CHAPTER 1

ROLE OF ACE, ACE2 AND NEPRILYSIN IN THE KIDNEY

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1.   INTRODUCTION

From the initial description of renin activity over a century ago, ongoing study of the renin-angiotensin-aldosterone system (RAAS) continues to yield novel findings that redefine the functional nature of this system, as well as reveal the complexity of the interplay among the various RAAS components. Indeed, the recent discoveries of angiotensin converting enzyme 2 (ACE2) (Crackower *et al* 2002; Donoghue *et al* 2000; Tipnis *et al* 2000); the renin receptor (Nguyen *et al* 2002) and the angiotensin-(1-7) [Ang-(1-7)] receptor (Santos *et al* 2002) represent important examples of our evolving concepts of the RAAS and cardiovascular regulation. Coupled with the emerging view that the RAAS is not defined as simply an endocrine system, these local or tissue systems may exhibit distinct functional and processing pathways (Chappell *et al* 1989; Chappell *et al* 2004; Paul *et al* 2006). The kidney is clearly an important target organ of the circulating RAAS, particularly the actions of Ang II and aldosterone to promote sodium and water reabsorption, as well as their influence on the progression of tissue injury and fibrosis (Harris 1999). The kidney also exhibits a local RAAS that expresses Ang II, Ang-(1-7), and multiple Ang receptor subtypes that mediate the distinct actions of these two peptides in both normal and pathophysiolgocial conditions such as hypertension or diabetes (Burns 2000; Carey & Siragy 2003; Navar *et al* 2000). Figure 1 illustrates one current view of the renal RAAS network that emphasizes the distinct synthetic pathways of Ang II and Ang-(1-7), as well as functional actions mediated by the AT_1, AT_2 and $AT_{(1-7)}$ receptors. Although the emergence of receptor subtypes distinguishes the distinct signaling pathways of Ang II and Ang-(1-7), the post-renin enzymes that form and degrade these peptides must be considered in lieu of the overall regulation of the functional RAAS within the kidney. The inclusion of Ang-(1-12) as a potential intermediate in Ang II formation via a renin-independent pathway reflects the recent
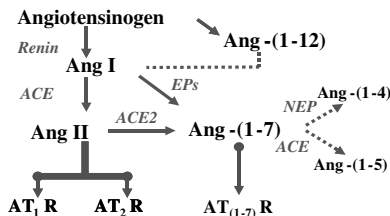
Figure 1. Scheme that depicts the processing pathways involved in the formation and degradation of angiotensin II (Ang II) and Ang-(1-7) within the kidney. Ang II binds to either AT_1 or AT_2 receptor (R) subtypes, while Ang-(1-7) recognizes an $AT_{(1-7)}$ R. ACE, angiotensin converting enzyme; EPs, endopeptidases; NEP, neprilysin

demonstration of endogenous levels of the peptide in the kidney, circulation and other tissues (Nagata *et al* 2006). In this chapter, the roles of ACE2, ACE and the endopeptidase neprilysin in the functional expression of the intrarenal hormones of the RAAS are reviewed.

2.   ANGIOTENSIN CONVRETING ENZYME

ACE may be considered the activation step in the catalytic cascade for the formation of Ang II from Ang I (Fig. 1). Although evidence of non-ACE pathways for biosynthesis of Ang II is evident (Sadjadi *et al* 2005a; Tokuyama *et al* 2002), ACE likely represents the major, if not sole enzyme responsible for Ang II formation under normal physiological conditions in humans and other species. This is not to imply that ACE has no other substrates than Ang I (see below), but that a primary role for ACE is the generation of Ang II. Indeed, the identification of ACE and the characterization of the enzymatic properties must be considered a pivotal achievement in our understanding of the RAAS and cardiovascular disease, as well as leading to the successful development of ACE inhibitors in the treatment for hypertension and renal disease. ACE is a metallopeptidase composed of a single monomeric protein. Somatic ACE contains two catalytic regions designated as the amino (N) and carboxy (C) domains. Selective inhibitors against both catalytic domains of somatic ACE are now available, however, the functional significance of the two domains is presently unknown (Dive *et al* 1999; Georgiadis *et al* 2003). The enzyme cleaves two residues from the carboxy end of various peptides and, hence, its description as a *dipeptidyl*-carboxypepitdase. Within the kidney, somatic ACE is primarily a glycosylphosphatidylinositol-anchored membrane protein and the majority of the enzyme including both catalytic regions faces the extracellular space. ACE is localized throughout the kidney with high concentrations in vascular endothelial cells, proximal tubules and interstitial cells. ACE is also released from the apical surface of epithelial cells into the proximal tubular fluid and likely contributes to the urinary levels of the enzyme (Hattori *et al* 2000). Indeed, the tubular fluid should be considered a distinct intrarenal compartment that contains RAAS processing enzymes and the peptide products may interact with Ang receptors

along the entire tubular area of the kidney. The release of ACE from the cell membrane is a specific process as releasing enzymes or "sheddases" have been identified that recognize a unique motif on the stalk region of the enzyme (Beldent *et al* 1993). The conversion of membrane-bound ACE to a soluble form does not appear to substantially alter the substrate preference or the catalytic properties of the enzyme. Although the significance of this event is not currently understood, enzyme shedding may underlie an endocrine process to transport ACE to more distal areas of the nephron that are deficient in this peptidase activity for the discrete production of Ang II. In this regard, Casarini and colleagues have presented intriguing data that the urinary excretion of the N-terminal domain of ACE may serve as a urinary marker in both humans and experimental hypertensive models (Marques *et al* 2003).

