

[27] Li, X. C.; Zhuo, J. L. Proximal tubule-dominant transfer of AT1a receptors induces blood pressure responses to intracellular angiotensin II in AT1a receptor-deficient mice. Am J Physiol Regul Integr Comp Physiol 2013;304(8): R588-R598.

[28] Davisson, R. L.; Ding, Y.; Stec, D. E.; Catterall, J. F.; Sigmund, C. D. Novel mechanism of hypertension revealed by cell-specific targeting of human angiotensinogen in transgenic mice. Physiol Genomics 1999;1:3–9.

[29] Bernstein, K. E.; Ong, F. S.; Blackwell, W. L.; Shah, K. H.; Giani, J. F.; Gonzalez-Villalobos, R. A.; Shen, X. Z.; Fuchs, S.; Touyz, R. M. A modern understanding of the traditional and nontraditional biological functions of angiotensin-converting enzyme. Pharmacol Rev 2013;65(1):1–46.

[30] Chappell, M. C.; Pirro, N. T.; Sykes, A.; Ferrario, C. M. Metabolism of angiotensin-(1-7) by angiotensin converting enzyme. Hypertension 1998;31(1 (Pt 2)):362–367.

[31] Yamada, K.; Iyer, S. N.; Chappell, M. C.; Ganten, D.; Ferrario, C. M. Converting enzyme determines the plasma clearance of angiotensin-(1-7). Hypertension 1998;98(32):496–502.

[32] Chappell, M. C. Nonclassical renin-angiotensin system and renal function. Compr Physiol 2012;2(4):2733–2752.

[33] Shaltout, H. A.; Westwood, B.; Averill, D. B.; Ferrario, C. M.; Figueroa, J.; Diz, D. I.; Rose, J. C.; Chappell, M. C. Angiotensin metabolism in renal proximal tubules, urine and serum of sheep: Evidence for ACE2-dependent processing of Angiotensin II. Am J Physiol Renal Physiol 2006;292:F82–F91.

[34] Park, S.; Bivona, B. J.; Kobori, H.; Seth, D. M.; Chappell, M. C.; Lazartigues, E.; Harrison-Bernard, L. M. Major role for ACE-independent intrarenal ANG II formation in type II diabetes. Am J Physiol Renal Physiol 2010;298(1):F37–F48.

[35] Caughey, G. H.; Raymond, W. W.; Wolters, P. J. Angiotensin II generation by mast cell alpha- and beta-chymases. Biochim Biophys Acta 2000;1480(1–2):245–257.

[36] Nagata, S.; Hatakeyama, K.; Asami, M.; Tokashiki, M.; Hibino, H.; Nishiuchi, Y.; Kuwasako, K.; Kato, J.; Asada, Y.; Kitamura, K. Big angiotensin-25: a novel glycosylated angiotensin-related peptide isolated from human urine. Biochem Biophys Res Commun 2013;441(4):757–762.

[37] Urata, H.; Kinoshita, A.; Misono, K. S.; Bumpus, F. M.; Husain, A. Identification of a highly specific chymase as the major angiotensin II-forming enzyme in the human heart. J Biol Chem 1990;265:22348–22357.

[38] Ahmad, S.; Simmons, T.; Varagic, J.; Moniwa, N.; Chappell, M. C.; Ferrario, C. M. Chymase-Dependent Generation of Angiotensin II from Angiotensin-(1-12) in Human Atrial Tissue. PLoS One 2011;6(12):e28501.

[39] Wintroub, B. U.; Kaempfer, C. E.; Schechter, N. M.; Proud, D. A human lung mast cell chymotrypsin-like enzyme: identification and partial characterization. J Clin Invest 1986;77:196–201.

[40] Semaan, W.; Desbiens, L.; Houde, M.; Labonte, J.; Gagnon, H.; Yamamoto, D.; Takai, S.; Laidlaw, T.; Bkaily, G.; Schwertani, A.; Pejler, G.; Levesque, C.; Desjardins, R.; Day, R.; D'Orleans-Juste, P. Chymase inhibitor-sensitive synthesis of endothelin-1 (1-31) by recombinant mouse mast cell protease 4 and human chymase. Biochem Pharmacol 2015;94(2):91–100.

[41] Caughey, G. H. Mast cell proteases as protective and inflammatory mediators. Adv Exp Med Biol 2011;716:212–234.

[42] Santos, C. F.; Paula, C. A.; Salgado, M. C.; Oliveira, E. B. Kinetic characterization and inhibition of the rat MAB elastase-2, an angiotensin I-converting serine protease. Can J Physiol Pharmacol 2002;80(1):42–47.

