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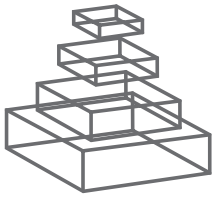

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BEYOND THE CONVENTIONAL RENIN ANGIOTENSIN SYSTEM

Topic Editor:
Walmor De Mello, University of Puerto Rico, USA

It is well known that the activation of the circulating renin angiotensin system is involved in cardiovascular pathology including hypertension, heart failure and is responsible for important organic changes induced by diabetes.

Evidence is now available that independently of the classical system, there are local renin angiotensin systems in different organs including the heart, circulatory vessels, kidney and probably brain and that components of these local systems participate in important aspects of physiology and pathology. Of particular interest is the presence of an intracellular component-the so called intracrine renin angiotensin system, which seems related to regulation of several cellular functions. A discussion of the different aspects of this important topic is of relevance to cell biology, endocrinology, physiology and pathology and justify a comprehensive presentation to the scientific community organized by experts in their respective fields.

Table of Contents

04 ***Beyond the Circulating Renin-Angiotensin Aldosterone System***
Walmor C. De Mello

06 ***Clinical Perspectives and Fundamental Aspects of Local Cardiovascular and Renal Renin-Angiotensin Systems***
Walmor C. De Mello and Edward D. Frohlich

15 ***Local Renin-Angiotensin System in the Reproductive System:***
Daniel Herr, IInga Bekes and Christine Wulff

22 ***Autophagy and the (Pro)Renin Receptor***
Katrina J. Binger and Dominik N. Muller

29 ***Contribution of the Local RAS to Hematopoietic Function: A Novel Therapeutic Target***
Kathleen E. Rodgers and Gere S. diZerega

36 ***A Role for the Brain RAS in Alzheimer's and Parkinson's Diseases***
John W. Wright, Leen H. Kawas and Joseph W. Harding

48 ***The Role of Tissue Renin-Angiotensin-Aldosterone System in the Development of Endothelial Dysfunction and Arterial Stiffness***
Annayya R. Aroor, Vincent G. DeMarco, Guanghong Jia, Zhe Sun, Ravi Nistala, Gerald A. Meininger and James R. Sowers

55 ***New Frontiers in The Intrarenal Renin-Angiotensin System: A Critical Review of Classical and New Paradigms***
Jia L. Zhuo, Fernanda M. Ferrao, Yun Zheng and Xiao C. Li

69 ***Update on the Angiotensin Converting Enzyme 2-Angiotensin (1-7)-Mas Receptor Axis: Fetal Programming, Sex Differences and Intracellular Pathways***
Mark C. Chappell, Allyson C. Marshall, Ebaa M. Alzayadneh, Hossam A. Shaltout and Debra I. Diz

82 ***The Clinical Relevance of Local Renin Angiotensin Systems***
Duncan J. Campbell

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Clinical perspectives and fundamental aspects of local cardiovascular and renal renin-angiotensin systems

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Evidence for the potential role of organ specific cardiovascular renin–angiotensin systems (RAS) has been demonstrated experimentally and clinically with respect to certain cardiovascular and renal diseases. These findings have been supported by studies involving pharmacological inhibition during ischemic heart disease, myocardial infarction, cardiac failure; hypertension associated with left ventricular ischemia, myocardial fibrosis and left ventricular hypertrophy; structural and functional changes of the target organs associated with prolonged dietary salt excess; and intrarenal vascular disease associated with end-stage renal disease. Moreover, the severe structural and functional changes induced by these pathological conditions can be prevented and reversed by agents producing RAS inhibition (even when not necessarily coincident with alterations in arterial pressure). In this review, we discuss specific fundamental and clinical aspects and mechanisms related to the activation or inhibition of local RAS and their implications for cardiovascular and renal diseases. Fundamental aspects involving the role of angiotensins on cardiac and renal functions including the expression of RAS components in the heart and kidney and the controversial role of angiotensin-converting enzyme 2 on angiotensin peptide metabolism in humans, were discussed.

Keywords: local renin-angiotensin systems, heart, arteries and kidney

INTRODUCTION

The presence of local organ specific renin–angiotensin systems (RAS) has been demonstrated for the heart, large arteries and arterioles, kidneys, and other organs and their activation lead to structural and functional changes, which are independent of those elicited by the classical renin–angiotensin endocrine system (1–4). Components of these local RAS, for instance, have been found in cells and tissues (5–8) and some of their local functions play an important role on cellular homeostasis.

In this review, we present several clinical circumstances involving certain cardiovascular diseases, which support the notion that the activation of local RAS plays an important role on the mechanisms of these pathological conditions. These vignettes cited also involve renal diseases because the renal glomerular and arteriolar alterations contribute to the development and progression of end-stage renal disease (ESRD).

CLINICAL CIRCUMSTANCES

MYOCARDIAL INFARCTION AND CARDIAC FAILURE

This first clinical cardiovascular local RAS example relates to the introduction of angiotensin-converting enzyme (ACE) inhibitors and later to angiotensin II (type 1) receptor blocking agents (ARBs) to patients hospitalized with an initial myocardial infarction. This innovative therapeutic intervention proved to reduce ventricular remodeling in naturally developing spontaneously hypertensive rats (SHRs) (9) and following myocardial infarction in rats (10) then later in a small number of hospitalized patients (11) and, ultimately, in a larger clinical trial involving patients enrolled in the survival and ventricular enlargement (SAVE) trial (12). Thus, in patients who were promptly treated with an ACE inhibitor, immediately following acute myocardial infarction, a significant reduction in death, development of heart failure, and subsequent repeated myocardial infarction were found. Several subsequent multicenter clinical trials, using other ACE inhibitors or the newer ARBs, confirmed the initial findings thereby demonstrating their beneficial effects on ventricular remodeling, reduction in the end-stage events of cardiac failure, and repeated myocardial infarction (13). The finding that these beneficial effects can occur independently of blood pressure supports the conclusion that the activation of local RAS contributes significantly to cardiovascular pathology (14).

HYPERTENSIVE HEART DISEASE

Similar evidence involving therapeutic intervention was demonstrated by the findings of the initial Veterans Administration Cooperative Study Treatment Group on Antihypertensive Agents (15, 16) and by the Framingham Heart Study's first demonstration of "Factors of Risk" underlying coronary heart disease (17). The existence that cardiac failure and left ventricular hypertrophy

Abbreviations: ACE, angiotensin-converting enzyme; ACE2, angiotensin-converting enzyme 2; Ang (1–7), angiotensin (1–7); Ang II, angiotensin II; ARB, angiotensin receptor blocker; ESRD, end-stage renal disease; $I_{Clswell}$, swelling-dependent chloride current; LV, left ventricle; LVH, left ventricular hypertrophy; NO, nitric oxide; RAS, renin–angiotensin system; SHR, spontaneous hypertensive rats.

(LVH), respectively, were first introduced by these two groups, to interdict in the major cardiac fatal and treatable complications of hypertensive heart disease (15–17). Subsequent reports later demonstrated that these two major complications of hypertensive heart disease were prevented by antihypertensive therapy. They also introduced the means to reduce left ventricular (LV) mass and its co-morbid events (18). In more recent years, increased LV mass and LVH were shown to be associated with extensive interstitial and perivascular fibrosis as well as by significant ischemia of both ventricles (18–20). Furthermore, when patients with LVH associated with hypertension (but not by co-existent occlusive coronary artery disease) were also treated with RAS inhibitors, the fibrosis and ischemia were significantly reduced (18–22). This, then, provided additional evidence of the beneficial value of local cardiac RAS inhibition.

The precise mechanisms underlying the development of LVH have usually been explained as an adaptive compensation by the LV to pressure overload by the hypertensive disease. Newer information has been introduced more recently concerning the development of fibrosis, apoptosis, aldosterone, and other induced cellular biochemical events in the LV. Others have suggested that angiotensin II (Ang II) causes hypertension and LVH through actions of AT1 receptors expressed by the kidney that reduce urinary sodium excretion (23) not involving Ang II-mediated aldosterone responses.

PROLONGED DIETARY SALT EXCESS

Two very different diseases involving local RAS in the heart as well as in the glomerular arterioles of the kidneys (24–26) support the important role of local RAS. SHRs receiving long-term dietary salt excess have shown remarkably similar pathophysiological expressions of disease similar to those which occurs in patients with hypertension having ventricular fibrosis, myocardial ischemia, and heart failure or with ESRD (27–29). These end-stage events occur in patients with hypertension and/or with diabetes mellitus having ESRD with intrarenal fibrosis and hyaline degeneration of the glomerulae and arterioles. As with the foregoing diseases that were shown by controlled multicenter drug trials using Ang II inhibitors described above, the progression of ESRD was also shown to be significantly retarded (30, 31). Interestingly, a recent report of SHR, given a prolonged dietary excess of salt, demonstrated a second local renal RAS (in addition to that of the juxtaglomerular apparatus) that produced a more plentiful production of angiotensinogen (32).

Finally, a word of speculation may be in order involving end-stage cardiac and renal diseases, the most common causes of hospitalization in geriatric hypertensive (or normotensive) patients from industrialized nations (33). These data suggest that a lifetime of excessively high salt intake together with these untoward outcomes may be intimately associated with the aging process (34). Indeed perhaps this may also relate to our new knowledge about local RAS in heart, arteries and arterioles of the kidneys.

FUNDAMENTAL ASPECTS BEHIND THE FOREGOING CLINICAL EXAMPLES

Experimental evidence supporting the notion that local RAS are present in different organs including the heart and kidney (3, 4, 35–40) has opened a new window into our understanding of how the local RASs contributes to local regulation of tissue and organ function. The synthesis of several components of the RAS in the heart (8, 41) or their uptake from plasma (8, 36, 41), for instance, makes it possible to explain the synthesis of Ang II locally (41). Furthermore, the presence of AT1 receptors, angiotensinogen and Ang II in different cells (8), supports the concept of local RAS. In the normal heart of pigs as much as 75% of cardiac Ang II is synthesized at tissue sites (42) whereas in human beings, the gradients of Ang II across the heart were increased in patients with congestive heart failure (5). Rapid internalization of the Ang II–AT1 receptor complex, contributes significantly to the intracellular levels of the peptide [see for review Ref. (43)] and the internalized AT1 receptor, is displaced to different organelles including the nucleus and mitochondria (43–47). Activation of AT1 receptor binding sites in renal nuclei has been found to elicit an increase in calcium (48) and in the expression of TGF-B1 and NHE-3 (46). Concerning the role of the local renal RAS on the generation of hypertension, recent studies revealed that the infusion of Ang II into mice lacking renal ACE, indicated no renal responses or hypertension in the knockout mice compared with wild-type control (49).

Transgenic mouse models developed to examine the role of the local RAS on cardiac remodeling, generated contradictory results revealing ventricular hypertrophy or fibrosis in some models but not in other (40, 50, 51) leading to the conclusion that cardiac remodeling is probably much more dependent on hemodynamic changes than on local Ang II levels. In hypertensive transgenic mouse lacking the synthesis of angiotensinogen, for instance, the local components of the RAS do not seem to be essential for the subsequent development of ventricular hypertrophy and fibrosis (41). The production of Ang II, in cardiac muscle caused by a αMHC promoter, increases the release of Ang II by 20-fold, but not hypertrophy was produced (51). On the other hand, in transgenic mouse lines over-expressing angiotensinogen by the heart, Ang II is increased in cardiac muscle but not in plasma (52) and ventricular hypertrophy was found despite no change in blood pressure. In these models, the hypertrophy was abolished by ACE inhibitors or AT1 blockers (53), again supporting the notion of a local RAS. Xu et al. (54) found that when hemodynamic loading conditions remain unchanged, cardiac Ang II does not elicit hypertrophy but in animals with hypertension, cardiac Ang II, acting via AT(1)R, increases oxidative stress, inflammation, ventricular hypertrophy, and cell death (probably via down regulation of PI 3 kinase and Akt).

These apparent discrepant results achieved with different transgenic models could be related to the use of different animal species or experimental conditions. Furthermore, the only parameter used to define cardiac remodeling in many of these studies was ventricular hypertrophy and other aspects of cellular remodeling like cell communication, fibrosis as well as expression and function of ionic channels were not considered.

Concerning the origin of cardiac renin, evidence is available that in the normal heart, cardiac renin is dependent on its uptake from plasma (6, 36, 42) but studies performed after myocardial infarction (55) or after stretch of cardiomyocytes (40) showed increased renin expression. Furthermore, a renin transcript that does not encode a secretory signal and remains inside the cell is

over-expressed during myocardial infarction (55, 56) suggesting that intracellular renin has functional properties. The cytosolic renin protein exerts functions different and even opposite to those of secretory renin, which increases necrotic death rates of cardiac cells, while the cytosolic renin isoform even protects cells from necrotic death (56). In adrenal gland, a local secretory RAS exists that may stimulate aldosterone production and elicits an amplification for circulating angiotensin (Ang II) (57). The regulation of the secretory adrenal RAS is clearly different from the regulation of the circulatory RAS because under potassium load, the activity of the renal and circulatory RAS is suppressed whereas activity of the adrenal RAS is stimulated (57).