Extensive evidence suggests that intrarenal ACE participates in the direct formation of Ang II from Ang I. The renal administration of ACE inhibitors reduces interstitial levels of Ang II and attenuates blood pressure. Moreover, in an animal model of tissue-depleted ACE that preserves circulating levels of the enzyme, renal Ang II is significantly reduced (Modrall *et al* 2003). Interestingly, intrarenal levels of Ang I were also markedly reduced in the tissue ACE null mouse while renal Ang-(1-7) concentrations were maintained (Modrall *et al* 2003). These data serve to emphasize that ACE participates in the metabolism of other peptide hormones (Skidgel & Erdos 2004). In the case of Ang-(1-7), ACE efficiently metabolizes the peptide to Ang-(1-5), a product which is presently not known to exhibit functional activity (Chappell *et al* 1998; Deddish *et al* 1998; Rice *et al* 2004). We have postulated that the formation of Ang-(1-7), particularly under prolonged activation of the RAAS, is considered to balance or attenuate the constrictor and prolifer-ative actions of Ang II (Chappell & Ferrario 1999; Ferrario *et al* 2005c). Indeed, Ang-(1-7) exhibits vasodilatory, natriuretic and anti-proliferative actions through the stimulation of nitric oxide and arachidonic acid metabolites (Sampaio *et al* 2007). Ang-(1-7) abrogates the Ang II-dependent activation of MAP kinase in primary cultures of proximal tubule epithelial cells (Su *et al* 2006). Moreover, the inhibitory actions of Ang-(1-7) were blocked by the Ang-(1-7) antagonist [D-Ala[7]]-Ang-(1-7) suggesting a receptor mediated pathway distinct from either AT_1 or AT_2 receptor subtypes (Su *et al* 2006). Similar effects of Ang-(1-7) were originally demonstrated in non-renal cells (Tallant *et al* 2005a). In the circulation, ACE inhibitors increase circulating levels of Ang-(1-7) and augment the *in vivo* half life of the peptide by almost 6 fold (Iyer *et al* 1998; Yamada *et al* 1998). The urinary excretion of Ang-(1-7) increases in both human and experimental hyper-tensive models following acute administration of ACE inhibitors (Ferrario *et al* 1998; Yamada *et al* 1999). The increased excretion of Ang-(1-7) most likely reflects the reduced intrarenal metabolism of the peptide and the efficient shunting of the Ang I pathway to formation of Ang-(1-7). Our recent studies in isolated sheep proximal tubules reveal that without prior inhibition of ACE, Ang-(1-7) derived from either Ang I or Ang II was rapidly converted to Ang-(1-5) (Shaltout *et al* 2007). Blockade of Ang-(1-7) partially reverses the beneficial actions of ACE inhibitors on blood pressure in hypertensive rats as an Ang-(1-7) monoclonal antibody or

the [D-Ala[7]]-Ang-(1-7) antagonist increase blood pressure (Iyer *et al* 1997; Iyer *et al* 2000). Moreover, studies by Benter and colleagues find that the renoprotective effects of exogenous Ang-(1-7) in LNAME-treated SHR were not further improved with the ACE inhibitor captopril (Benter *et al* 2006a).

Apart from Ang II and Ang-(1-7), renal ACE may also participate in the metabolism of other peptides including kinins, substance P and the hematopoietic fragment acetyl-Ser-Asp-Lys-Pro (Ac-SDKP). Bradykinin-(1-9) is very rapidly metabolized by ACE in a two -step process to the inactive fragments bradykinin-(1-7) and bradykinin-(1-5). ACE inhibition is associated with increased circulating and tissue levels of bradykinin-(1-9) and the renal content of kinin is higher in the tissue ACE null mouse (Campbell *et al* 2004). In general, bradykinin is a potent vasodilator and inhibitor of cell growth through stimulation of nitric oxide, as well as exhibiting natriuretic actions within the kidney (Scicli & Carretero 1986). Interestingly, Santos and colleagues have reported that the functional activity of Ang-(1-7), under certain conditions, is dependent on the increased release of bradykinin (Fernandes *et al* 2001). Moreover, the kinin B2 receptor antagonist HOE140 blocked nitric oxide release by the non-peptide Ang-(1-7) agonist AVE0991 (Wiemer *et al* 2002).

Similar to Ang-(1-7), circulating levels of the Ac-SDKP are markedly increased with ACE inhibition and the enzyme cleaves the Lys-Pro bond of the tetrapeptide (Azizi *et al* 1997; Raousseau *et al* 1995). Although current evidence does not support a role for Ac-SDKP in the regulation of blood pressure, the peptide does exhibit potent anti-fibrotic and anti-inflammatory actions (Peng *et al* 2003). Indeed, exogenous administration of Ac-SDKP attenuates proteinuria and improves renal function in several models of renal injury and hypertension (Omata *et al* 2006). Interestingly, Ang-(1-7) and Ac-SDKP may be the only known endogenous substrates that are exclusively cleaved by the N-terminal catalytic domain of human ACE (Raousseau *et al* 1995; Deddish *et al* 1998). Moreover, prolyl (oligo)endopeptidase, an enzyme that processes Ang I or Ang II to Ang-(1-7) in endothelial and neural cells (Chappell *et al* 1990; Santos *et al* 1992), may also convert thymosin-β_2 to Ac-SDKP in plasma and tissue (Cavasin *et al* 2004). The unusual specificity of the N-domain of ACE for Ang-(1-7) and Ac-SDKP suggests an overlap of the activities of these two peptide systems within the kidney as well. Although elucidation of the signaling mechanisms and receptors for Ang-(1-7) and Ac-SDKP is at an early stage, future studies should consider whether there is a basis for the functional similarities between these peptides.