[43] Santos, C. F.; Oliveira, E. B.; Salgado, M. C.; Greene, A. S. Molecular cloning and sequencing of the cDNA for rat mesenteric arterial bed elastase-2, an angiotensin II-forming enzyme. J Cardiovasc Pharmacol 2002;39(5):628–635.

[44] Santos, C. F.; Caprio, M. A.; Oliveira, E. B.; Salgado, M. C.; Schippers, D. N.; Munzenmaier, D. H.; Greene, A. S. Functional role, cellular source, and tissue distribution of rat elastase-2, an angiotensin II-forming enzyme. Am J Physiol Heart Circ Physiol 2003;285(2):H775–H783.

[45] Santos, C. F.; Greene, A. S.; Salgado, M. C.; Oliveira, E. B. Conversion of renin substrate tetradecapeptide to angiotensin II by rat MAB elastase-2. Can J Physiol Pharmacol 2004;82(11):1000–1005.

[46] Campbell, D. J. Angiotensin II generation in vivo: does it involve enzymes other than renin and angiotensin-converting enzyme? J Renin Angiotensin Aldosterone Syst 2012;13(2):314–316.

[47] Corti, R.; Burnett, J. C. J.; Rouleau, J. L.; Ruschitzka, F.; Luscher, T. F. Vasopeptidase inhibitors: a new therapeutic concept in cardiovascular disease? Circulation 1909;104(15):1856–1862.

[48] Cataliotti, A.; Boerrigter, G.; Chen, H. H.; Jougasaki, M.; Costello, L. C.; Tsuruda, T.; Lee, S. C.; Malatino, L. S.; Burnett, J. C., Jr. Differential actions of vasopeptidase inhibition versus angiotensin-converting enzyme inhibition on diuretic therapy in experimental congestive heart failure. Circulation 2002;105(5): 639–644.

[49] Ferrario CM, Averill DB, Brosnihan KB, Chappell MC, Iskandar SS, Dean RH, Diz DI. Vasopeptidase inhibition and angiotensin-(1-7) in the spontaneously hypertensive rat. Kidney Intl 2002;62(4):1349-1357.

[50] Tikkanen, I.; Tikkanen, T.; Cao, Z.; Allen, T. J.; Davis, B. J.; Lassila, M.; Casley, D.; Johnston, C. I.; Burrell, L. M.; Cooper, M. E. Combined inhibition of neutral endopeptidase with angiotensin converting enzyme or endothelin converting enzyme in experimental diabetes. J Hypertens 2002;20:707–714.

[51] Cao, Z.; Burrell, L. M.; Tikkanen, I.; Bonnet, F.; Cooper, M. E.; Gilbert, R. E. Vasopeptidase inhibition attenuates the progression of renal injury in subtotal nephrectomized rats. Kid Int 2001; 2:715-721.

[52] Kubota, E.; Dean, R. G.; Hubner, R. A.; Casley, D. J.; Johnston, C. I.; Burrell, L. M. Differential tissue and enzyme inhibitory effects of the vasopeptidase inhibitor omapatrilat in the rat. Clin Sci 2003;105:339–345.

[53] Hubers, S. A.; Brown, N. J. Combined angiotensin receptor antagonism and neprilysin inhibition. Circulation 2016;133(11):1115–1124.

[54] Chappell, M. C. Biochemical evaluation of the renin-angiotensin system: the good, bad, and absolute? Am J Physiol Heart Circ Physiol 2016;310(2):H137–H152.

[55] Iyer SN, Chappell MC, Averill DA, Diz DI, Ferrario CM. Vasodepressor actions of Angiotensin-(1-7) unmasked during combined treatment with lisinopril and losartan. Hypertension 1998:31:699-705.

[56] Westwood, B. M.; Chappell, M. C. Divergent pathways for the angiotensin-(1-12) metabolism in the rat circulation and kidney. Peptides 2012;35(2):190–195.

[57] Wilson, B. A.; Cruz-Diaz, N.; Marshall, A. C.; Pirro, N. T.; Su, Y.; Gwathmey, T. M.; Rose, J. C.; Chappell, M. C. An angiotensin-(1-7) peptidase in the kidney cortex, proximal tubules, and human HK-2 epithelial cells that is distinct from insulin-degrading enzyme. Am J Physiol Renal Physiol 2015;308(6):F594–F601.