The function of intracellular renin and Ang II was demonstrated when renin or Ang II was dialyzed into cardiac myocytes from the failing heart. Renin, and particularly Ang II, decreased cell communication and increased the inward calcium current (37, 58, 59). The decrease of gap junction conductance leads to a decrease of electrical coupling and mechanical desynchronization as well as the generation of slow conduction and cardiac arrhythmias (60, 61). Recent studies performed on the intact ventricle of normal rats revealed that intracellular renin causes a depolarization of ventricular fibers and a decreased action potential duration at 50 and 90% repolarization, respectively while the cardiac refractoriness was significantly decreased with consequent generation of triggered activity (59). The intimate mechanism by which intracellular renin alters cardiac excitability involves changes of potassium current, which is responsible for repolarization of the action potential (59). The possible role of an intracellular renin receptor (62), which is activated by renin (62, 63), cannot be discarded and further studies will be needed to support this idea. The pathophysiological significance of intracellular renin is far from clear and further studies will be needed to clarify this point.

RECENT DEVELOPMENTS

Our view of the RAS has been changed dramatically in recent years with studies demonstrating that Ang II can be hydrolyzed by angiotensin-converting enzyme 2 (ACE2), angiotensinases as well as neprilysin generating angiotensin (1–7) [Ang (1–7)], Ang A, Ang IV, and Ang III (64–66) and that new receptors for Ang (IV) (AT4), prorenin [(pro)renin receptor (PRR)], and Mas receptor for Ang (1–7) have been identified (67–70). Interestingly, the activation of prorenin receptor is able not only to catalyze prorenin to Ang II but also to induce cellular responses not related to the peptide (71, 72). Of particular interest is the recent finding that not all the peptides from RAS are derived from Ang I. The plasma levels of Ang (1–12), initially isolated from the rat intestine and present in heart, aorta, and kidney (73, 74) are not altered by renin inhibition or bilateral nephrectomy, which suggests a local effect of Ang (1–12) in tissues independently of the systemic circulation (73, 74). Chymase seems to be the most important enzyme involved in the metabolism of Ang (1–12), at least in the heart (75). Other studies of Ang (1–12) metabolism indicated that in the plasma of normal or hypertensive rats, ACE has a role generating Ang I from Ang (1–12) (76).

A new component of the RAS is amantadine, which is a heptapeptide possessing functions similar to those of Ang (1–7) and found in human plasma particularly in patients with ESRDs (77). The vasodilation caused by amantadine was not inhibited in Mas-deficient mice (77) suggesting its interaction with another Mas receptor. The precise role of this compound on cardiovascular disease is not known.

AT2 RECEPTORS

Although it is known that the effect of Ang II on cardiac and vascular remodeling involves the activation of AT1 receptors, recent studies revealed that the AT2 receptor activation causes vasodilation and its agonist C21 is able to decrease myocardial fibrosis and vascular injury in SHRs [see Ref. (66, 78)]. The role of AT2 receptors on cardiac remodeling is supported by studies using AT2-knockout mice and the results indicated that this receptor plays an essential role in the development of ventricular hypertrophy induced by pressure overload (79) [see Ref. (80)]. AT2 receptor activation seems to inhibit inflammation and apoptosis (81), attenuates cardiopulmonary injury by decreasing pulmonary inflammation (82) and in obese animals, long-term activation of AT2 receptors increases ACE2 activity and contributes to natriuresis and blood pressure reduction (83). The natriuresis is probably related to Ang III (84).

Myocardial fibrosis impairs ventricular relaxation and is an important cause of diastolic heart failure. The presence of fibrosis is not limited to the left ventricle and is found in the right ventricle as well as in the interventricular septum, suggesting that hypertension is not the only factor involved but also local production of Ang II is involved (85). The fibrotic action of the peptide within the heart seems to depend on fibroblast hyperplasia as well as activation of collagen biosynthesis and suppression of collagen degradative pathways. Activation of pathways related to AT1 receptors as well as MAP/endoplasmic reticulum (ER) kinase pathway activation play a key role of the generation of fibrosis and recently, evidence has been provided that Ang II AT2 receptors prevent cardiac remodeling after myocardial infarction and improve cardiac function (86).

THE (PRO)RENIN RECEPTOR

The PRR (71), mainly located intracellularly (62), is a new member of the RAS, originally considered as involved in the regulation of blood pressure. Recent observations using transgenic animals over-expressing PRR did not provide a clear answer to this question but demonstrated different aspects of PRR biology. It is now clear that PRR is an accessory protein of V-ATPase (87) playing an important role on the regulation of several cellular homeostatic processes including autophagy (88).

A knockout model generated by Kinouchi et al. (89) showed death within 3 weeks and an accumulation of vesicles and autophagosomes in cardiomyocytes indicating a change in autophagic flux. The role of PRR on the etiology of cardiovascular diseases, however, is not clear and further studies will be needed to clarify this point.

ADIPOCYTES AND REGULATION OF ANG II PLASMA LEVELS

The presence of a local RAS in adipocytes is supported by recent findings showing that RAS is activated during obesity in humans and that obesity-prone rats show increased levels of Ang II and hypertension (84). In mice over-expressing angiotensinogen in

adipocytes, the plasma levels of Ang II are increased as well as the systolic blood pressure (90). On the other hand, adipocyte-specific deficiency of angiotensinogen prevented the obesity-induced increase in plasma levels of Ang II (84) indicating an important role of adipocytes on the regulation of Ang II plasma levels and on ulterior consequences including hypertension and vascular remodeling.

INTRACRINE ACTION OF ANGIOTENSIN II IN THE HEART AND MESENTERIC ARTERIES

The concept of an intracrine renin–angiotensin aldosterone system (RAAS) in the heart has been substantially supported (3, 5–7, 37). When eplerenone was administered chronically to the failing heart, the intracellular action of Ang II on the inward calcium current (91) was abolished, an effect reversed by aldosterone and related to a decrease of intracellular AT1 receptor levels (91). The activation of the intracrine RAAS might be involved in the generation cellular hypertrophy (92, 93), cardiac arrhythmias (60), and on regulation of vascular tone (94).

Of particular interest was the recent finding that intracellular administration of Ang II to arterial myocytes isolated from mesenteric arteries of Sprague Dawley rats increased the total potassium current and the resting potential, whereas extracellular administration of Ang II reduced total potassium current and elicited depolarization of smooth muscle cells (94). These effects of intracellular Ang II on potassium current and membrane potential were inhibited by dialyzing a PKA inhibitor inside the cell together with Ang II (94). Because it is well known that the resting potential is a determinant factor on the regulation of vascular tone (95), these results might indicate that endogenous or internalized intracellular Ang II in vascular resistance vessels counteracts the effect of extracellular Ang II and plays an important role on the regulation of vascular tone and peripheral resistance (94).

MITOCHONDRIA AND INTRACRINE RENIN-ANGIOTENSIN SYSTEM

A revealing finding was that in the ER, renin cleaves angiotensinogen to Ang I, which is subsequently processed to Ang II by ACE (96). Different components of the RAS including the processing enzymes, angiotensins, and their receptors can be transported intracellularly via secretory vesicles to the cell surface, to mitochondria, or to the nucleus.

Activation of the mitochondrial Ang system is coupled to mitochondrial nitric oxide (NO) production and the binding of Ang II to mtAT2Rs stimulates NO formation through mtNOS, suppressing mitochondrial oxygen consumption. Nuclear Ang II can stimulate NO formation (via AT2Rs) or Ca^{2+} and phosphoinositol 3 kinase (PI3K) via AT1Rs (96). The pathophysiological meaning of the presence of renin or Ang II in mitochondria is not known, but considering the role of Ang II on oxidative stress, it is possible to think that activation of AT1 or AT2 receptors in mitochondria might be involved in the etiology of heart or kidney failure.

CELL VOLUME CHANGES EVERYTHING. MECHANICAL SENSITIVITY OF HEART MUSCLE AND CARDIAC REMODELING

One of the important limitations on studies of cellular functions is the assumption that cell volume is constant. It is known that preservation of cell volume is fundamental for cell function and survival and that several mechanisms are working constantly in order to maintain cell volume. Changes in metabolism and the transport of osmotically active substances across the cell membrane are important causes of cell volume variations. It is well known that metabolic pathways are sensitive to changes in cell volume and that glycolysis is inhibited by cell swelling [see Ref. (97)]. Cell swelling, which activates several ionic channels at the cell membrane, changes the action potential duration and alters cardiac excitability.

Recently, it has been shown that that the RAAS is involved in the regulation of cell volume in normal as well as in the failing heart (98). Indeed, in myocytes isolated from the failing ventricle and exposed to renin plus angiotensinogen or to Ang II, an increase of cell volume was seen concurrently with the inhibition of the sodium pump (98). The activation of the Na–K–2Cl cotransporter is involved in the effect of Ang II because bumetanide abolished the swelling induced by the peptide (98). Ang II also increases the swelling-dependent chloride current ($I_{Clswell}$) in the failing and in the normal heart (98), while Ang (1–7), which has been found to counteract many effects of Ang II (99), reduces the heart cell volume and decreases the swelling-activate chloride current ($I_{Clswell}$) (98). This effect of the heptapeptide might be involved in the beneficial effect of Ang (1–7) by decreasing the incidence of cardiac arrhythmias during ischemia/reperfusion (65, 100, 101).

Experimental studies using low doses of aliskiren in hypertensive TGR(mRen2) 27 rats, revealed a decreased structural and electrical cardiac remodeling independently of blood pressure (102) supporting the notion that the renin inhibitor has a direct effect on the heart. The beneficial effect of aliskiren was related to a decrease of AT1 receptor levels. Because AT1 receptors are mechanosensors (103) independently of Ang II [see also Ref. (104)], it is reasonable to think that mechanical stress is able to produce cardiac remodeling even in absence of the peptide. These findings leads to the hypothesis that cardiac remodeling elicited by pressure overload, depends upon the mechanical sensitivity of the cardiac muscle to mechanical stimulation (102) determined by the expression of mechanosensors like AT1 receptors.

ON THE ROLE OF ACE2

Angiotensin-converting enzyme 2 is a newly discovered enzyme having a high homology to ACE and able to hydrolyze Ang II to the peptide Ang (1–7) (105, 106). Ang (1–7) counteracts the pressor effects of Ang II as well as the proliferative and profibrotic effects of the peptide (65, 69, 70, 99, 100, 107, 108), reduces the incidence of heart failure after myocardial infarction in rats (99) and humans (109), and enhances the cardiac function, coronary perfusion, and aortic endothelial function (99). Previous studies have shown that Ang (1–7) increases the conduction velocity in the failing heart (100, 101) and decreases the incidence of slow conduction and reentry. Recently, evidence has been provided that the activation of the ACE2-Ang (1–7)-Mas receptor axis is involved in the regulation of heart cell volume (110) as well as in the magnitude of the swelling-activated chloride current $I_{(Clswell)}$. This effect of Ang (1–7) was inhibited by ouabain, supporting the view that the heptapeptide activates the sodium pump. Ang II, on the other hand, had an opposite effect on heart cell volume causing cell swelling and increasing the swelling-activated

chloride current (110). During myocardial ischemia, cell swelling elicited by the inward movement of water increases $I_{(Clswell)}$ with consequent decrease of cardiac refractoriness. These observations support the notion that the activation of the ACE2-Ang (1–7)-Mas receptor axis is of benefit reducing the cell volume and the incidence of cardiac arrhythmias during ischemia-reperfusion (110). In other studies, it was found that the loss of ACE2 accelerates the maladaptive LV remodeling after myocardial infarction (111). Interestingly, perinuclear immunostaining of the Ang (1–7) was found in mesangial cells (112) and very low concentrations of Ang (1–7) stimulated NO release opening the possibility that intracellular Ang (1–7) has also an intracrine effect.

Although compelling evidence has been presented supporting the view that ACE2 activation counteracts the effects of Ang II in ventricular muscle, some fundamental aspects of the biological significance of ACE2-Ang (1–7)-Mas receptor axis remain unclear. Overexpression of ACE2 in the failing heart, for instance, does not prevent the progression of human heart failure (109). In human coronary circulation, the levels of Ang (1–7) were found to be linked to those of Ang I not Ang II, indicating no role of ACE2 on Ang II metabolism (113). This finding is not in agreement with previous studies on human heart failure showing that ACE2 plays an important role on Ang II metabolism (109).

In the kidney, evidence has been presented that Ang (1–7) causes vasodilation in renal tubuli and counteracts the effect of AT1 receptor activation in several renal diseases such as tubulointerstitial fibrosis, diabetic nephropathy and glomerulonephritis. Under some experimental conditions, however, Ang (1–7) may be harmful by exacerbating renal injury [see Ref. (114)]. This suggests that the state of activation of local RAS, the involvement of non-Mas receptor mediated pathways, or even the dose might explain the discrepant results (65).

ON THE ROLE OF ALDOSTERONE AND MINERALOCORTICOID RECEPTORS

There is experimental and clinical evidence that aldosterone causes fibrosis in the cardiovascular system. The RALES trial, for instance, indicated a beneficial effect of spironolactone on morbidity and mortality in patients with heart failure mainly related to the decrease of fibrosis (115) RALES. The contribution of aldosterone to the effect of local RAS activation has been supported by several studies [see Ref. (3)] and justifies the concept of a local RAAS. Although the expression of aldosterone synthase as well as the synthesis of aldosterone seems unlikely in the normal heart, it has been reported an enhanced synthesis of aldosterone in the failing heart (116, 117). Furthermore, elevated plasma aldosterone levels are associated with increased incidence of heart attack and stroke (118).