The role of RAAS enzymes including ACE and renin has been primarily emphasized for their catalytic properties; however, compelling evidence now reveals receptor-like properties for these two enzymes. Indeed, a renin receptor was recently cloned by Nyguen and colleagues with significant concentrations of the protein in the glomerulus and vascular smooth muscle cells. (Diez-Freire *et al* 2006; Nguyen *et al* 2002). Receptor-bound renin exhibits increase proteolyitc activity for Ang I formation, but both pro-renin and renin also induce distinct signaling pathways following binding. In isolated mesangial cells, exogenous renin increased TGF-β expression and other matrix proteins including plasminogen activator inhibitor (PAI-1) and fibronectin

that was apparently independent of Ang II synthesis (Huang *et al* 2006). ACE inhibitors may also induce cell-specific signaling by inducing conformational changes in membrane-bound ACE without alterations in Ang II or other peptides (Benzing *et al* 1999). Two kinases, c-Jun kinase and MAP kinase kinase 7 associate with the intracellular portion of ACE. Moreover, ACE inhibitors increase the phosphorylation and nuclear trafficking of phosphorylated cJun kinase (Kohlstedt *et al* 2002). This aspect of ACE-dependent activation of various kinases has been demonstrated in human endothelial cells and the question remains as to what extent this occurs in other cells or tissues. In addition, ACE inhibitors or the angiotensin peptides Ang-(1-9) and Ang-(1-7) induce the association of ACE and the bradykinin B2 receptor that prevents the rapid down-regulation of the ligand-receptor complex, thus potentiating the actions of bradykinin (Burckle *et al* 2006; Chen *et al* 2005).

3.　　ANGIOTENSIN CONVERTING ENZYME 2

Almost 50 years following the discovery of ACE, a new homolog of the enzyme termed ACE2 was identified by two separate groups (Donoghue *et al* 2000; Tipnis *et al* 2000). ACE2 activity is not attenuated by ACE inhibitors nor does the enzyme share the same catalytic properties. In this regard, ACE2 contains a single zinc-dependent catalytic site that corresponds to the C-terminal domain of somatic ACE. ACE2 exhibits carboxypeptidase activity cleaving a single amino acid residue at the carboxyl terminus of various peptides. The original studies assessed Ang I as the peptide substrate for ACE2, given the similar homology to ACE and the existing evidence for ACE-independent pathways; however, ACE2 converted Ang I to the nonapeptide Ang-(1-9) (Donoghue *et al* 2000). This product is currently not known to exhibit functional activity, but may serve as a substrate for the further processing to Ang II or Ang-(1-7) (Li *et al* 2004). The subsequent kinetic studies of over 120 peptides found that the conversion of Ang II to Ang-(1-7) was much preferred over that for Ang I (Vickers *et al* 2002). Indeed, ACE2 exhibits an approximate 500-fold greater kcat/Km for Ang II versus Ang I and has the highest efficiency among the known Ang-(1-7)-forming enzymes (Fig. 2). These studies also revealed that other peptides including apelin 13 and dynorphin are cleaved by ACE2 at a similar or slightly greater efficiency than Ang II (Vickers *et al* 2002). At this time, the majority of studies have focused on the role of ACE2 in the metabolism of angiotensins (see below), principally Ang II to Ang-(1-7), and the role of ACE2 in the processing of apelin or other peptides has not been sufficiently addressed.

Similar to ACE, ACE2 exists in both soluble and membrane-associated forms with high expression in the kidney, heart, brain, lung and testes (Harmer *et al* 2002). Although there is significant circulating ACE activity in various species, plasma levels of ACE2 are quite low, but may vary among species (Elased *et al* 2006; Rice *et al* 2006). Recent studies in the sheep reveal appreciable ACE2 in the plasma, albeit the activity was significantly lower than that for ACE (Fig. 3, inset) (Shaltout *et al* 2007). For this assessment, we compared the enzyme activities using the endogenous substrates for both ACE and ACE2 (Ang I and Ang II, respectively)
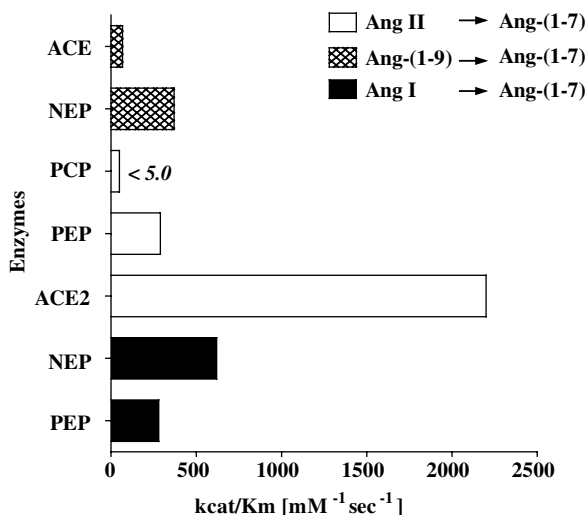
Figure 2. Comparison of the efficiency constants for the formation of Ang-(1-7) from Ang II, Ang-(1-9) and Ang I. Peptidase abbreviations: ACE, angiotensin-converting enzyme; NEP, neprilysin; PCP, prolyl carboxypeptidase; PEP, prolyl (oligo) endopeptidase
Source: Kinetic data from Rice et al 2004 & Welches et al 1993
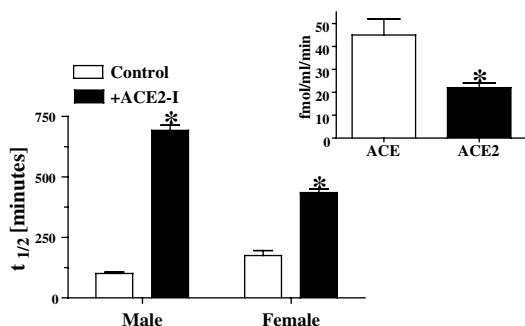