[58] Zhuo, J. L.; Li, X. C. Novel roles of intracrine angiotensin II and signalling mechanisms in kidney cells. J Renin Angiotensin Aldosterone Syst 2007;8(1):23–33.

[59] Bkaily, G.; Nader, M.; Avedanian, L.; Choufani, S.; Jacques, D.; D'Orleans-Juste, P.; Gobeil, F.; Chemtob, S.; Al-Khoury, J. G-protein-coupled receptors, channels, and Na+-H+ exchanger in nuclear membranes of heart, hepatic, vascular endothelial, and smooth muscle cells. Can J Physiol Pharmacol 2006;84(3-4):431–441.

[60] Re, R. N. Intracellular renin and the nature of intracrine enzymes. Hypertension 2003;42(2):117–122.

[61] Tipnis, S. R.; Hooper, N. M.; Hyde, R.; Karran, E.; Christie, G.; Turner, A. J. A human homolog of angiotensin-converting enzyme. Cloning and functional expression as a captopril-insensitive carboxypeptidase. J Biol Chem 2000;275:33238–33243.

[62] Turner, A. J.; Hooper, N. M. The angiotensin-converting enzyme gene family: genomics and pharmacology. TIPS 2002;23(4):177–183.

[63] Donoghue, M.; Hsieh, F.; Baronas, E.; Godbout, K.; Gosselin, M.; Stagliano, N.; Donovan, M.; Woolf, B.; Robinson, K.; Jeyaseelan, R.; Breitbart, R. E.; Acton, S. A novel angiotensin-converting enzyme-related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1-9. Circ Res 2000;87:E1–E9.

[64] Rice, G. I.; Thomas, D. A.; Grant, P. J.; Turner, A. J.; Hooper, N. M. Evaluation of angiotensin-converting enzyme (ACE), its homologue ACE2 and neprilysin in angiotensin peptide metabolism. Biochem J 2004;383(Pt 1):45–51.

[65] Vickers, C.; Hales, P.; Kaushik, V.; Dick, L.; Gavin, J.; Tang, J.; Godbout, K.; Parsons, T.; Baronas, E.; Hsieh, F.; Acton, S.; Patane, M.; Nichols, A.; Tummino, P. Hydrolysis of biological peptides by human angiotensin-converting enzyme-related carboxypeptidase. J Biol Chem 2002;277(17):14838–14843.

[66] Garabelli, P. J.; Modrall, J. G.; Penninger, J. M.; Ferrario, C. M.; Chappell, M. C. Distinct roles for angiotensin converting enzyme 2 and carboxypeptidase A in the processing of angiotensins in the murine heart. Exp Physiol 2008;96:613-621.

[67] Rice, G. I.; Jones, A. L.; Grant, P. J.; Carter, A. M.; Turner, A. J.; Hooper, N. M. Circulating activities of angiotensin-converting enzyme, its homolog, angiotensin-converting enzyme 2, and neprilysin in a family study. Hypertension 2006;48:914–920.

[68] Yamaleyeva, L. M.; Gilliam-Davis, S.; Almeida, I.; Brosnihan, K. B.; Lindsey, S. H.; Chappell, M. C. Differential regulation of circulating and renal ACE2 and ACE in hypertensive mRen2.Lewis rats with early-onset diabetes. Am J Physiol Renal Physiol 2012;302(11):F1374–F1384.

[69] Epelman, S.; Shrestha, K.; Troughton, R. W.; Francis, G. S.; Sen, S.; Klein, A. L.; Tang, W. H. Soluble angiotensin-converting enzyme 2 in human heart failure: relation with myocardial function and clinical outcomes. J Card Fail 2009;15(7):565–571.

[70] Tikellis, C.; Bialkowski, K.; Pete, J.; Sheehy, K.; Su, Q.; Johnston, C.; Cooper, M.; Thomas, M. ACE2 deficiency modifies renoprotection afforded by ACE inhibition in experimental diabetes. Diabetes 2008;57:1018–1025.

[71] Bradford, C. N.; Ely, D. R.; Raizada, M. K. Targeting the vasoprotective axis of the renin-angiotensin system: a novel strategic approach to pulmonary hypertensive therapy. Curr Hypertens Rep 2010;12(4):212–219.

[72] Chappell, M. C. Emerging evidence for a functional angiotensin-converting enzyme 2-angiotensin-(1-7) mas receptor axis; more than regulation of blood pressure? Hypertension 2007;50(4):596–599.