In vascular smooth muscle as well as in immune cells, the local RAAS plays an important role on endothelial dysfunction and contributes to the production of arterial stiffness. In humans with obesity and diabetes, the RAAS is associated with enhanced oxidative stress and inflammation in the vascular tissue supporting the view that the mineralocorticoid receptors play a role on generation of insulin resistance (119). Indeed, basic and clinical studies have demonstrated that elevated plasma aldosterone levels predict the development of insulin resistance by interfering with insulin signaling in vascular tissues. Aldosterone suppresses insulin signaling via the downregulation of insulin receptor substrate-1 in vascular smooth muscle cells (120, 121).

Recent observations indicated that spironolactone enhances the beneficial effect of aliskiren on cardiac structural and electrical remodeling in TGR(mRen2)27 rats (122) and that chronic administration of eplerenone to the failing heart reduces the cardiac effect of Ang II on inward calcium current through a decline in AT1 receptor level at the surface cell membrane (91). Because AT1 receptor is a mechanosensor involved in cardiac remodeling, it is reasonable to think that part of the beneficial effect of spironolactone in the failing heart is related to a smaller sensitivity of cardiac muscle to mechanical stress.

SUMMARY

Recent findings that Ang II can be hydrolyzed by ACE2 and neprisylin as well the evidence of new receptors for Ang (IV), Ang (1–7), and Ang III, and the possibility that Ang (1–12) might be the mother of all angiotensins are other evidences of how complex is the RAS. The observation that in human coronary circulation, the levels of Ang (1–7) are related to those of Ang I, but not of Ang II, lead to the question whether many aspects of Ang (1–7) pharmacology are different in humans.

The presence of a local RAS in adipocytes and the observation that the RAAS is activated during obesity in humans, seem to demonstrate how important is this local system on the generation of obesity and hypertension.

The relevance of cell volume and mechanical stretch as a regulators of chloride or potassium channels and the role of AT1 receptors as mechanosensors independently of Ang II indicates that during myocardial ischemia or heart failure, abnormalities on the electrical properties of the heart and cardiac remodeling can be produced independently of the RAS but able to alter the effect of Ang II and Ang (1–7). The recent observation that intracellular Ang II counteracts the effects of extracellular Ang II on potassium current and resting potential in mesenteric arteries leads to the question whether internalized or endogenous levels of Ang II in vascular resistance vessels represent an important factor on the regulation of peripheral resistance and arterial blood pressure. Furthermore, evidence that components of the RAS are present in mitochondria and in the nucleus raises the possibility that the activation of AT1 and AT2 receptors in these organelles influences gene expression and oxidative stress, which is an important cause of cellular dysfunction and the cause of several diseases. Further studies on all these areas will provide opportunity to prevent and treat cardiovascular and renal diseases. The possible role of PRRs on the regulation of cellular homeostasis including autophagy as well as the importance of Ang II AT2 receptors on ventricular hypertrophy needs to be clarified.

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Local renin-angiotensin system in the reproductive system

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The renin-angiotensin system (RAS) is well known as regulator of electrolytes and blood pressure. Besides this function, there are numerous studies supporting the idea of a local tissue RAS. This system controls the local activity of the different RAS family members, especially of the functional proteins Angiotensin II and Angiotensin (1–7). Those antagonistically acting proteins have been described to be expressed in different organ systems including the human reproductive tract. Therefore, this local RAS has been suspected to be involved in the control and regulation of physiological and pathological conditions in the female reproduction tract. This review of the available literature summarizes the physiological influence of the RAS on the follicular development, ovarian angiogenesis, and placental- and uterine function. In addition, in the second part the role of the RAS concerning ovarian- and endometrial cancer becomes elucidated. This section includes possible novel therapeutic strategies via inhibition of RAS-mediated tumor growth and angiogenesis. Looking at a very complex system of agonistic and antagonistic tissue factors, it may be supposed that the RAS in the female reproduction tract will be of rising scientific interest in the upcoming years.

Keywords: endometrial cancer, endometrium, ovarian cancer, ovary, renin-angiotensin system, reproductive tract, local

INTRODUCTION

The Renin-Angiotensin system (RAS) is of paramount importance for the perpetuation of the circular flow, regulating the electrolyte metabolism thus the blood pressure (1–4). This considerable function of the RAS is mediated by the systemic RAS-pathway. The latter consists of a cascade of peptides, acting as precursors which become transformed by different enzymes into the bioactive end products (5). The main protein of this system is Angiotensinogen, which is synthesized in the liver (6). Following the pathway, Angiotensinogen becomes converted into Angiotensin I, catalyzed by Renin, which is of renal origin. Subsequently, Angiotensin I can be further modulated by the angiotensin-converting enzyme (ACE) I to Angiotensin II or by ACE II to Angiotensin (1–9).

Angiotensin (1–9) then becomes transformed by ACE or neutral endopeptidase (NEP) into Angiotensin (1–7) (7–9). Those two bioactive effector molecules, Angiotensin II and Angiotensin (1–7) act in an antagonistic way by binding to different receptors: angiotensin receptor type 1 (AT1R) and type 2 (AT2R) or Mas-receptor (10). The G-coupled Mas-receptor mediates vasodilatory and anti-proliferative effects and antagonizes actions of the AT1R (11). In recent years, attention has also focused on the evidence of a widespread local tissue RAS (12). Expression of elements of this local RAS has been described in different parts of the human reproductive tract. Apparently, the both antagonistic bioactive proteins of the RAS, in particular Angiotensin II and Angiotensin (1–7) can result from the local tissue RAS (13, 14). This local production of the bioactive peptides is not necessarily dependent on the local expression of all components of the local tissue RAS, since it is also possible to take up components from the circulation, such as renin. Furthermore, besides presence of Angiotensin II and Angiotensin (1–7), expression of AT1R, AT2R, and Mas-receptor human reproductive tissue is needed in order to mediate the local impact of the RAS for physiological and pathological processes, including follicle maturation, fine-tuning of the regulation of reproduction, angiogenesis as well as tumor cell proliferation (15–18). An influencing effect on cancer has been described for different tumor types already during the last two decades (19, 20).

MATERIALS AND METHODS

We performed a systematic literature review concerning presence and function of the RAS in the female reproduction tract. This was based on the medical databases Medline, Embase, BIOSIS, and CINHAL. Literature analysis was conducted without a timeframe on all existing publications including 2013. All manuscripts were sighted based on the title and abstract and any duplicate manuscripts occurring in the literature search were excluded. After fulfilling the inclusion criteria (content-related, experimental, and clinical studies, in the case of experimental studies dividing into studies in humans and/or animals) the manuscripts were reviewed and analyzed. Thereby, the data was extracted and content-related articles allocated into two different groups:

- Physiological role of the RAS in the reproductive tract?
- Role of the RAS in gynecologic cancers.

PHYSIOLOGICAL ROLE OF THE RAS IN THE REPRODUCTIVE TRACT

OVARY AND FOLLICULAR DEVELOPMENT

Definitely, a local tissue RAS is present in the adult ovary (18). In the human ovary, all family members of the RAS have been proven at the protein level, whereas in the bovine, porcine, and rat ovary only single compounds of the RAS are expressed (12, 21–23). It has been speculated, that the attendance of RAS compounds is significantly involved in the regulation of fetal development since RAS expression can be observed in the porcine ovary already around 45 days of gestation: AT1R and AT2R have been detected in granulosa cells of primordial, primary, and secondary follicles (23). In addition, Angiotensin II and its receptors AT1R/AT2R seem to have regulatory effects in the ovary regarding oocyte nuclear maturation and ovulation (24–27). This regulative function has mainly been investigated in antral follicles, but also in porcine granulosa cells of earlier stages of follicular development (28). Obviously, there are significant differences between the species. When bovine Cumulus oocyte complexes (COCs) were cultivated with Angiotensin II, nuclear maturation of the oocyte was induced (12, 26). Furthermore, Ferreira et al. indicated that Ang II may have an impact on bovine ovulation via AT2R (25). In addition, functional studies have demonstrated that inhibition of the AT2R prevents bovine ovulation significantly (29). In rats, AT1R is expressed in healthy follicles (30) and AT2R-expression is obviously involved in follicular atresia through apoptosis (31, 32).

Unfortunately, the published data concerning involvement of the RAS in the regulation of the hormonal regulation of the ovary is scanty and sometimes inconsistent. For example, for the bovine corpus luteum, it has been shown, that tissue levels of Angiotensin II do not change throughout the cycle, indicating that steroids may have no influence on tissue RAS (33). In conflict with this finding, a significant influence of the RAS on progesterone synthesis has been described (34, 35). The observed increase of progesterone and soluble and membrane-bound aminopeptidase A was accompanied by a decrease of membrane-bound aminopeptidases B/N (RAS-regulating enzymes) due to inhibition alpha 1-adrenergic receptors in rats (35). In addition, the data concerning gonadotropin-dependent expression of RAS-proteins is disputed: it has been shown that application of hCG in case of early pregnancy has the capacity to activate the local RAS in the ovary (36), whereas our own group observed a significant hCG-dependent decrease of Angiotensin II in human granulosa lutein cells *in vitro* (37). This result goes in line with the perception that the antagonistically to Angiotensin II acting Angiotensin (1–7) and its receptor Mas were found to be increased after gonadotropin stimulation in the rat ovary (22). Basically, the role of Angiotensin (1–7) seems to be of increasing interest: Angiotensin (1–7), Mas-receptor, and ACE 2 were identified in all stages of follicular development in humans (38) and functional studies indicate a role of the Angiotensin (1–7)-pathway in the rodent *in vivo* (39) suggesting to be a mediator of gonadotropin functions in the ovulatory cascade (40).

OVARIAN VASCULATURE FUNCTION AND ANGIOGENESIS

The most outstanding data in the literature has been published concerning the regulatory character of the RAS on vascular function and angiogenesis in the ovary. The invoking effects on the vessels are thereby first of all adapted from the Angiotensin II-AT1R-pathway (41–44). To be contrary to this, the restitution of the luteal vasculature is mediated by the AT2R-pathway (45). Anyway, Angiotensin II obviously influences the microvascular endothelial function in the corpus luteum (42). Hayashi et al. demonstrated that microvascular endothelial cells (MVE) in the corpus luteum express ACE and are capable to convert Angiotensin I into Angiotensin II. The Angiotensin II production thereby increases significantly under stimulation with estradiol in combination with vascular endothelial growth factor (VEGF) (41). MVE furthermore possess AT1R and AT2R (41, 42). Interestingly, the expression of those two receptors differ throughout the cycle: AT1R remains constant but AT2R-expression is lowest during the mid luteal phase and highest during the late luteal phase (41, 46). The regulation of angiogenic processes is urgently needed to ensure the constant flow of growth, maturation, and demise of the corpus luteum. It has been shown by our group, human granulosa lutein cells collected during *in vitro* fertilization (IVF) are expressing several components of the RAS (47). In addition, we demonstrated that exogenous Angiotensin II stimulation increased VEGF synthesis via AT1R signaling *in vitro* (47). This data may implicate the regulatory effect of the RAS on angiogenesis in the corpus luteum. In agreement with its meaning concerning control of systemic blood pressure, the individual family members of the local RAS also regulate perfusion and vascular tone in the ovary (36, 44).

PLACENTA

The human placenta is one of the most interesting tissues in the reproductive tract, because the utero-placental unit provides a transposition of nutritive substances and oxygen between mother and fetus. It has been assumed by many authors that the RAS influences the placental function (48–52), since all different components of the RAS are expressed in human placenta cell lines (53) as well in placental tissue (54, 55). However, functional data of the placental RAS is very rare. Obviously, the different RAS-proteins are expressed differentially in the various areas of this organ: angiotensinogen, Renin, Angiotensin I, Angiotensin II, ACE, AT1R, and AT2R were localized to maternal decidua (56, 57) and Angiotensin II and ACE were additionally found in pericytes of endometrial spiral arteries. However, Angiotensinogen and renin also have been detected in fetal capillaries (58). The AT1R, which is predominantly expressed in the placenta, was found in cytotrophoblast and syncytiotrophoblast cells as well as in fetal capillaries, while little is known concerning localization of the AT2R (59, 60). The antagonistic proteins to Angiotensin II, namely Angiotensin (1–7) and ACE2 were found to be expressed in syncytiotrophoblast, cytotrophoblast, and vascular smooth muscle cells of primary and secondary villi (58). The above mentioned members of the RAS family can be detected from 6 weeks of gestation until birth. Obviously, there are some variations in the course of pregnancy: it has been shown that mRNA of ACE is increasing during gestation but decreases near term. In addition, AT1R mRNA and AT1R protein levels are rising throughout the entire pregnancy, reaching highest levels at the end (61). Since a direct connection between Angiotensin II and AT1R has been observed in the placenta, it has been supposed that this fact indicates a

regulating effect of Angiotensin II on the AT1R expression (62). From a more clinical point of view, there are several references that the placental RAS is involved in trophoblast invasion and angiogenesis (63, 64), being a possible cause of defect for the development of conditions with disordered utero-placental perfusion, namely preeclampsia (see below).