Figure 3. The ACE2 inhibitor MLN-4760 (ACE2-I) increases the half life ($t_{1/2}$) of Ang II in the serum of male and female sheep. Inset: comparison of ACE and ACE2 activities in female serum. ACE and ACE2 were determined by the conversion of Ang I to Ang II and Ang II to Ang-(1-7), respectively, by HPLC analysis in the absence or presence of the ACE2 MLN-4760 and the ACE inhibitor lisinopril. Data are n = 4-5, mean ± SEM;* $P < 0.05$ vs. control

at equimolar concentrations under identical incubation conditions. Interestingly, as shown in Fig. 3, male sheep exhibited higher ACE2 activity than females that likely contributes to the lower half-life ($t_{1/2}$) of serum Ang II in males (Westwood *et al* 2006). Addition of the specific ACE2 inhibitor abolished the conversion of Ang II to Ang-(1-7) as measured by a HPLC-^{125}I-detector and markedly increased the Ang II-[$t_{1/2}$] in both male (6 fold) and female (3 fold) sheep (Fig. 3). These

ex vivo data in sheep serum demonstrate that circulating ACE2 constitutes a major pathway in the metabolism of Ang II and support the increase in circulating Ang II levels in the ACE2 null mouse (Crackower *et al* 2002). Furthermore, we did not find that soluble ACE2 in the serum contributed to the direct conversion of Ang I to Ang-(1-9) even in the presence of complete ACE inhibition (Shaltout *et al* 2007).

Within the kidney, ACE2 is primarily localized to the apical aspect of the proximal tubule epithelium. Indeed, expression of ACE2 in the renal MDCK cell line revealed exclusive trafficking of the enzyme to the apical side, while the distribution of expressed ACE was different, trafficking to the basolateral and luminal aspects of the cell (Guy *et al* 2005). Consistent with the apical expression of ACE2 in the renal epithelium, we found significant urinary ACE2 activity that converted Ang II to Ang-(1-7), but did not process Ang I to Ang-(1-9) (Shaltout *et al* 2007). The glycosylated form of ACE2 is approximately 120,000 Daltons and the filtration of the enzyme into the tubular fluid is highly unlikely (Shaltout *et al* 2007). In this regard, Lambert and colleagues report that the metallopeptidase ADAM 17 may function as a secretase to release ACE2 from extracellular side of the cell membrane (Lambert *et al* 2005). Interestingly, ADAM 17 does not release ACE suggesting that the regulation for the secretion for ACE and ACE2 is distinct. The localization of ACE2 in the proximal tubule epithelium along with other elements of the RAS (ACE, angiotensinogen, Ang receptors) supports a role for the enzyme in the processing of angiotensin peptides. In the rat kidney, Burns and colleagues found no evidence that ACE2 or other peptidases metabolize Ang II in proximal tubule preparations or in perfused proximal tubule segments isolated from male Sprague Dawley rats (Li *et al* 2004). However, ACE2 activity was clearly evident in the rat tubules as the conversion of exogenous Ang I to Ang-(1-9) was sensitive to the ACE2 peptide inhibitor DX-600 (Li *et al* 2004). Ang-(1-9) was subsequently converted to Ang-(1-7) by ACE, a pathway similar to that reported for Ang I metabolism in isolated cardiomyocytes (Donoghue *et al* 2000). In contrast to the rat, we found that ACE2 was the predominant activity to convert Ang II to Ang-(1-7) in sheep proximal tubules (Shaltout *et al* 2007). The addition of the non-peptide ACE2 inhibitor MLN-4760 significantly attenuated the metabolism of Ang II at early time points. However, as shown in Fig. 4, the significant ACE and neprilysin activities required prior inhibition to protect Ang-(1-7) from rapid degradation in the proximal tubules. We could not demonstrate that ACE2 participated in the direct metabolism of Ang I, particularly under conditions where other enzymatic pathways were blocked (Shaltout *et al* 2007). Indeed, Ang I was directly converted to Ang II and Ang-(1-7) via ACE and neprilysin, respectively. The preferred conversion of Ang II to Ang-(1-7) by ACE2 in the sheep kidney is entirely consistent with kinetic studies on various peptide substrates by the human enzyme (Rice *et al* 2004; Vickers *et al* 2002), as well studies in membrane fractions of mouse kidney and rat renal cortex that demonstrated ACE2-dependent conversion of Ang II to Ang-(1-7) (Elased *et al* 2006; Ferrario *et al* 2005b). An explanation for the discrepancy in the metabolism studies for angiotensin metabolism is not readily apparent; however, if the rat exhibits different kinetic properties for Ang I and Ang II than sheep or human, then the role of ACE2 is likely to be quite different among species. Additionally, these studies have important
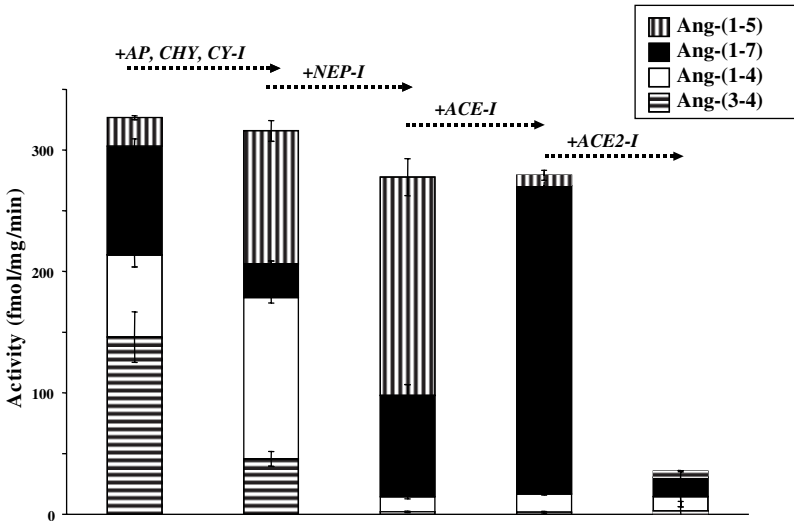
Figure 4. ACE2 inhibition blocks the conversion of Ang II to Ang-(1-7) in isolated proximal tubules from female sheep. Sequential addition of peptidase inhibitors on the formation of Ang II metabolites include: AP, amniopeptidase (amastatin, bestatin); CHY, chymase, carboxypeptidase A (chymostatin, benzyl succinate); CY, cysteine protease (PCMB); NEP, neprilysin (SCH3977), ACE, angiotensin converting enzyme (lisinopril); ACE2 (MLN-4760). Data are n = 4, mean ± SEM