[73] Soler, M. J.; Wysocki, J.; Ye, M.; Lloveras, J.; Kanwar, Y.; Batlle, D. ACE2 inhibition worsens glomerular injury in association with increased ACE expression in streptozotocin-induced diabetic mice. Kid Int 2007;72:614–623.

[74] Oudit, G. Y.; Herzenberg, A. M.; Kassiri, Z.; Wong, D.; Reich, H.; Khokha, R.; Crackower, M. A.; Backx, P. H.; Penninger, J. M.; Scholey, J. W. Loss of angiotensin-converting enzyme-2 leads to the late development of angiotensin II-dependent glomerulosclerosis. Am J Pathol 2006;168(6):1808–1820.

[75] Crackower, M. A.; Sarao, R.; Oudit, G. Y.; Yagil, C.; Kozieradzki, I.; Scanga, S. E.; Oliveira-dos-Santo, A. J.; da Costa, J.; Zhang, L.; Pei, Y.; Scholey, J.; Bray, M. R.; Ferrario, C. M.; Backx, P. H.; Manoukian, A. S.; Chappell, M. C.; Yagil, Y.; Penninger, J. M. Angiotensin-converting enzyme 2 is an essential regulator of heart function. Nature 2002;417:822–828.

[76] Oudit, G. Y.; Kassiri, Z.; Patel, M. P.; Chappell, M.; Butany, J.; Backx, P. H.; Tsushima, R. G.; Scholey, J. W.; Khokha, R.; Penninger, J. M. Angiotensin II-mediated oxidative stress and inflammation mediate the age-dependent cardiomyopathy in ACE2 null mice. Cardiovasc Res 2007;75(1):29–39.

[77] Chhabra, K. H.; Xia, H.; Pedersen, K. B.; Speth, R. C.; Lazartigues, E. Pancreatic angiotensin-converting enzyme 2 improves glycemia in angiotensin II-infused mice. Am J Physiol Endocrinol Metab 2013;304(8):E874–E884.

[78] Oudit, G. Y.; Liu, G. C.; Zhong, J.; Basu, R.; Chow, F. L.; Zhou, J.; Loibner, H.; Janzek, E.; Schuster, M.; Penninger, J. M.; Herzenberg, A. M.; Kassiri, Z.; Scholey, J. W. Human recombinant ACE2 reduces the progression of diabetic nephropathy. Diabetes 2010;59(2):529–538.

[79] Zhong, J.; Basu, R.; Guo, D.; Chow, F. L.; Byrns, S.; Schuster, M.; Loibner, H.; Wang, X. H.; Penninger, J. M.; Kassiri, Z.; Oudit, G. Y. Angiotensin-converting enzyme 2 suppresses pathological hypertrophy, myocardial fibrosis, and cardiac dysfunction. Circulation 2010;122(7):717–28, 18.

[80] Wysocki, J.; Ye, M.; Rodriguez, E.; Gonzalez-Pacheco, F. R.; Barrios, C.; Evora, K.; Schuster, M.; Loibner, H.; Brosnihan, K. B.; Ferrario, C. M.; Penninger, J. M.; Batlle, D. Targeting the degradation of angiotensin II with recombinant angiotensin-converting

enzyme 2: prevention of angiotensin II-dependent hypertension. Hypertension 2010;55(1):90–98.

[81] Ye, M.; Wysocki, J.; Gonzalez-Pacheco, F. R.; Salem, M.; Evora, K.; Garcia-Halpin, L.; Poglitsch, M.; Schuster, M.; Batlle, D. Murine recombinant angiotensin-converting enzyme 2: effect on angiotensin II-dependent hypertension and distinctive angiotensin-converting enzyme 2 inhibitor characteristics on rodent and human angiotensin-converting enzyme 2. Hypertension 2012;60(3):730–740.

[82] Huentelman, M. J.; Grobe, J. L.; Vazquez, J.; Stewart, J. M.; Mecca, A. P.; Katovich, M. J.; Ferrario, C. M.; Raizada, M. K. Protection from angiotensin II-induced cardiac hypertrophy and fibrosis by systemic lentiviral delivery of ACE2 in rats. Exp Physiol 2005;90(5):783–790.

[83] Diez-Freire, C.; Vasquez, J.; Correa de Adjounian, M. F.; Ferrari, M. F. R.; Yuan, L.; Silver, X.; Torres, R.; Raizada, M. K. ACE2 gene transfer attenuates hypertension-linked pathophysiological changes in the SHR. Physiol Genomics 2006;27:12–19.