FALLOPIAN TUBE

Data concerning the RAS and the oviduct is rare. Any clinical relevant findings have not been published. However, Angiotensin II has been localized in blood vessel endothelium and in stromal cells. Both binding and Angiotensin II type-2 receptor mRNA were detected at high levels, but no differences in receptor concentration could be detected in fallopian tubes ipsilateral or contralateral to the corpus luteum (65).

UTERUS

Outline above, the data of uterine RAS are of descriptive nature and mostly limited to the endometrium. Studies investigating the functional relevance of the RAS in the uterus are rare.

Being an indispensible part of the reproductive tract, the endometrium underlies a cyclic change of growth and degradation. Basically, all elements of the local tissue RAS are expressed in the endometrium (66), however this expression diversifies during the cycle (46, 67): angiotensin II underlies cyclic variances within the endometrium and is increased during the proliferative phase, and decreased during the secretory phase (68). Angiotensin-(1–7) and its receptor MAS is also present throughout the menstrual cycle but increases in the glandular endometrium in the mid and late secretory phase. Although AT1R and AT2R are expressed in the endometrium, expression of AT2R is more frequent and varying (68) and it is down-regulated during pregnancy (69). The AT2R-expression is thereby most prominent in the myometrium (up to 90%) as compared to AT1R expression (up to 10%). Unfortunately, data concerning functional effects of the RAS in the uterus is rare. Since endometrium is controlled by female sex hormones, it has been supposed that the RAS might also be influenced by those hormones. This assumption is supported by the finding, that the local expression and production of renin is increased after stimulation with progesterone (70).

PATHOPHYSIOLOGICAL ROLE OF THE RAS IN THE REPRODUCTIVE TRACT

During the past few years, the primary small number of publications concerning pathophysiologic aspects of the RAS has been markedly increased. This affects aspects of reproduction, in essence preeclampsia, as well as of the role of the RAS controlling gynecological cancers. Thereby, the most resilient data is available regarding the regulatory aspects on tumor cell proliferation, vascular function, and angiogenesis (71).

RAS AND REPRODUCTION

In patients with polycystic ovary syndrome, the intra-follicular renin, which is needed for synthesis of the bioactive peptides of the RAS, affects maturation and oocyte quality (43). Follicles with high levels of renin indicating a high local RAS activity, were associated with better oocyte quality and showed higher VEGF concentrations during IVF procedures (43). Furthermore, a strong activation of the local ovarian RAS by beta-hCG has been observed during IVF treatment. This process was also associated with an increased VEGF concentration. Consequently, it has been assumed, that the activation of the ovarian RAS and consecutive high levels of VEGF might act synergistically during pathogenesis of ovarian hyperstimulation syndrome (OHSS).

Although the role of the RAS concerning invasion of trophoblast and placentation is poorly investigated, there is evidence that dysfunction of this system may cause hypertension and preeclampsia (56, 72, 73, 74): patients with preeclampsia present with increased expression of Angiotensin II and AT1R in maternal decidua cells and in the placenta itself (74, 75). In pre-eclamptic pregnancies Angiotensin II and AT1R was been observed to be increased, whereas levels of Angiotensin I, Angiotensin (1–7), ACE, and ACE2 were normal as compared to healthy pregnancies (48).

Current data describe a relevant clinical link between RAS and preeclampsia: women with a male fetus who developed gestational hypertension had increased Angiotensin (1–7) levels at 15 weeks gestation compared with women with normal pregnancies, suggesting that these women were on an early trajectory for the development of hypertension. Therefore, the authors proposed measurement of Ang-(1–7) during early pregnancy in order to predict new-onset hypertension (76).

In addition Valdes et al. also reported elevated Angiotensin (1–7) concentrations in spontaneously aborted and ectopic early pregnancy placentas, which lead the authors to hypothesize that the ACE2–Angiotensin-(1–7) axis plays a functional role in placental development (77). Further recent data underlines the connection between RAS and preeclampsia, since there is an association with a polymorphism of Angiotensinogen in Chinese women. This finding might cause disordered vasculogenesis contributing to the development of preeclampsia (78).

OVARIAN CANCER

The published data apropos RAS and invasive epithelial ovarian cancer provides a role concerning proliferation and dissemination of cancer cells and tumor-angiogenesis. Ovarian cancer cells express Angiotensin II and AT1R (79) but there is still missing evidence of the other components of the local RAS in ovarian cancer. It has been shown that levels of AT1R are higher in borderline lesions and in invasive epithelial ovarian cancer as compared with normal ovaries (80). Being in line with this finding, ovarian cancer patients presenting with high levels of AT1R, have a worse prognosis in comparison with tumors lacking the AT1R expression. Obviously, the Angiotensin II → AT1R pathway is able to influence ovarian cancer cell proliferation (80). Since it has been shown that levels of VEGF as well as rates of angiogenesis are increased due to Angiotensin II, the link between the RAS and Angiogenesis has been established in epithelial ovarian cancer cells (80). At least *in vivo*, this connection even acts quantitatively: stimulation of the ovarian cancer cell line Scov 3 with Angiotensin II caused increased VEGF expression (80) and high levels of AT1R are associated with significantly increased VEGF production and micro-vessel density (MVD) (79). All those findings might indicate a therapeutical approach. Therefore, inhibition of AT1R has been performed in mice with peritoneal carcinomatosis, leading to a significant decrease of ascites and peritoneal

tumor cell dissemination. In patients, current data at least indicate that agonistic auto antibodies against AT1R may be associated with advanced progression of early ovarian cancer (81). These findings implicate that AT1-AA might be selected as a detectable biomarker and potential therapeutic target in diagnosis and treatment of EOC patients. Summing up, it appears that two substantial mechanisms, increased tumor cell proliferation and angiogenesis are mediated by the RAS. Therefore, targeting the Angiotensin II → AT1R pathway could provide a future treatment strategy for invasive epithelial ovarian cancer.

ENDOMETRIAL CANCER

Similarly to ovarian cancer, there are proofs for a possible influence of the local RAS concerning endometrial cancer. It has been published that in endometrial cancer, prognosis, tumor cell proliferation, and angiogenesis are affected by the RAS (82). According to the situation in the ovary, again increased local levels of Angiotensin II are associated with poorer prognosis in endometrial cancer patients (82, 83). This finding might be due to the fact, that those higher levels of Angiotensin II were found in patients with an advanced tumor stage (82). In this study, 81.9% were positive for Ang II and 59.6% positive for the AT1R. However, it seems as not only progression of disease but also an increased risk for developing endometrial cancer might be mediated by the RAS: a polymorphism of ACE has been described to be associated earlier onset of this disease (84). Again, the Angiotensin II → AT1R axis increases VEGF and thereby angiogenesis in a dose-dependent way (83). Recently, it has even speculated that Angiotensin II modulates the VEGF type-2 receptor KDR via AT1R (85). Inversely regarded, the connection of the RAS with angiogenesis is supported by the fact that low Angiotensin II activity is associated with less VEGF and a decreased MVD. This relation was basis for functional experiments: the treatment with the AT1R-blocker Losartan has a anti-proliferative effect in endometrial cancer tissue *in vitro* (86). In summary, high activity of the local RAS in endometrial cancer is associated with higher incidences, earlier onset, and increased rates of angiogenesis. The roles of Ang-(1–7) and the AT2R as well as clinical randomized study data is completely lacking and need to be further investigated.

CONCLUSION

This review summarizes the available literature concerning the local tissue RAS in the reproductive tract with regards to physiological and pathological clinical situations. The majority of the published studies remain on a non-functional descriptive level, but nevertheless, a role of the local tissue RAS as regulator in the human reproductive tract van be supposed. Obviously, the RAS affects oocyte maturation and quality, endometrial lining as well as hormone production and may therefore be considered as important system for regulation of physiologic pathways. Furthermore, the published data indicates a potential involvement of the local RAS in affecting physiological angiogenesis in the reproductive tract. Currently, pathologic conditions are better investigated than physiology. The Angiotensin II → AT1R pathway promotes tumor growth and angiogenesis in malignancies, arising new treatment strategies by inhibition of the AT1R. Data concerning stimulation of the antagonistic pathways such as the AT2R or Angiotensin (1–7) pathway as treatment modality for ovarian- or endometrial cancer is lacking. Due to current data, it is clear that most conclusions made are speculative since only a negligible number of functional studies have been conducted and clinical randomized data is missing completely. However, regarding a very complex and variable system of agonistic and antagonistic tissue factors, it may be hypothesized that the RAS in the female reproduction tract will be of increasing interest in the near future.

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Autophagy and the (pro)renin receptor

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The (pro)renin receptor (PRR) is a newly reported member of the renin-angiotensin system (RAS); a hormonal cascade responsible for regulating blood pressure. Originally, identification of PRR was heralded as the next drug target of the RAS, of which such therapies would have increased benefits against target-organ damage and hypertension. However, in the years since its discovery, several conditional knockout mouse models of PRR have demonstrated an essential role for this receptor unrelated to the RAS and blood pressure. Specific deletion of PRR in podocytes or cardiomyocytes resulted in the rapid onset of organ failure and subsequently animal mortality after only a matter of weeks. In both cell types, loss of PRR resulted in the intracellular accumulation of autophagosomes and misfolded proteins, indicating a disturbance in autophagy. In light of the fact that the majority of PRR is located intracellularly, this molecular function appears to be more relevant than its ability to bind to high, non-physiological concentrations of (pro)renin. This review will focus on the role of PRR in autophagy and its importance in maintaining cellular homeostasis. Understanding the link between PRR, autophagy and how its loss results in cell death will be essential for deciphering its role in physiology and pathology.

Keywords: renin-angiotensin system, cardiovascular disease, Wnt signaling, vacuolar H^+-ATPase, proteostasis, autophagosome, rapamycin

PRR AND THE RENIN-ANGIOTENSIN SYSTEM

The discovery of the (pro)renin receptor (PRR) by Ngyuen et al. (1) came at a time in the field of hypertension and cardiovascular disease when the search was on for a new therapeutic target of the renin-angiotensin-system (RAS). Normally, this hormone system is responsible for the regulation of blood pressure and water retention. Under pathological conditions, overactivity of the RAS results in the downstream accumulation of angiotensin (ang) II, an octapeptide which binds to specific receptors in target organs such as the kidney and heart. Pathologically high levels of ang II leads to hypertension and injury to target organs, resulting in disease (2). Numerous therapeutic interventions currently in use considerably reduce levels of ang II and are effective at lowering blood pressure (3). However, these drugs are not completely protective against target-organ damage, and as such, there is continued interest in the development of therapeutics against other components of the RAS which may have increased protection against disease.

The RAS is classically initiated by the proteolytic removal of the prosegment of prorenin to form active renin. Renin then starts the RAS cascade by cleaving angiotensinogen to form the decapeptide angiotensin I, which is further cleaved by the angiotensin-converting enzyme to form ang II. Upon discovery of PRR, an additional non-classical activation of prorenin was identified. Here, upon binding PRR, a conformational change in prorenin is thought to be induced, resulting in the removal of the prorenin prosegment from the active enzymatic cleft and hence, the non-proteolytic activation of prorenin into renin and initiation of the RAS cascade (1). This was the first study to show a molecular function for PRR and demonstrate its putative role in activation of the RAS. It should be noted however that the concentrations of prorenin used to observe this effect are considerably higher than those levels detected *in vivo* (4).

In addition to the activation of the RAS and generation of ang II, an alternative mechanism was proposed by which (pro)renin binding to the PRR directly contributes to disease. Human mesangial cells treated *in vitro* with recombinant human or rat renin showed an increase in transforming growth factor (TGF)-β and plasminogen activator inhibitor (PAI)-1 levels. In the presence of an ang II receptor blocker (ARB), the increase of TGF-β and PAI-1 was not affected, indicating that this result was independent of the RAS and ang II generation (5). In a human monocyte cell line, stimulation with recombinant renin in the presence of an ARB resulted in an activation of extracellular signal-related kinases (ERK) 1/2 (6). This again suggested that the increase in phospho-ERK 1/2 is directly due to the binding of prorenin to the PRR, and unrelated to the initiation of the RAS. Several other studies have also shown the activation of signaling pathways upon (pro)renin stimulation in an ang II independent manner (7–9). The identification of this second function for PRR led to the hypothesis that (pro)renin binding to the PRR causes pathology independently of the RAS *via* the induction of inflammatory and pro-fibrotic signaling cascades.

A MURKY PICTURE OF PRR FUNCTION EMERGES

THERAPEUTIC BLOCKADE OF PRR PREVENTS TARGET-ORGAN DAMAGE?

The aforementioned studies laid the framework for the development of inhibitors of PRR. To date, only one putative inhibitor of PRR has been published: the handle-region peptide (HRP). HRP is a short pentapeptide comprised from the prorenin prosegment (11P–15P). This putative inhibitor was developed

by Suzuki et al. who screened antibodies raised against various epitopes of prorenin that would induce the non-proteolytic activation of prorenin (10). They identified two regions of prorenin, the "handle" (11P–15P) and "gate" (15P–26R) from which they deduced that these two regions are most important in the non-proteolytic activation of prorenin. The "handle" region was decided by the authors to be the most exposed epitope of prorenin and thus more likely to directly interact with PRR (10).