implications on the role of ACE as well, particularly whether ACE is involved in the formation (Li *et al* 2004) or degradation of Ang-(1-7) (Chappell *et al* 1998; Chappell *et al* 2000; Yamada *et al* 1998). Although Campbell and colleague demonstrate significant quantities of endogenous Ang-(1-9) in the rat kidney (Campbell *et al* 1991), chronic ACE inhibition or combined ACE/AT$_1$ blockade (Chappell, unpublished observations) did not attenuate renal Ang-(1-7) levels in the rat. In addition, Ang-(1-7) levels within the kidney were maintained in tissue ACE knockout mice (Modrall *et al* 2003). Thus, these *in vivo* studies do not strongly support an ACE2-ACE cascade leading to the formation of Ang-(1-9) and Ang-(1-7) in the kidney.

The molecular studies utilizing ACE2 knockout mice provide additional evidence for the enzyme's role to balance the expression of Ang II and Ang-(1-7). We originally showed that ACE2 null mice exhibit higher circulating and tissue levels of Ang II (Crackower *et al* 2002). Indeed, the increased ratio of renal Ang II to Ang-(1-7) may contribute to the renal pathologies observed in older ACE2 null mice (Oudit *et al* 2006). Furthermore, the incidence of glomerulosclerosis and proteinuria in the male mice was markedly attenuated by AT$_1$ receptor blockade. Several hypertensive models including the spontaneously hypertensive rat (SHR), stroke-prone SHR and Sabra salt sensitive rat exhibit lower mRNA levels and protein expression for ACE2 in the kidney than normotensive controls (Crackower *et al* 2002; Zhong *et al* 2004), as well as human prehypertensives (Keidar *et al* 2006). Tikellis and colleagues find that renal ACE2 expression is actually higher in the SHR than WKY normotensive controls at day one following