[84] Shenoy, V.; Ferreira, A. J.; Qi, Y.; Fraga-Silva, R. A.; Diez-Freire, C.; Dooies, A.; Jun, J. Y.; Sriramula, S.; Mariappan, N.; Pourang, D.; Venugopal, C. S.; Francis, J.; Reudelhuber, T.; Santos, R. A.; Patel, J. M.; Raizada, M. K.; Katovich, M. J. The angiotensin-converting enzyme 2/angiogenesis-(1-7)/Mas axis confers cardiopulmonary protection against lung fibrosis and pulmonary hypertension. Am J Respir Crit Care Med 2010;182(8): 1065–1072.

[85] Yamazato, M.; Yamazato, Y.; Sun, C.; Diez-Freire, C.; Raizada, M. K. Overexpression of angiotensin-converting enzyme 2 in the rostral venterolateral medulla causes long-term decrease in blood pressure in the spontaneously hypertensive rats. Hypertension 2007;49:926–931.

[86] Hernandez Prada, J. A.; Ferreira, A. J.; Katovich, M. J.; Shenoy, V.; Qi, Y.; Santos, R. A.; Castellano, R. K.; Lampkins, A. J.; Gubala, V.; Ostrov, D. A.; Raizada, M. K. Structure-based identification of small-molecule angiotensin-converting enzyme 2 activators as novel antihypertensive agents. Hypertension 2008;51(5):1312–1317.

[87] Qi, Y.; Zhang, J.; Cole-Jeffrey, C. T.; Shenoy, V.; Espejo, A.; Hanna, M.; Song, C.; Pepine, C. J.; Katovich, M. J.; Raizada, M. K. Diminazene aceturate enhances angiotensin-converting enzyme 2 activity and attenuates ischemia-induced cardiac pathophysiology. Hypertension 2013;62(4):746–752.

[88] De Maria, M. L.; Araujo, L. D.; Fraga-Silva, R. A.; Pereira, L. A.; Ribeiro, H. J.; Menezes, G. B.; Shenoy, V.; Raizada, M. K.; Ferreira, A. J. Anti-hypertensive effects of diminazene aceturate: an angiotensin- converting enzyme 2 activator in rats. Protein Pept Lett 2016;23(1):9–16.

[89] Macedo, L. M.; Souza, A. P.; De Maria, M. L.; Borges, C. L.; Soares, C. M.; Pedrino, G. R.; Colugnati, D. B.; Santos, R. A.; Mendes, E. P.; Ferreira, A. J.; Castro, C. H. Cardio-

protective effects of diminazene aceturate in pressure-overloaded rat hearts. Life Sci 2016;155:63–69.

[90] Shenoy, V.; Gjymishka, A.; Jarajapu, Y. P.; Qi, Y.; Afzal, A.; Rigatto, K.; Ferreira, A. J.; Fraga-Silva, R. A.; Kearns, P.; Douglas, J. Y.; Agarwal, D.; Mubarak, K. K.; Bradford, C.; Kennedy, W. R.; Jun, J. Y.; Rathinasabapathy, A.; Bruce, E.; Gupta, D.; Cardounel, A. J.; Mocco, J.; Patel, J. M.; Francis, J.; Grant, M. B.; Katovich, M. J.; Raizada, M. K. Diminazene attenuates pulmonary hypertension and improves angiogenic progenitor cell functions in experimental models. Am J Respir Crit Care Med 2013;187(6):648–657.

[91] Velkoska, E.; Dean, R. G.; Griggs, K.; Burchill, L.; Burrell, L. M. Angiotensin-(1-7) infusion is associated with increased blood pressure and adverse cardiac remodelling in rats with subtotal nephrectomy. Clin Sci (Lond) 2011;120(8):335–345.

[92] Haber, P. K.; Ye, M.; Wysocki, J.; Maier, C.; Haque, S. K.; Batlle, D. Angiotensin-converting enzyme 2-independent action of presumed angiotensin-converting enzyme 2 activators: studies in vivo, ex vivo, and in vitro. Hypertension 2014;63(4): 774–782.

[93] Velkoska, E.; Dean, R. G.; Burchill, L.; Levidiotis, V.; Burrell, L. M. Reduction in renal ACE2 expression in subtotal nephrectomy in rats is ameliorated with ACE inhibition. Clin Sci (Lond) 2010;118(4):269–279.