Administration of this peptide *in vivo* showed protection in several animal models of disease, specifically in diabetic microvascular complications (8, 11–13) and in a rat model of spontaneous hypertension (14). The authors did not identify a precise concentration at which HRP was most effective. Several other groups attempted to reproduce these studies however they were unable to show any efficacy (6, 15–17). A study by Wilkinson-Berka et al. developed a sensitive radioimmunoassay to detect plasma levels of HRP. These authors were not able to detect HRP in the plasma of SD rats infused with 1 mg/kg/day of HRP by mini-pump for 7 days, indicating the rapid metabolism of the peptide (13). This raises the question as to whether HRP is able to effectively traffic through the body to specifically inhibit PRR at the target-organ of interest.

It is important to note that HRP was developed at a time in which high-resolution structural information on prorenin was not known. Morales et al. have since solved the structure of prorenin and provide some explanation for the discrepancy between the various HRP studies (18). Based on their structural information, they show that the "handle" region is not an exposed epitope, as originally thought, but instead buried in the prorenin molecule. This is supported by a recent study which has shown that HRP does not specifically bind PRR with high affinity (19). This is in agreement with a study done by us, where we demonstrate non-specific binding of fluorescently labeled HRP to cells lacking PRR (15).

OVEREXPRESSION OF PRR LEADS TO HYPERTENSION?

As described above, the contribution of PRR to cardiovascular disease and hypertension was hypothesized to be due to the binding of prorenin to PRR, resulting in its non-proteolytic activation and/or the induction of signaling pathways which directly lead to pathology. The development of transgenic animal models over-expressing PRR were expected to shed more light on this, as with more PRR available it was hypothesized that more binding of (pro)renin would occur and thus more of these pathogenic processes would occur. Unfortunately, these animal models did not give a clear indication as to the molecular contribution of PRR to cardiovascular disease. Rats constitutively over-expressing human PRR had normal blood pressure and ang II levels but developed renal nephropathy (20). In contrast, in a different model in which transgenic rats overexpressed PRR solely in smooth muscle cells, these animals had elevated systolic blood pressure but normal renal function (21). The differing results from these two models have been proposed to be due to differences in the uncontrolled overexpression of PRR in these animals (22).

GENETIC ABLATION OF PRR PREVENTS HYPERTENSION AND CARDIOVASCULAR DISEASE?

In consideration of the confusing results from the overexpression models and HRP studies, the field next turned to the development of knockout models to establish if loss of PRR would be protective against cardiovascular disease. The first attempt at generation of a PRR knockout mouse model was a failure. Injection of PRR knockout embryonic stem cells into host blastocysts did not generate chimeras, indicating an essential function of PRR in cellular development and survival (23). This is in contrast to other members of the RAS in which knockout mice have been successfully generated (24). Also of note is that renin expression begins well after PRR [in the mouse not until E14 (25)], again implying a non-RAS role for PRR. It is important to note here that when PRR was first discovered by Nguyen et al. it was shown to be identical to that of a protein called M8–9, a truncated protein of the vacuolar H^+-ATPase (V-ATPase) (1). As the V-ATPase is a multifunctional protein essential for cellular homeostasis and development (26), these initial PRR knockout reports gave great support to the notion that PRR has functions separate to the RAS. Several other animal models have confirmed that loss of PRR has a profound effect on development, including zebrafish (27), *Xenopus* (28, 29), and *Drosophila* (28, 30). PRR also appears to be important for human development, as it has been identified that humans with mutations in PRR have intellectual disabilities and epilepsy (31). However, this singular study needs to be reassessed and validated in light of the information now available from whole genome sequencing (32).

Due to the developmental effects described for PRR, an alternative approach at understanding the role of PRR in cardiovascular disease was undertaken by Kinouchi et al. (33). Here, a conditional knockout model was generated in which PRR was specifically deleted in cardiomyocytes ($ATP6AP2^{lox/Y}$; αMHC-Cre). These mice had a severe cardiac phenotype and died only 3 weeks after birth. Upon closer inspection of the PRR knockout cardiomyocytes, an accumulation of vesicular bodies was observed. Additionally, autophagosomes comprising of electron dense material were evident. By western blotting the authors show an accumulation of LC3B II and p62 in myocardial tissue from PRR knockout mice, indicating a disturbance in autophagic flux (33). In terms of the original question posed, this study did not give any insight as to how the binding of (pro)renin to PRR may contribute to cardiovascular disease. More recently, we and another group have generated podocyte-specific PRR knockout mice ($ATP6AP2^{lox/Y}$; Podocin-Cre) (34, 35). In these two studies, knockout mice had again severe phenotypes and mortality after only 3 weeks. Similarly to PRR knockout in cardiomyocytes, the study by our group also detected autophagosomes within these podocytes and identified alterations in levels of LC3B (35), confirming loss of PRR disturbs autophagic flux.

In summary, in the 11 years since its discovery the contribution of PRR to the pathogenesis of cardiovascular disease and hypertension remains unclear. However, one clear and striking result from the above-mentioned conditional knockout studies in cardiomyocytes and podocytes is that the loss of PRR results in a disturbance in cellular autophagic flux and homeostasis (33–35).

This is in addition to knockout models in other organisms where the loss of PRR disturbs cellular function and development. This is confirmed by the description of new tissue-specific PRR knockout model (ATP6AP2$^{lox/Y}$; Hoxb7-Cre), which also has severe developmental effects (36). Taken together, the regulation of autophagy and cellular homeostasis is thus looking more likely to be the true cellular function of PRR and is the focus of this review.

AUTOPHAGY

Autophagy is derived from the Greek names of *auto* "self" and *phagein* "to eat," and describes the process by which the cell literally eats itself. This is an essential and evolutionary conserved process utilized by all cell types to maintain cell homeostasis. Under normal conditions, a basal level of autophagy is required to maintain protein quality control and remove damaged proteins, organelles, and lipids, which may otherwise harm normal cellular function (37). With aging, the rate of basal autophagy is thought to decline and it has been proposed that this reduction in autophagic flux results in the accumulation of damaged proteins and the induction of neurodegenerative and cardiovascular diseases (38, 39). The level of basal autophagy has been shown to vary between different cell types with terminally differentiated cell types, such as neurons, having very high levels of autophagic flux (40).

Autophagy also has an essential role in response to conditions of cellular nutrient deprivation or starvation. Under these conditions, macroautophagy (hereafter referred to as autophagy) is induced as a way in which to replenish nutrients and prevent cell death, *via* the degradation of cellular proteins and organelles to generate amino acids. Due to its important role in maintaining cellular homeostasis the process of autophagy is extremely tightly regulated. This process involves multiple steps and the converging of several signaling pathways, requiring the coordinated action of literally hundreds of proteins. The specific details involved in the regulation, initiation, action, and resolvement of autophagy have been discussed extensively elsewhere (41, 42). For the purpose of this review, a brief summary is shown in **Figure 1**.

The induction of autophagy is controlled by an atypical serine kinase, the mammalian target of rapamycin (mTOR). Under normal conditions, where the cell is in a nutrient rich state or with a lack of stress signals, mTOR is active and inhibits autophagy. However, once in a nutrient-deprived or stressed state, the activity of mTOR is reduced/inhibited, and the process of autophagy is initiated.

Briefly, autophagy begins with the nucleation of the autophagosome membrane. Upon a decrease in mTOR activity, the autophagy-related protein (Atg) 13 is dephosphorylated, allowing it to form an activating complex with another "Atg" protein, Atg1. The assembly of this complex initiates the formation of the autophagosome membrane, driven by Beclin-1 and a phophoinositide-3 kinase (PI3K) complex (**Figure 1**). The membrane is then extended around the contents to be degraded. The extension of this membrane involves numerous other Atg proteins but the most important, due to its common use in histology and western blotting to detect autophagy, is the protein light-chain (LC) 3B (in yeast known as Atg8). LC3B is normally present in the cytoplasm (LC3B I) and it is only upon its post-translational modification, where a lipid group (phosphatidylethanolamine)

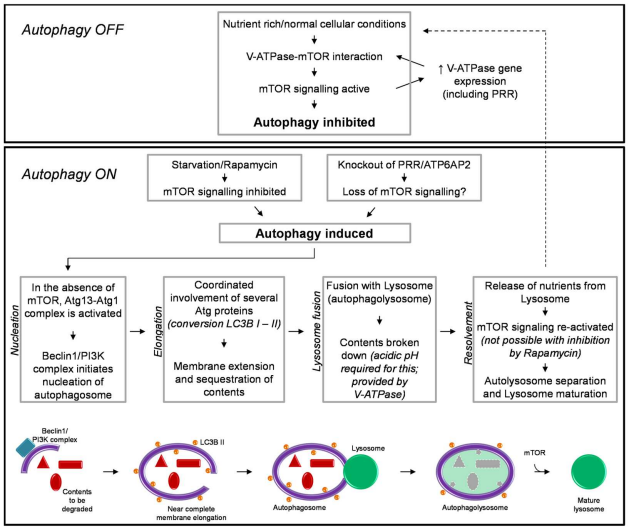

FIGURE 1 | A brief schematic of the regulatory processes involved in autophagy (see text for detail). Also shown is our hypothesized mechanism by which knockout of PRR/ATP6AP2 results in a loss of mTOR activity and the induction of autophagy.

is added (LC3B II) that it associates with the membrane of the autophagosome. This modification is commonly used as a marker for autophagy as the more hydrophobic LC3B II runs at a different molecular weight than LC3B I when analyzed by western blotting (43, 44). When the cellular contents to be degraded are completely enclosed by the autophagosome, the next step of autophagy involves the fusion of this body with a lysosome; thus forming an autophagolysosome. The lysosome is comprised of various proteases which require acidic pH for their activity. This pH is provided by the activity of the vacuolar H^+-ATPase (V-ATPase; discussed further in the next section). After degradation of its contents and the release of amino acids, the autophagolysosome separates and autophagy is attenuated. Importantly, the separation and maturation of lysosomes back to their normal state has been shown to also be regulated by mTOR (**Figure 1**) (45). Thus an important self-regulatory negative feedback mechanism is established in which after the restoration of cellular nutrients, mTOR is reactivated and both inhibits the induction of further autophagy and is involved in the resolvement of cellular homeostasis, *via* the removal of autophagosomes from the cell and promoting the maturation of lysosomes and return to their normal morphology (45).

Modulation of autophagy regulatory networks can have many different effects, depending on the level at which the autophagy pathway is inhibited, the cell type and/or disease in question in which such a modulation is utilized. Rapamycin is a chemical derived from *S. hygroscopicus* and is a potent inhibitor of mTOR activity and thus is an inducer of autophagy (46). Incubation of a number of different cell types with rapamycin results in the induction of autophagy and eventual death, due to a failure of the cells to attenuate and resolve autophagy (47–51). However, in some situations this can be of benefit. Rapamycin is currently approved

for clinical use to treat certain types of cancers such as breast, colorectal, and renal. Here, rapamycin potently inhibits cancer cell proliferation and is able to induce the death of cancerous cells and even inhibit angiogenesis (52). However rapamycin is not an effective drug for all clinical situations, as induction of autophagy can also have detrimental effects to the function of non-diseased cells (53). Thus for the use and further development of therapeutics such as this, a thorough understanding of the requirements of different cells for autophagy is crucial.

THE VACUOLAR H^+-ATPase AND ITS ROLE IN AUTOPHAGY

The "discovery" of PRR was eventually realized not to be the discovery of a *novo* protein after all. It is now well established that PRR is actually *ATP6AP2*; a gene product identified as an accessory protein of the V-ATPase (54). PRR has been shown to co-localize and immunoprecipitate with the V-ATPase, indicating a functional association (54, 55). In zebrafish, the loss of PRR results in a loss in pigmentation phenotype which directly mimics that of loss of a V-ATPase subunit (27). Additionally, the phenotype of the cardiomyocytes-specific PRR knockout model was attributed by the authors to a loss of V-ATPase function (33).

The V-ATPase is an essential multi-subunit complex present in nearly all cell types. It is responsible for establishing and maintaining intracellular pH gradients. Thus, its importance in maintaining cellular homeostasis is considerable. These duties range from the acidification of the lysosome, endocytosis, and recycling of membrane proteins, secretion and processing of hormones such as insulin, and basic cellular trafficking including the fusion of vesicular membranes (29, 56, 57).

It has recently been shown that the V-ATPase is important for the above-described mTOR signaling pathway, involved in regulating autophagy (**Figure 1**) (58). Addition of specific inhibitors of V-ATPase to the culture media of HEK293 cells inhibited the activity of mTOR. Additionally, immunoprecipitation studies identified an interaction between V-ATPase subunits and the Rag-Regulator complex, which interacts with mTORC1 to form the active mTOR signaling complex. This study proposed a mechanism by which the V-ATPase is thus crucial in maintaining mTOR activity by sensing the nutrient state of the cell and modulating the interaction between mTORC1 and the Rag-Regulator complex (58).

An important regulatory network between mTOR and the expression of V-ATPase subunits has also been identified (59). In a cell line with a genetically abnormally increased activity of mTOR, the expression of V-ATPase subunits was also increased. Of note is that PRR/ATP6AP2 was one such subunit identified to have increased expression under these circumstances. It was therefore concluded that mTOR regulates the expression of V-ATPase subunits (59). Considering the previous study by Zoncu et al. (58), this results in the development of a positive feedback loop whereas V-ATPase subunits are essential for maintaining mTOR activity and *vice versa* (**Figure 1**).

PRR: A PROTEIN IMPORTANT FOR REGULATING AUTOPHAGY?