birth, similar at 42 days and then dramatically declines in adult SHR by 80 days (Tikellis *et al* 2006). ACE activity, however, was markedly lower in the SHR kidney at all time points measured and declined in both strains at 80 days. Apart from the interesting pattern of development for ACE2 in the kidney, these data emphasize the need to at least consider alterations in both ACE and ACE2 in characterizing the functional output of the RAAS. Moreover, parallel studies to document the changes in renal Ang II and Ang-(1-7) during this developmental period are critical to establish the relevance to altered ACE and ACE2. It is clear that not all hypertensive models exhibit reduced ACE2 in the kidney. Our studies in the male mRen2.Lewis rat, a model of tissue renin expression with increased renal Ang II, found no difference in renal cortical ACE2 activity as compared to the normotensive Lewis strain, although cardiac activity was indeed lower in the hypertensive rats (Ferrario *et al* 2005a; Ferrario *et al* 2005b; Pendergrass *et al* 2006). Chronic blockade with either an ACE inhibitor or AT_1 antagonist increased ACE2 activity in the kidneys of both the mRen2.Lewis and Lewis rats, but enzyme activity was significantly higher in the normotensive strain following treatment (Jessup *et al* 2006). This may reflect the situation where RAAS blockade does not completely reverse the extent of renal injury in the male mRen2.Lewis model. In this regard, the reduced ACE2 and elevated renal Ang II in the injured kidney of albumin-loaded rats was associated with increased NF-κB expression (Takase *et al* 2005). In contrast, ACE2 and its product Ang-(1-7) increase in the kidney of the rat during pregnancy (Brosnihan *et al* 2003). It is well known that the RAAS is activated during pregnancy, yet blood pressure is not altered in normal pregnancy, and it will be of interest to determine whether ACE2 expression within the kidney is altered with pre-eclampsia. Diabetic nephropathy is clearly dependent on an activated RAAS and both ACE inhibitors and AT_1 receptor antagonists are effective in attenuating the progression of injury. Indeed, the renal expression of ACE2 is reduced in the proximal tubules of the streptozotocin-induced model of type I diabetes (Tikellis *et al* 2003; Wysocki *et al* 2006). Moreover, the attenuation of renal injury in this model by ACE inhibition is associated with increased ACE2 expression. A protective role for renal ACE2 is also evident from the findings that chronic ACE2 inhibition in the diabetic *db/db* mice exacerbates the extent of albuminuria almost 3-fold (Ye *et al* 2006). Although angiotensin content was not measured, the *db/db* mice exhibited increased glomerular expression of ACE and reduced ACE2 as compared to the control *db/dm* mice. Interestingly, the localization studies revealed distinct patterns of staining for ACE2 and ACE within the glomerulus – ACE2 in podocytes and ACE in the endothelial cells (Liebau *et al* 2006). Ang-(1-7) or the nonpeptide agonist AVE0991 attenuates proteinuria and improves renal vascular activity in the streptozoticin Type 1 diabetic rat, but did not reverse the urinary excretion of lysozyme, a marker of tubulointerstitial damage (Benter *et al* 2007). Moreover, the ratio of Ang-(1-7) to Ang II formed from exogenous Ang I was lower in glomeruli isolated from the kidneys of diabetic rats, however, the identity of the Ang-(1-7)-forming activity was not determined in this study (Singh *et al* 2005). Thus, in addition to the proximal tubule epithelium, the

glomerulus may be a second key site within the kidney where ACE2 may influence the local expression of angiotensin peptides and renal function.

4.   NEPRILYSIN

In the kidney, the endopeptidase neprilysin constitutes significant peptidase activity, particularly within the brush border region of the proximal tubules. Similar to ACE and ACE2, neprilysin is a zinc-dependent metallopeptidase that is anchored to the apical or extracellular region of the membrane, but is apparently resistant to enzymatic shedding. Although neprilysin was initially recognized for its enkephalin-degrading activity and frequently referred to as "enkephalinase", studies now reveal that this enzyme contributes to the metabolism of various peptides with cardiovascular actions including adrenomedullin, angiotensins, kinins, endothelins, substance P and the natriuretic peptides (Skidgel & Erdos 2004). Indeed, the development of neprilysin inhibitors, and more recently, dual or mixed inhibitors that target ACE as well remain potential therapies in cardiovascular disease (Veelken & Schmieder 2002). In general, these dual inhibitors were either equally or more effective in lowering blood pressure and reducing renal injury as compared to monotherapy with an ACE or neprilysin inhibitor (Kubota *et al* 2003; Tikkanen *et al* 2002). However, two large clinical trials (OCTAVE, OVERTURE) with the mixed inhibitor omapatrilat revealed an increased incidence of angioedema. Moreover, the drug was no more effective than an ACE inhibitor alone (Kostis *et al* 2004; Packer *et al* 2002). A subsequent experimental study has shown that omapatrilat inhibits amniopeptidase P and, although less potent than its actions against ACE and neprilysin, this may further augment the local concentrations of kinins or substance P to exacerbate vascular permeability (Sulpizio *et al* 2005). In this aspect, the development of more selective inhibitors against ACE and neprilysin may be of clinical benefit.

The rationale for neprilysin inhibition primarily resides in preserving the "cardioprotective" peptides bradykinin and ANP or BNP. However, neprilysin readily metabolizes Ang II to the inactive fragment Ang-(1-4) which undergoes further hydrolysis to the dipeptides Asp-Arg and Val-Tyr. Neprilysin also cleaves endothelin, although it is not clear to what extent reduced intrarenal levels of endothelin are beneficial given the functional diversity of the endothelin A and B receptor subtypes within the kidney (Schiffrin 1999). The additional ACE inhibition would prevent the accumulation of Ang II and further contribute to the protection of kinins, as well as possibly reduce endothelin release. One possible caveat to this approach is that neprilysin is the major Ang-(1-7)-forming activity from Ang I or Ang-(1-9) in the circulation (Campbell *et al* 1998; Yamamoto *et al* 1992). Indeed, acute administration of the potent neprilysin inhibitor SCH3977 reduced circulating levels of Ang-(1-7) and increased blood pressure in the SHR chronically treated with the ACE inhibitor lisinoropril (Iyer *et al* 1997). Although plasma levels of neprilysin are low to non-detectable, the enzyme is appropriately localized to the ectocellular surface of endothelial and smooth muscle cells to contribute to the formation of Ang- (1-7) within the vasculature (Llorens-Cortes *et al* 1992).