[94] Raffai, G.; Khang, G.; Vanhoutte, P. M. Angiotensin-(1-7) augments endothelium-dependent relaxations of porcine coronary arteries to bradykinin by inhibiting angiotensin-converting enzyme 1. J Cardiovasc Pharmacol 2014;63(5):453–460.

[95] Wilson, B. A.; Marshall, A. C.; Alzayadneh, E. M.; Chappell, M. C. The ins and outs of angiotensin processing within the kidney. Am J Physiol Regul Integr Comp Physiol 2014;307(5):R487–R489.

[96] Marshall, A. C.; Shaltout, H. A.; Pirro, N. T.; Rose, J. C.; Diz, D. I.; Chappell, M. C. Antenatal betamethasone exposure is associated with lower ANG-(1-7) and increased ACE in the CSF of adult sheep. Am J Physiol Regul Integr Comp Physiol 2013;305(7):R679–R688.

[97] Marshall, A. C.; Shaltout, H. A.; Pirro, N. T.; Rose, J. C.; Diz, D. I.; Chappell, M. C. Enhanced activity of an angiotensin-(1-7) neuropeptidase in glucocorticoid-induced fetal programming. Peptides 2014;52:74–81.

[98] Marshall, A. C.; Pirro, N. T.; Rose, J. C.; Diz, D. I.; Chappell, M. C. Evidence for an angiotensin-(1-7) neuropeptidase expressed in the brain medulla and CSF of sheep. J Neurochem 2014;130(2):313–323.

[99] Cruz-Diaz, N.; Wilson, B. A.; Pirro, N. T.; Brosnihan, K. B.; Marshall, A. C.; Chappell, M. C. Identification of dipeptidyl peptidase 3 as the angiotensin-(1-7) degrading peptidase in human HK-2 renal epithelial cells. Peptides 2016;83:29–37.

[100] Alzayadneh, E. M.; Chappell, M. C. Angiotensin-(1-7) abolishes AGE-induced cellular hypertrophy and myofibroblast transformation via inhibition of ERK1/2. Cell Signal 2014;26(12):3027–3035.

[101] Lew, R. A. HPLC in the analysis of peptide metabolism. Methods Mol Biol 2004;251:275–290.

[102] Velez, J. C.; Ierardi, J. L.; Bland, A. M.; Morinelli, T. A.; Arthur, J. M.; Raymond, J. R.; Janech, M. G. Enzymatic processing of angiotensin peptides by human glomerular endothelial cells. Am J Physiol Renal Physiol 2012;302(12):F1583–F1594.

[103] Hildebrand, D.; Merkel, P.; Eggers, L. F.; Schluter, H. Proteolytic processing of angiotensin-I in human blood plasma. PLoS One 2013;8(5):e64027.

[104] Schwacke, J. H.; Spainhour, J. C.; Ierardi, J. L.; Chaves, J. M.; Arthur, J. M.; Janech, M. G.; Velez, J. C. Network modeling reveals steps in angiotensin peptide processing. Hypertension 2013;61(3):690–700.

[105] Suski, M.; Gebska, A.; Olszanecki, R.; Stachowicz, A.; Uracz, D.; Madej, J.; Korbut, R. Influence of atorvastatin on angiotensin I metabolism in resting and TNF-alpha - activated rat vascular smooth muscle cells. J Renin Angiotensin Aldosterone Syst 2014;15(4):378–383.

[106] Chappell, M. C.; Tallant, E. A.; Brosnihan, K. B.; Ferrario, C. M. Conversion of angiotensin I to angiotensin-(1-7) by thimet oligopeptidase (EC 3.4.24.15) in vascular smooth muscle cells. J Vasc Med Biol 1994;5(4):129–137.

[107] Alghamri, M. S.; Morris, M.; Meszaros, J. G.; Elased, K. M.; Grobe, N. Novel role of aminopeptidase-A in angiotensin-(1-7) metabolism post myocardial infarction. Am J Physiol Heart Circ Physiol 2014;306(7):H1032–H1040.

[108] Grobe, N.; Elased, K. M.; Cool, D. R.; Morris, M. Mass spectrometry for the molecular imaging of angiotensin metabolism in kidney. Am J Physiol Endocrinol Metab 2012;302(8):E1016–E1024.

[109] Zisman, L. S.; Keller, R. S.; Weaver, B.; Lin, Q.; Speth, R.; Bristow, M. R.; Canver, C. C. Increased angiotensin-(1-7)-forming activity in failing human heart ventricles: evidence for upregulation of the angiotensin-converting enzyme Homologue ACE2. Circulation 2003;108(14):1707–1712.