The best insight into what is the precise contribution of PRR to autophagy comes from analysis of the recent studies by Riediger et al. (35) and Oshima et al. (34). These studies both generated mice with PRR specifically deleted in podocytes; a specialized cell of the kidney. As mentioned briefly above, loss of PRR in this cell type resulted in animal mortality approximately 3 weeks after birth (**Table 1**) (34, 35). The cause of this severe phenotype was due to the animals developing nephritic syndrome and acute kidney injury; identified by proteinuria, glomerulosclerosis, and the accumulation of proteaceous casts in tubules. Upon inspection by transmission electron microscopy, the accumulation of vesicles was observed in addition to the presence of large autophagosomes, also after only 3 weeks. Additionally, an accumulation of LC3B was detected, indicating that the deletion of PRR in podocytes results in a disturbance in autophagic flux (34, 35). This mirrors what Kinouchi et al. observed in their cardiomyocyte-specific PRR knockout model, as described above (33). Eventually this disturbed autophagic flux led to podocyte death, as indicated by a decrease in Wilms tumor-1 signal (34, 35). This did not correlate with the activation of apoptotic pathways, indicating that the gross disturbance in autophagy was the main cause of cell loss (35).

Two separate conditional knockout studies in the podocyte have been generated to specifically investigate the role of autophagy in this cell type (**Table 1**). Atg5 is a protein important for the elongation of the autophagosome membrane and sequestration of contents (**Figure 1**). In contrast to PRR, conditional deletion of *ATG5* in podocytes resulted in severe kidney disease only after 24 months, with no animal mortality at this time (60). The presence of autophagosomes within Atg5 knockout podocytes was evident only after 8–10 months (**Table 1**). In this study, the authors proposed that loss of Atg5 results in a gradual decrease in the cells ability to remove unwanted and damaged cellular material (60). In this case, the late onset of disease can be attributed to the cells lacking a functioning *basal* autophagy and so, first a certain threshold of cellular stress must be reached (i.e., by aging) before the phenotype is evident.

In clear contrast to this is the study by Cina et al. who generated podocyte-specific *mTOR* knockout mice (61). Here, these mice developed proteinuria, glomerulosclerosis, and other hallmarks of acute kidney injury after only 3 weeks. Like both the *PRR* and *ATG5* knockout studies, autophagosomes were detected within the podocytes, however, more similar to the PRR knockout model, these were evident after only 2 weeks (**Table 1**). The authors propose in this study that the loss of mTOR in podocytes has a twofold effect (61). Firstly, loss of mTOR results in the induction of autophagy, as indicated by the presence of autophagosomes and accumulation of LC3B II. Secondly, the authors demonstrate that loss of mTOR results in a failure of negative feedback loops to stop the induction of further autophagy and resolve this process. Hence, they propose that the severe and acute nephritic syndrome in mTOR podocyte-specific knockout mice is due to the disruption of the autophagic cycle at two points: induction and resolvement (**Figure 1**).

What is striking is the similarity in the severity of the phenotype observed between the conditional knockout of PRR and mTOR in these podocyte studies (**Table 1**). This strongly suggests that PRR is important in mTOR function, either due to a specific interaction or indirectly *via* its association with the

Table 1 | Comparison of the time of onset of various parameters in PRR and autophagy-related podocyte-specific knockout mouse models.

	(Pro)renin receptor (34, 35)	Atg5 (60)	mTOR (61)
Genotype	ATP6AP2$^{lox/Y}$; Pod-Cre	ATG5$^{flox/flox}$; Pod-Cre	mTOR$^{flox/del}$; Pod-Cre
Mortality	3 weeks	No effect (mice live >24 months)	Not analyzed
Proteinuria (albumin/creatinine)	2 weeks	Mild at 8–12 months, severe at 20–24 months	3 weeks
Glomerulosclerosis	2 weeks	24 months	4 weeks
Proteinaceous casts in tubules	2 weeks	24 months	2–4 weeks
Podocyte number	Decreased at 2 weeks	Decreased at 22 months	Not analyzed
Podocin expression	Decreased at 3 weeks (not analyzed by Riediger et al.)	No change at 24 months	Decreased at 3 weeks
Podocyte foot effacement	2 weeks	24 months	3 weeks
Autophagosome formation within podocytes	2 weeks	8–12 months	2 weeks
Alteration in LC3B processing	Accumulation of LC3B positive cells (immunofluorescence)	Not analyzed	Increased LC3B II conversion (western blot)

V-ATPase (58). In support of this concept is that ubiquitous mTOR knockout mouse models also have similarities to that of PRR, where mTOR knockout embryonic stem cells have limited proliferation resulting in early lethality (E5.5), indicating an essential role for mTOR in cellular development (62, 63). The comparison of future PRR and mTOR conditional knockout models in other cell types will give more insight into the molecular mechanism by which PRR is important for mTOR activity.

WHERE TO NEXT?

There is now clear evidence that PRR has an essential role in maintaining cellular homeostasis, specifically due to its involvement in autophagy. This will undoubtedly result in shift in research focus away from the contribution of PRR to cardiovascular disease, toward understanding its general role in the biology, homeostasis, proliferation, and development of all cell types.

It must be acknowledged that this paradigm change was initiated by the study by Cruciat et al. (29). This group essentially stumbled across PRR as being important in canonical Wnt signaling as part of their large research study to identify new genes of importance to this signaling pathway. In this study, they showed a clear link between the association of PRR with the V-ATPase and the activation of protein receptors important for the induction of Wnt signaling (29). It has also been established in *Drosophila* that PRR is important for another Wnt signaling pathway, the planar-cell polarity (PCP) pathway (30). More recently, this group has dissected the mechanism by which PRR is important for PCP signaling. Here, loss of PRR affected the co-localization and endocytosis of receptors important for PCP, and resulted in defects in the degradation of other receptors such as Notch and E-Cadherin (64). As discussed in this review, it also appears likely that PRR has an important role in the signaling pathways important for regulating autophagy. We therefore propose that PRR is essential for proteostasis, where the loss of this protein results in the disturbance of multiple signaling pathways, resulting in severe defects in cellular homeostasis. This could be due to its role in regulating autophagy, in which loss of PRR results in the disturbance of multiple signaling pathways, due to the induction of autophagy and lack of resolvement of this process, resulting in eventual cellular death. It is also possible, as with the study described by Cruciat et al. that PRR specifically interacts with proteins important for signal transduction. Of note is that the signaling pathways of Wnt and autophagy are closely intertwined (65). Deciding which of these two hypotheses is correct will be made easier once the precise molecular mechanism by which PRR contributes to V-ATPase activity is determined. This is a particularly important concept to understand in light of the fact that lower organisms which lack PRR (e.g., yeast) still have a functioning V-ATPase (33). The answers to these questions will lead to new insight into the regulatory pathways essential for maintaining cellular homeostasis.

CONCLUSION

It is now clear that PRR has an important role in the regulation and maintenance of cellular homeostasis, most probably *via* signaling pathways important for autophagy. In consideration of this new information, it would be interesting to revisit the original studies describing the involvement of PRR with the RAS and regulation of blood pressure, to distinguish the role of V-ATPase function, and autophagy in these systems. More recent papers, which have identified a role for PRR in various signaling pathways, should also be carefully re-examined to deduce whether these effects are directly mediated by PRR, or indirectly by the gross disturbance of cellular homeostasis.

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Contribution of the local RAS to hematopoietic function: a novel therapeutic target

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The renin-angiotensin system (RAS) has long been a known endocrine system that is involved in regulation of blood pressure and fluid balance. Over the last two decades, evidence has accrued that shows that there are local RAS that can affect cellular activity, tissue injury, and tissue regeneration. There are locally active ligand peptides, mediators, receptors, and signaling pathways of the RAS in the bone marrow (BM). This system is fundamentally involved and controls the essential steps of primitive and definitive blood-cell production. Hematopoiesis, erythropoiesis, myelopoiesis, thrombopoiesis, formation of monocytic and lymphocytic lineages, as well as stromal elements are regulated by the local BM RAS. The expression of a local BM RAS has been shown in very early, primitive embryonic hematopoiesis. Angiotensin-converting enzyme (ACE-1, CD143) is expressed on the surface of hemangioblasts and isolation of the CD143 positive cells allows for recovery of all hemangioblast activity, the first endothelial and hematopoietic cells, forming the marrow cavity in the embryo. CD143 expression also marks long-term blood-forming CD34+ BM cells. Expression of receptors of the RAS is modified in the BM with cellular maturation and by injury. Ligation of the receptors of the RAS has been shown to modify the status of the BM resulting in accelerated hematopoiesis after injury. The aim of the present review is to outline the known functions of the local BM RAS within the context of primitive and definitive hematopoiesis as well as modification of BM recovery by administration of exogenous ligands of the RAS. Targeting the actions of local RAS molecules could represent a valuable therapeutic option for the management of BM recovery after injury as well as neoplastic disorders.

Keywords: renin-angiotensin system, bone marrow, stem cells, angiotensin 1-7, CD143, hematopoiesis, myelosuppression

INTRODUCTION

The first evidence that there are effects of the renin-angiotensin system (RAS) on bone marrow (BM) and hematopoiesis resulted from clinical use of therapeutics that modify the production/action of angiotensin II (A-II). From the early 1980s, studies showed that there was a small reduction in hematocrit in patients receiving angiotensin-converting enzyme (ACE) inhibitors, particularly with enalapril (1). Further, enalapril use was associated with anemia in renal transplant patients as well as normal rats (2, 3). Prior to the use of Epogen to treat anemia in patients on long-term dialysis, the majority of patients receiving captopril developed dose dependent anemia that was reversed upon discontinuation of captopril treatment (4). Erythrocytosis occurs after renal transplantation. In these patients, there are anecdotal reports and studies showed that ACE inhibitors, and angiotensin receptor blockers (ARBs) reduce hematocrit levels in post-transplantation erythrocytosis (5–7). In two prospective studies, a reduction in hemoglobin concentrations was reported in hypertensive patients treated with ARBs compared with patients treated with β-blockers or calcium antagonists (8, 9). These observations led to the hypothesis in the mid-1990s that there is a local RAS in the BM that is involved in the regulation of hematopoiesis. These early observations were thought to be the result of increased levels of an inhibitor of hematopoiesis, the acetylated tetrapeptide AcSKPD, that is hydrolyzed by ACE or due to interactions of the RAS directly with the hematopoietic system. Activation of the RAS was shown to enhance erythropoietin production (10). Similarly, administration of ACE inhibitors reduces plasma erythropoietin levels, exacerbating anemia (11). Administration of A-II to patients after hemorrhage leads to an increase in plasma erythropoietin levels (12). This work was recently reviewed by Vlahakos (13). All of these studies suggested that the RAS directly modifies erythropoiesis.

THE RENIN-ANGIOTENSIN SYSTEM IN BONE MARROW AND HEMATOPOIESIS

RAS COMPONENTS AND RAS KNOCKOUT MICE

Research over the last few decades has confirmed the presence of a local, integrated RAS within several tissues. Every known component of the RAS is contained within BM cells, including mRNA for angiotensinogen, renin, ACE, AT_{1a}, AT_2, Mas, and ACE_2 [(14, 15), **Figure 1**]. Transgenic mice carrying both human renin

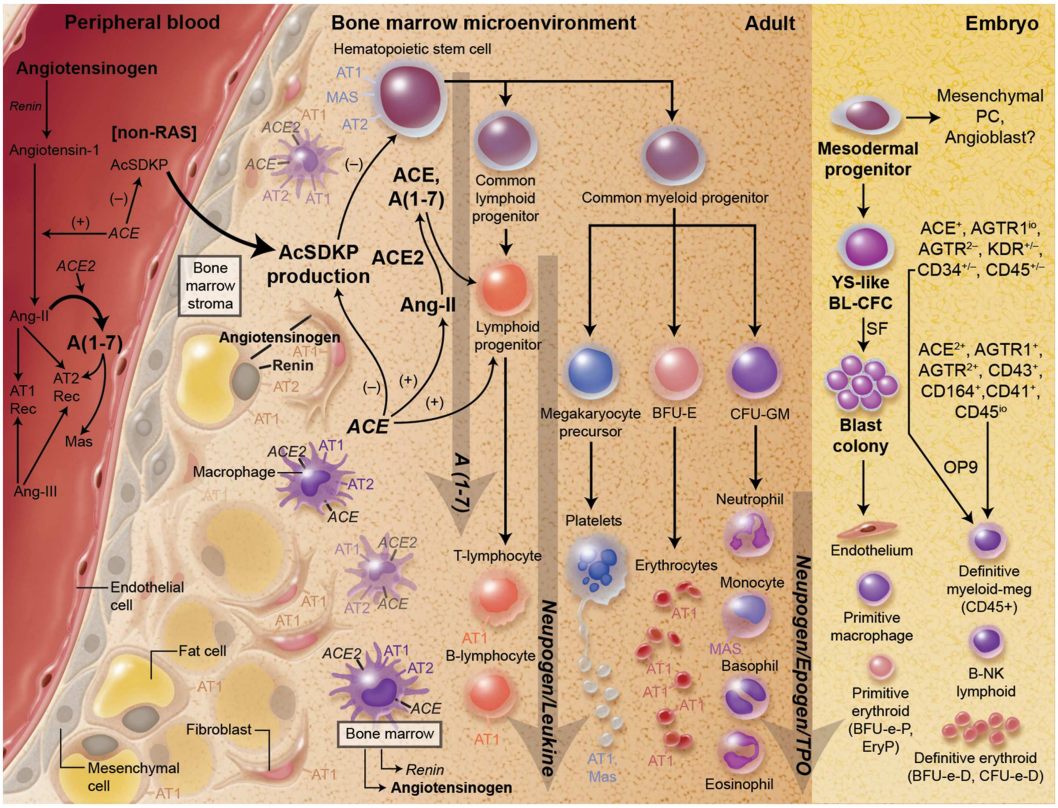

FIGURE 1 | There is substantial evidence for a key role for the RAS both primitive and definitive hematopoiesis as well as the development of hematopoietic progenitor cells. Every component of the RAS is present in the local environment of the bone marrow.