In the kidney, neprilysin may contribute to both the formation as well as the degradation of the Ang-(1-7) (Allred *et al* 2000). Neprilysin cleaves the Pro[7]-Phe[8]

bond of Ang I to Ang-(1-7), but the very high levels of the enzyme in the kidney may continue to metabolize Ang-(1-7) at the Tyr^5-Ile^6 bond to form Ang-(1-4) and Ang-(5-7) (Allred *et al* 2000; Chappell *et al* 2001). Indeed, the mixed inhibitor omapatrilat augmented the urinary levels of Ang-(1-7) in both human hypertensives and the SHR model (Ferrario *et al* 2002a; Ferrario *et al* 2002b). The clinical study revealed a strong correlation between the reduction in blood pressure and increased excretion of Ang-(1-7) with the dual peptidase inhibitor (Ferrario *et al* 2002a). Interestingly, chronic treatment of male SHR with omapatrilat (2 weeks, 30 mg/kg daily) was also associated with the increased renal expression of ACE2. As shown in Fig. 5, immunocytochemical studies demonstrate enhanced expression
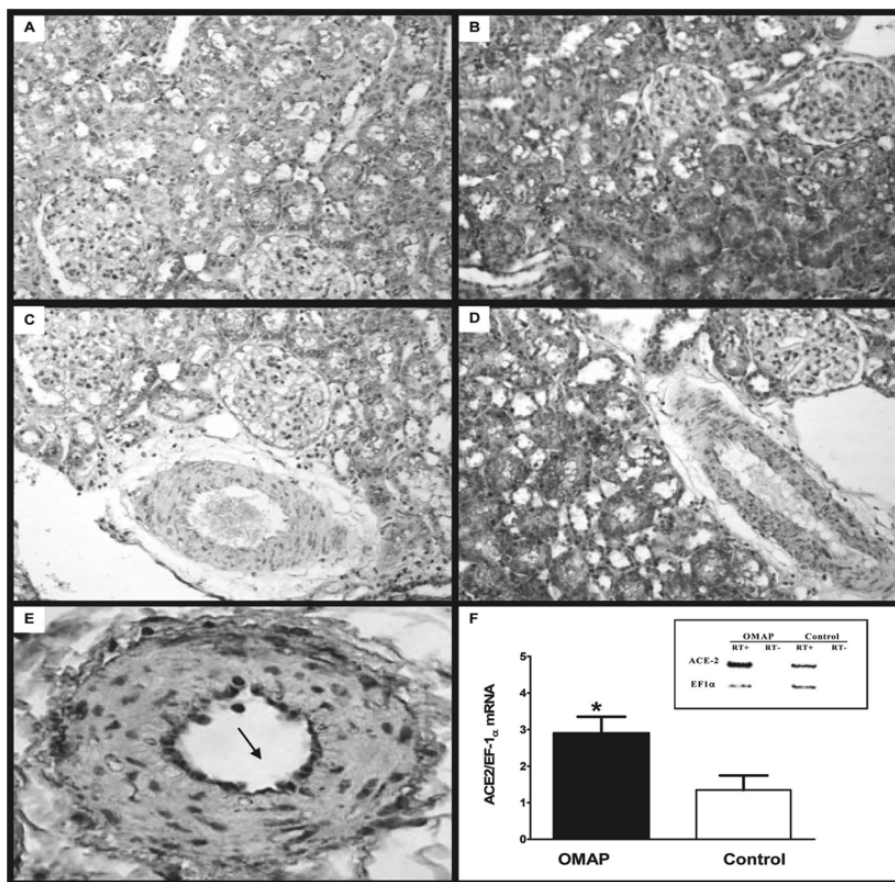

Figure 5. Increased expression of Ang-(1-7) and ACE2 in the renal cortex of SHR following treatment with omapatrilat. Immunocytochemical staining for Ang-(1-7) in control (A) and treated (B) SHR; ACE2 staining in control (C) and treated (D), group. ACE2 staining in renal artery of treated SHR; arrow indicates intimal layer (E). Renal cortical ACE2 mRNA levels are significantly increased 2-fold following omapatrilat treatment (F); inset: ACE2 and EF-1α bands in the presence of the specific RT primers (RT+). Data are n = 7-8, mean ± SEM

of both ACE2 and Ang-(1-7) within the renal cortex of the treated-SHR (Chappell *et al* 2002). Omapatrilat treatment also revealed the renal vascular expression of ACE2 with staining evident in the intimal, medial and adventitial regions of the renal artery (Fig. 5E); vascular staining for the enzyme was undetectable in the untreated SHR group (Fig. 5C). Cortical mRNA of ACE2 expressed as a ratio to EF-1α increased 2-fold suggesting that transcriptional regulation contributes to the enhanced expression of ACE2 within the kidney (Fig. 5F). These studies are of interest as they reveal an additional mechanism of the vasopeptidase inhibitor that may result in the enhanced conversion of Ang II to Ang-(1-7) by ACE2, as well as protecting Ang-(1-7) from both neprilysin- and ACE-dependent degradation within in the kidney. Furthermore, these data suggest an important ability of the dual peptidase inhibitor (as well as the administration of other RAAS inhibitors alone) to restore ACE2 levels in the hypertensive kidney which may mitigate against the Ang II-AT$_1$ receptor axis of the RAAS. Indeed, Raizada and colleagues show that lenti-viral expression of ACE2 has amelioratory effects on blood pressure and cardiac fibrosis in the SHR, although the renal effects of enhanced enzyme activity were not ascertained (Diez-Freire *et al* 2006). Their data clearly demonstrate that ACE2 can markedly alter the balance of an activated RAAS pathway towards a normotensive phenotype. Further study is required to determine the extent that the beneficial actions of increased ACE2 reflect the greater inhibition of Ang II or the increased accumulation of Ang-(1-7) in the kidney or other tissue.