and angiotensinogen genes have increased hematocrits in animals intact for AT1, but not AT1 null animals (16). Further, mice that are deficient in individual components of the RAS have been shown to have impairment of hematopoiesis. Direct evidence for the role of RAS in hematopoiesis independent of ACE hydrolysis of AcSDKP came from animal models (ACE Knock out (KO)) and *in vitro* studies. In one strain of ACE-KO mice, there were increases in circulating levels of AcSDKP that was accompanied by a 35 mm Hg decrease in blood pressure, renal insufficiency, and unexpected normocytic anemia associated with an increase in circulating erythropoietin in response to anemia (17). A similar anemia was also present in another strain of genetically engineered mice expressing a truncated form of secreted ACE (18). In these mice, plasma ACE activity was reduced without evidence of renal insufficiency indicating that the anemia was not the consequence of the renal failure, but the result of a reduction in red-cell mass. The degree of anemia in these two mouse strains was similar, despite a significant difference between plasma AcSDKP levels. This finding again suggests that AcSDKP was not the primary cause of anemia. To evaluate the role of A-II in hematopoiesis, hematocrit was measured before and after A-II infusion for 2 weeks. The hematocrit level was corrected in ACE-deficient mice to near wild-type levels, strongly suggesting that the lack of A-II in these mice was the direct cause of the anemia. Further, several studies have shown that A-II, in the presence of erythropoietin, will increase erythroid progenitors *in vitro* (19–21).

In another strain of ACE-KO mice, abnormalities in myelopoiesis were seen. These abnormalities were characterized by increased BM myeloblasts and myelocytes, as well as extramedullary myelopoiesis (22). Further, neutrophils (banded and segmented) and erythroid elements were reduced approximately one third in the BM. Increases in splenic CD11b+ Gr1dim cells in these mice together with the increase in BM myelocytes indicate a block in myeloid differentiation at the post-GMP stage of development. A-II, through AT1, stimulates myeloid differentiation and function. In these mice, plasma levels of A-II were decreased by approximately 10-fold. Part of these effects was thought to be due to up-regulation of C/EBPα, a central transcription factor of myelopoiesis. Macrophages derived from ACE-KO mice had depressed C/EBPα expression and treatment with A-II restored expression of this transcription factor (23). Function of the myeloid cells that did develop in these mice was also impaired (22). Peritoneal macrophages from ACE-KO mice were deficient in the production of effector molecules, such as tumor necrosis factor-α, interleukin-12p40, and CD86 when stimulated with lipopolysaccharide and interferon-γ. ACE-KO mice were more susceptible to *Staphylococcus aureus* infection showing a reduction in host resistance.

RAS IN HEMATOPOIESIS

Observations in KO mice provides evidence that points to a significant role for the RAS in the regulation of hematopoiesis and the development of hematopoietic progenitor cells (23–25). The central role of the RAS in regulating early hematopoiesis has been the focus of several reviews (23, 26–28). In addition to reduced/delayed hematopoiesis in ACE-KO mice, is the observation that expression of ACE demarcates early events in hematopoiesis both in the fetus and the adult. The earliest marker for the isolation of hemangioblasts, hematopoietic stem cells, and epidermal stem cells is ACE_1 (CD143) (26, 29–31). ACE_1 was also shown to be expressed in all presumptive and developing blood-forming tissues of the human embryo and fetus: para-aortic splanchnopleura, yolk sac, aorta-gonad-mesonephros, liver, and BM (32). This is expanded upon further below.

The role of ACE and A-II in adult hematopoiesis led to investigations of the possible role of the RAS in primitive hematopoiesis. The first studies were done in avian models. In these models, primitive hematopoiesis occurs prior to intra-embryonic hematopoiesis (33). Extra-embryonic blood islands differentiate into endothelial and hematopoietic cells (34). ACE gene expression and protein was detected in the yolk-sac endoderm before the beginning of blood-island differentiation, when the circulation is not yet established between the yolk sac and the embryo (35). At 30 h development in the avian model, the other components of the RAS, renin and angiotensinogen, were present in the vicinity of the blood islands, which strongly suggests a role for the RAS in erythropoiesis. Administration of ACE inhibitors or ARBs to 2-day-old embryos resulted in a significantly lower hematocrit in treated embryos than in control embryos. These results show that the RAS modulates blood-island differentiation during the primitive yolk-sac erythropoiesis (35). Since these observations, studies were done to evaluate the expression of the RAS components in mammalian embryos, especially in humans. These studies confirm a role for RAS in mammalian primitive hematopoiesis.

Studies in mammalian hematopoiesis were facilitated by the development of the monoclonal antibody, BB9. BB9 is specific for the somatic isoform of surface ACE (CD143). As outlined above, the first observation was that a protein immunologically similar to ACE is expressed in the embryonic para-aortic splanchnopleura, where blood-cell progenitors are generated (36), suggesting that a local RAS exists within the intra-embryonic sites of definitive hematopoiesis in the mammal. ACE expression was also identified emerging hematopoietic cells from both $CD34^-$ and $CD34^+$ areas of human yolk sac, intra-embryonic splanchnopleura, and hemogenic endothelium of the aorta-gonad-mesonephros region and fetal liver (FL) (29). The embryonic pattern of human ACE expression is consistent with a dorsal emigration of ACE^+CD34^- hemangioblasts from the para-aortic splanchnopleura, and subsequent colonization of the ventral aspect of the dorsal aorta to give rise to $CD34^+$ hemogenic endothelial cells. Using BB9, a primitive subset of $CD34^+$ multi-lineage hematopoietic stem cell that could engraft NOD SCID mice was identified in adult BM, mobilized peripheral blood, and umbilical cord blood (37).

In 2008, Zambidis and others reported that ACE is a novel marker for identifying hemangioblasts differentiating from human embryonic stem cells (hESC). Cells developed from hESC that expressed ACE ($ACE^+CD45^-CD34^\pm$) were hemangioblasts that were progenitors for not only endothelium but also both primitive and definitive human lymphohematopoietic cells. Thrombopoietin and basic fibroblast growth factor were identified as critical factors for the proliferation of human hemangioblasts. Furthermore, ACE and receptors for A-II, AT1, and AT2 directly regulated hemangioblast expansion and differentiation. ACE enzymatic activity was required for hemangioblast expansion. Further, differentiation toward either endothelium or multipotent hematopoietic progenitors is modified by exposure to AT1 and AT2 antagonists. In this study, AT2 function was necessary for expansion of hemangioblast colonies into multipotent hematopoietic progenitors; whereas blockade of AT2 by PD 123319 abolished hematopoietic differentiation and sent the hemangioblasts toward an endothelial lineage. Conversely, inhibition of AT1 by losartan augmented differentiation of hemangioblast colonies into multipotent hematopoietic progenitors.

The influence of modification of the RAS in mesenchymal stem cells (MSC) has also been evaluated (38). Expression of the AT1 receptor in MSC was initially observed in 2000 (21). MSC can be differentiated into a number of cell types, including those involved in fibrosis and adipogenesis. Differentiation of human MSCs into adipocytes resulted in increased expression of renin and AT2 and a decrease in angiotensinogen and ACE expression with a net increase in endogenous cellular A-II production. Incubation of MSC with A-II with and without an AT1 antagonist inhibited adipogenesis, whereas A-II and an AT2 antagonist abolished the inhibition of adipogenesis. MSC can also be differentiated into renin-producing cells in the kidney, juxtaglomerular cells (39, 40). Increased numbers or activation of these cells is important in the initiation of pathological effects of chronic overexpression of A-II through increase production of renin, the rate-limiting enzyme in the production of A-II.

RAS: A NOVEL TARGET IN BONE MARROW INJURY AND MYELOSUPPRESSION

Multi-lineage suppression of BM progenitors occurs following myelosuppressive chemotherapy, as well as radiation, resulting in cytopenias of their formed elements in the peripheral circulation. Myelosuppression and the more severe myeloablation (requiring hematopoietic stem cell support for recovery) can be the result of exposure to chemotherapy or radiation therapy for neoplastic disease, to diagnostic radiation exposure or due to radiation exposure as a result of nuclear accident or terrorism. The manifestations of myelosuppression include anemia, thrombocytopenia, lymphopenia, and neutropenia. In the setting of chemo or radiation therapy, myelosuppression is often managed with a delay and/or a dose reduction in the next scheduled cycle of chemotherapy, to allow recovery of BM and circulating formed elements. However, such modifications to the chemotherapy regimen result in lower relative dose intensity (the ratio of delivered dose intensity to planned dose intensity). Numerous studies, particularly in breast cancer and NHL, have established that long-term survival may be compromised if the total dose or relative dose intensity falls below a threshold value (41–45).

Prolonged or severe myelotoxic effects may reflect a diminished hematopoietic reserve, which may occur with aging, age-related comorbidity, intensive chemotherapy, combined radiation therapy/radiation therapy, or previous exposures to myelosuppressive therapies. Therefore the risk of myelosuppression leading to modifications of chemotherapy is higher in older patients and patients with recurrent neoplasms. Even when the chemotherapy regimen is relatively benign and myelotoxicity is limited, elderly patients tend to be more vulnerable than younger patients (46).

The most widely utilized hematopoietic stimulants (erythropoietin, filgrastim, or sargramostim) act on later stage precursors and usually induce proliferation, differentiation, and mobilization of single cell lineages from the BM into the peripheral circulation. For this reason, they do not individually impact chronic and progressive multi-lineage cytopenias that commonly occur after myelosuppression. This finding suggests that a treatment which stimulates proliferation and differentiation of hematopoietic stem/progenitor cells should reduce incidence of clinically significant cytopenias.

ANGIOTENSIN 1-7 AS A THERAPEUTIC IN BONE MARROW RECOVERY

Because of the increased sensitivity of immature cells compared with more mature cells of a given cellular lineage to the proliferative effects of angiotensin peptides, therapeutic opportunities exist to enhance tissue regeneration, particularly the repair of injuries in the BM associated with chemotherapy and radiation. Potential populations include cancer patients receiving antineoplastic or radiation therapy with myelosuppressive side effects, stem cell transplant patients after myeloablative conditioning, patients with conditions resulting in ineffective myelopoiesis and apoptosis of hematopoietic progenitors and individuals exposed to nuclear radiation.

Peptides of the RAS are potent stimulators of progenitor cell proliferation (21, 24, 47–49). RAS receptors are increased after injury (50–53). Preclinical studies have shown that hematopoietic progenitor cells are sensitive to Angiotensin 1-7 [A(1-7)] stimulation and the effect of this biologically active member of the endogenous protective arm of the RAS *in vivo* is most robust after injury (24, 47–49, 54). A(1-7) has multi-lineage effects on hematopoietic progenitors *in vitro* and *in vivo* (24, 48, 52, 54–56) and is undergoing clinical development for the treatment of myelosuppression and to increase hematopoietic stem cell transplantation.

Angiotensin 1-7 treatment following 5-fluorouracil (5FU) administration resulted in a higher number of progenitors in the myeloid, megakaryocyte, and erythroid lineage in murine BM (48). However, the most extensive preclinical data set with A(1-7) is following myelosuppression due to total body irradiation (TBI) (24, 47). An early increase and sustained expansion in early mixed, myeloid, erythroid, and megakaryocytic progenitors was observed in A(1-7)-treated animals compared to controls. At 30 days after TBI, A(1-7) treatment increased early mixed progenitors (three- to fivefold), megakaryocyte (two- to threefold), myeloid (three- to sixfold), and erythroid (two- to fivefold) progenitors in the BM and reduced radiation-induced thrombocytopenia (RIT) (up to twofold). Consistent with clinical results below, it is important to initiate treatment with this peptide once the damage resulting from the myelotoxic exposure has ceased. For example, improvement in BM progenitors following TBI were better at higher doses of A(1-7) when drug was initiated at 48 h post-TBI as opposed to 24 h post-TBI.

In the A(1-7)-treated animals, the nadir in BM progenitors was not as low as in the control animals and accelerated recovery was observed. The multi-lineage BM response resulted in platelet and white blood-cell recovery after TBI (24, 47). Initiation of A(1-7) treatment could be delayed up to 5 days following TBI with continued improvement of hematopoietic recovery both in the BM and formed elements in the peripheral blood (24).