5.   REGULATION OF THE INTRARENAL RAAS

The positive influence of ACE2 in the SHR kidney following blockade of ACE and neprilysin emphasizes the complex regulation of RAAS components within the kidney (see Fig. 6). We have also shown that ACE inhibition alone or AT$_1$ receptor antagonism increases either renal ACE2 mRNA or activity (Igase *et al* 2005; Ferrario *et al* 2005b). Consistent with these data in the intact animal, Gallagher
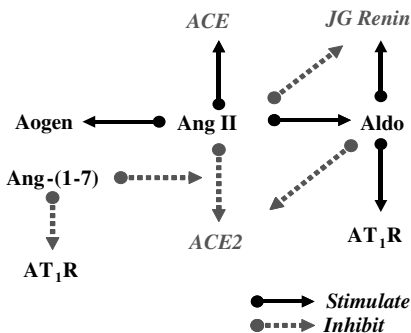

Figure 6. A potential regulatory scheme for the stimulatory and inhibitory pathways of the renin-angiotensin-aldosterone system within the kidney. ACE, angiotensin converting enzymes; Aogen, angiotensinogen; Aldo, aldosterone; AT$_1$R, angiotensin type 1 receptor; JG, juxtaglomerular

and colleagues demonstrate that Ang II directly down regulates ACE2 through activation of the AT_1 receptor (Gallagher *et al* 2006). Although Ang-(1-7) alone did not influence the basal expression of ACE2 in these cells, the peptide attenuated the inhibitory effects of Ang II on ACE2 via a receptor-dependent mechanism (Gallagher *et al* 2006). In contrast to the negative influence on ACE2, Ang II increases ACE expression within the kidney (Harrison-Bernard *et al* 2002; Sadjadi *et al* 2005b). Ang II also positively influences the expression of its precursor protein angiotensinogen (Kobori *et al* 2001; Zhang *et al* 2002), and in selective areas of the kidney, either maintains or up regulates the AT_1 receptor as well (Harrison-Bernard *et al* 2002). This effect on the AT_1 receptor may also lead to increased renal levels of Ang II via receptor mediated uptake and stable sequestration of the circulating peptide (Ingert *et al* 2002). In contrast, Ang-(1-7) can down regulate the AT_1 receptor through stimulation of a cylocoxygenase pathway (Clark *et al* 2003; Clark *et al* 2001). There are few studies on the regulation of the Ang-(1-7) receptor, although chronic ACE or AT_1 blockade reduced *mas* mRNA expression in the renal cortex of the Ren2 Lewis congenic rat (Jessup *et al* 2006). Consistent with the positive feedback concept, the current evidence suggests that aldosterone down regulates ACE2 (Keidar *et al* 2005; Tallant *et al* 2005b) while the mineralo-corticoid increases expression of ACE, AT_1 receptor, renin and intrarenal Ang II (Bayorh *et al* 2006; Klar *et al* 2004; Schiffrin 2006). Thus, the renal RAAS appears to function in a positive regulatory manner on these components to promote or maintain Ang II content. In this regard, ACE2 may serve as an important mechanism to break or reduce the positive gain of the system for Ang II production or enhanced signaling in the kidney. Although Ang II potently reduces juxtaglomerular (JG)-derived release and expression of renin, renin is not suppressed but increases in the collecting duct and distal tubules (Prieto-Carrasquero *et al* 2004). The negative feedback by Ang II on JG renin may also be balanced by renin-independent pathways that contribute to the formation of Ang I. Alternatively, Nagata and colleagues (Nagata *et al* 2006) find significant concentrations of the novel peptide Ang-(1-12) in the kidney and other tissues that may not require renin for the peptide's synthesis (see Fig. 1). The infusion of Ang-(1-12) produced an immediate increase in blood pressure that was abolished by either an ACE inhibitor or an AT_1 receptor antagonist (Nagata *et al* 2006). These data suggest that following formation of Ang-(1-12), the peptide or its intermediate is converted to Ang II by ACE. The elucidation of the enzyme(s) responsible for the formation of Ang-(1-12) and the factors that influence its expression may greatly contribute to our understanding the regulation of the intrarenal RAAS, particularly under pathophysiological conditions.

6. CONCLUSIONS

The majority of experimental studies on the RAAS and the regulation of blood pressure have utilized male animals. As there is overwhelming evidence for sex differences in the extent of hypertension and cardiovascular injury, the consideration

of gender in the regulation of the renal RAAS enzyme cascade should be carefully considered (Bachmann *et al* 1991; Brosnihan *et al* 1999; Reckelhoff *et al* 2000). For example, the presence of renal damage in the male ACE2 knockout mice was not evident in the estrogen replete female littermates and possibly there is greater expression of RAAS components in the males with the loss of ACE2 (Oudit *et al* 2006). We and others have shown that estrogen depletion is associated with altered expression of renin, AT_1 receptors, ACE and NOS isoforms, as well as exacerbates hypertension and salt-sensitive renal injury (Bayorh *et al* 2001; Brosnihan *et al* 1997; Chappell *et al* 2003; Chappell *et al* 2006; Harrison-Bernard *et al* 2003; Roesch *et al* 2000; Yamaleyeva *et al* 2007). Moreover, in lieu of the negative outcomes for estrogen or combined hormone replacement in older women (HERS, WHI), the influence of aging on the response of the intrarenal RAAS and other systems may be of equal importance. Clearly, understanding the regulation and interplay of ACE, ACE2 and neprilysin within the kidney, as well as other areas including the heart, brain and vascular beds are critical to treating the burgeoning problem of cardiovascular disease.

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