It is hypothesized that A(1-7) acts to stimulate hematopoiesis through the Mas receptor. The expression of Mas in normal BM is low. However, as with injuries to other tissues, Mas expression in hematopoietic progenitors in the BM is increased by myelosuppression (52). The ability of A(1-7) to accelerate BM recovery was blocked by administration of the Mas antagonist, A779; whereas losartan, an antagonist of AT1 (the constitutively expressed receptor for A-II) did not (24, 55). In order to ascertain the receptor that A(1-7) acts through to stimulate hematopoietic recovery, antagonists of the type I receptor (losartan), type 2 receptor (PD123319), or Mas receptor were co-administered with A(1-7). Administration of the antagonists had no effect on the recovery of platelets while the Mas antagonist blocked the acceleration of platelet recovery by A(1-7) (55). Of interest, RAS receptors, AT1, AT2, and Mas, are G coupled protein receptors that are capable of distinguishing small changes in peptide sequence and provide novel targets for modulation of hematopoiesis.

The kinetics of changes in hematopoietic progenitors in BM with A(1-7) treatment after TBI were evaluated. There was an early increase (up to fivefold by Day 7) in myeloid and erythroid progenitors that continued to expand more rapidly than in control animals through Day 14. The number of megakaryocytes in the BM was measured by CD41+ (platelet glycoprotein IIb of IIb/IIIa complex) expression. In contrast to myeloid and erythroid progenitors, the nadir for megakaryocyte number after TBI was Day 8. In control animals, recovery did not start until Day 14 and plateaued at Day 22. In the A(1-7)-treated animals, the nadir was not as low as the control animals and recovery was seen at Day 10. By Day 14, the megakaryocyte number doubled in the treated animals and was comparable to that observed in the control animals at Day 22. After Day 22 recovery in the control animals reached a plateau, and recovery of the number of megakaryocytes continued through Day 30 (the last time point measured) in the animals treated with A(1-7). Changes were also observed in thrombopoietin, a key regulator in platelet generation, production, and utilization that are consistent with these observations. At the nadir of platelet levels, there was increased utilization of thrombopoietin. However, later, when megakaryocyte levels and maturity were comparable to non-irradiated controls in A(1-7)-treated animals, there was an increase in circulating thrombopoietin levels. These data suggest that the primary action of A(1-7) is at the level of the progenitor cell.

In all studies with repeat bleeding, a nadir was found in platelet number after TBI even with A(1-7) treatment. However, the nadir was diminished at an early time point. It is hypothesized that the mechanism by which this occurs is A(1-7) reducing consumption of platelets as well as increasing their production. Platelet

consumption occurs through bleeding at sites of injury due to radiation (such the gastrointestinal tract or the cerebral cortex) or through the formation of thrombus (in part due to endothelial dysfunction). As will be shown below, A(1-7) reduced mucosal injury. Further, A(1-7) reduces oxidative stress after injury, which would contribute to endothelial dysfunction.

SYNERGY AND MULTI-LINEAGE EFFECTS AFTER COMBINING A(1-7) WITH COLONY STIMULATING FACTORS

Combining A(1-7) with commonly used growth factors [filgrastim (Neupogen®), and erythropoietin (Epogen®)], in C57Bl/6 mice post-chemotherapy increased the numbers of BM progenitor cells and formed elements in the peripheral circulation (52, 57). A(1-7) administered in combination with suboptimal doses of Neupogen® throughout the post-myelosuppressive interval increased the number of progenitors and circulating WBC concentration to a greater extent than occurred with either drug alone (52). These studies indicate that the A(1-7) effects on progenitors can enhance the concentration of formed elements in the peripheral blood in the presence of appropriate differentiating agents.

Administration of A(1-7) with filgrastim (the latter given only 3 days starting at the white blood-cell nadir) decreased 10-fold the amount of filgrastim needed for optimal recovery of BM progenitors (CFU-GEMM, CFU-GM, CFU-Meg, and BFU-E) and circulating formed elements (WBC, platelets). In addition to the synergistic effects of combining A(1-7) and Neupogen on white blood-cell and neutrophil recovery, combining these therapies increased platelet concentrations above those observed with A(1-7) alone.

In addition, combination of A(1-7) with erythropoietin slightly increased (not significantly) red blood-cell concentrations above those achieved by erythropoietin alone. However, in this model, A(1-7) or A(1-7) in combination with erythropoietin increased all erythroid progenitors with the largest effect on early erythroid progenitors (immature BFU-E). As before with Neupogen, combining A(1-7) with Epogen has hematological effects outside of the erythroid lineage in that the concentration of circulating neutrophils was increased with this combination. In conclusion, filgrastim and erythropoietin acted synergistically with A(1-7) to increase the concentration of myeloid, megakaryocytic, and erythroid progenitor cells in the BM following chemotherapy, suggesting that A(1-7)'s multi-lineage effect on early progenitors in the marrow facilitates proliferation in response to lineage specific growth factors.

CLINICAL DEVELOPMENT OF A(1-7) FOR HEMATOPOIESIS

Multi-lineage suppression of marrow precursors occurs following myelosuppressive radiotherapy and chemotherapy resulting in cytopenias of one or more of the mature formed elements of blood. As described above, preclinical studies of A(1-7) demonstrated an increase in multiple lineages of early hematopoietic precursors from the BM and the peripheral blood of mice, and *in vitro* exposure of cells from human cord blood.

A Phase I/IIa prospective, blinded, randomized, dose-escalation study of a clinical formulation of A(1-7) was conducted in breast cancer subjects receiving three cycles of adjuvant doxorubicin and cyclophosphamide following surgical tumor reduction (54). The study compared the effects of up to 100 μg/kg of A(1-7) to filgrastim ($n = 5$) as a comparator arm for safety and response variables. A(1-7) was found to be safe and was well-tolerated. No dose-limiting toxicity was observed following subcutaneous administration of up to 100 μg/kg of A(1-7) for periods of up to 14 days. No A(1-7)-treated patients experienced any NCI Grade 1–4 platelet reductions compared to 60% of the controls. Further, patients had a lower incidence of lymphopenia, anemia, and mucositis. Additionally, following the completion of the third cycle of chemotherapy, recovery of hemoglobin, lymphocytes, leukocytes, neutrophils, and platelets was superior in A(1-7)-treated subjects. A(1-7) also reduced filgrastim use, as well as development of mucositis. Further, anemia was reduced even though two of the five control patients and one of 15 treated patients [in the lowest A(1-7) dose group] received erythropoietin for anemia. There were no A(1-7) treatment related serious adverse events (SAE) reported in this study.

A Phase 2b study evaluating the safety and efficacy of a clinical formulation of A(1-7) in reducing the incidence and severity of thrombocytopenia in subjects receiving a combination of gemcitabine and platinum therapy for ovarian carcinoma for six cycles was conducted. The primary endpoint of this study was the severity and incidence of thrombocytopenia as determined by the number of chemotherapy cycles during which the platelet count was below 50,000/mm^3. A significant reduction of Grade 4 thrombocytopenia was seen in the 100 μg/kg group (56). In addition, there was a significant increase in the maximal percent increase in platelet count and delivery of scheduled chemotherapy dose on time in subjects treated with 100 μg/kg/day versus placebo-treated control. Maintenance of the scheduled chemotherapy at the desired dose has been linked with improved tumor control and long-term survival. No dose-limiting toxicity was observed during the course of the study and no investigational product-related SAEs or deaths occurred.

Benefit: data from clinical studies confirm and extend the results seen in preclinical models. These findings are consistent with the species homology of A(1-7). Overall, the observed increase in sensitivity of immature stem/early progenitor cells to the proliferative and regenerative effects of angiotensin peptides offers unique therapeutic opportunities including significantly enhanced hematopoietic recovery after chemotherapy as well as the potential to facilitate BM regeneration.

CONCLUSION

A central role for the RAS in BM development and recovery has been the focus of two decades of research. ACE_1 is a pivotal component in hematopoiesis in that expression marks early cells involved in primitive and definitive hematopoiesis. Reduction of ACE activity either through ACE inhibitors or ACE-KO results in abnormal hematopoiesis, particularly in the erythroid and myeloid lineages. While all components of the RAS are expressed in BM cells, fetal receptors of RAS, such as AT2, are expressed only on very early progenitors and are down regulated during differentiation. However, injury to the BM or myelosuppression modified RAS expression and up-regulates receptors of the protective RAS, Mas, and AT2.

Preclinical studies of A(1-7) suggest a potential to increase multiple lineages of early hematopoietic precursors cultured from the BM and the peripheral blood of mice. These studies have shown an increase in the number of progenitors and formed elements in the peripheral blood after treatment with A(1-7). Studies in murine models show that A(1-7) prevents or mitigates thrombocytopenia following myelotoxic chemotherapy. This benefit is supported by clinical data from multiple trials which shows decreased incidence of thrombocytopenia and increased BM recovery in A(1-7) treated patients following myelotoxic chemotherapy. Additional preclinical data in TBI models show that A(1-7) treatment also prevents or mitigates thrombocytopenia even when treatment initiation is delayed up to 48 h post exposure. It is anticipated that demonstration of the benefit of A(1-7) administration in these models will be translatable to humans for the indication of mitigating thrombocytopenia following TBI.

Clinical studies also suggest that exposure to A(1-7) maintains the pre-chemotherapy health of the BM by restoring the various hematopoietic parameters to baseline values and allows the maintenance of chemotherapy dose intensity (54, 56). This consistent A(1-7)-mediated return to baseline hematopoietic values may be due to an increase in the number of hematopoietic progenitor cells thereby pharmacologically replenishing the hematopoietic system.

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A role for the brain RAS in Alzheimer's and Parkinson's diseases

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The brain renin-angiotensin system (RAS) has available the necessary functional components to produce the active ligands angiotensins II (AngII), angiotensin III, angiotensins (IV), angiotensin (1–7), and angiotensin (3–7). These ligands interact with several receptor proteins including AT_1, AT_2, AT_4, and Mas distributed within the central and peripheral nervous systems as well as local RASs in several organs. This review first describes the enzymatic pathways in place to synthesize these ligands and the binding characteristics of these angiotensin receptor subtypes. We next discuss current hypotheses to explain the disorders of Alzheimer's disease (AD) and Parkinson's disease (PD), as well as research efforts focused on the use of angiotensin converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARBs), in their treatment. ACE inhibitors and ARBs are showing promise in the treatment of several neurodegenerative pathologies; however, there is a need for the development of analogs capable of penetrating the blood-brain barrier and acting as agonists or antagonists at these receptor sites. AngII and AngIV have been shown to play opposing roles regarding memory acquisition and consolidation in animal models. We discuss the development of efficacious AngIV analogs in the treatment of animal models of AD and PD. These AngIV analogs act via the AT_4 receptor subtype which may coincide with the hepatocyte growth factor/c-Met receptor system. Finally, future research directions are described concerning new approaches to the treatment of these two neurological diseases.

Keywords: angiotensin II, angiotensin IV, hepatocyte growth factor, angiotensin receptors, c-Met receptor, Mas receptor, Alzheimer's disease, Parkinson's disease

As life expectancy has increased the incidences of dementia and Parkinson's disease (PD) have also increased. The number of Alzheimer's disease (AD) patients in the U.S. is presently estimated to be 4.5 million, with approximately 37 million worldwide (1, 2). By 2040 the worldwide number is predicted to reach 81 million with 4.6 million new patients diagnosed per year (3). There is a 3% occurrence of AD between the ages of 65–74 years, and upwards of 50% for those 85 years of age and older (4). Beyond the cost associated with treatment (estimated range from $70 to 150 billion annually in the U.S. alone) are the personal hardships and sacrifices suffered by family members and other care givers accompanied by the frustrations experienced by the patient and health care professionals as cognitive abilities continue to slowly deteriorate with no efficacious drug treatment available. It is clear that the brain renin-angiotensin system (RAS) is a potential contributor to dementia and blockade of this system has been shown to be important (5–9). However, the precise role(s) played by the brain RAS is unclear and somewhat convoluted given that the octapeptide angiotensin II (AngII) has been shown to disrupt learning and memory; while the hexapeptide angiotensin IV (AngIV) facilitates memory acquisition and consolidation. A second major neurodegenerative disease, PD, was first described by James Parkinson in 1867 and now affects about 10 million people in the U.S. Around the world PD impacts approximately 1% of the population over 50 years of age and 1.5% over 65 years (10). There is accumulating evidence that the brain RAS is important in the etiology of PD as well, and this recently discovered link with the RAS will be discussed.

This review initially describes the presently identified angiotensin ligands and their interaction with specific receptor proteins (AT_1, AT_2, and AT_4). The AT_1 and AT_2 receptor subtypes have been well characterized (11, 12); however, the AT_4 subtype has only been partially sequenced (13). Next we discuss the current hypotheses offered to explain the causes of AD and PD, and the drugs thus far developed to treat these dysfunctions. The role of angiotensins in memory formation and PD is discussed, followed by current attempts to develop new and efficacious treatments for AD and PD. Related to these efforts we describe an AngIV related analog effective in delaying or reversing symptoms in animal models of AD and PD. We conclude with thoughts concerning future directions in these important clinical areas of research.

FORMATION OF ANGIOTENSIN LIGANDS

Angiotensin peptides are derived from the precursor protein angiotensinogen via several enzymatic conversion pathways [**Figure 1**; Ref. (14–16)]. Briefly, the decapeptide angiotensin I (AngI) is formed by renin (EC 3.4.23.15) acting upon the amino terminal of angiotensinogen. AngI serves as a substrate for angiotensin converting enzyme (ACE; EC 3.4.15.1) that hydrolyzes the carboxy terminal dipeptide His-Leu to form the octapeptide

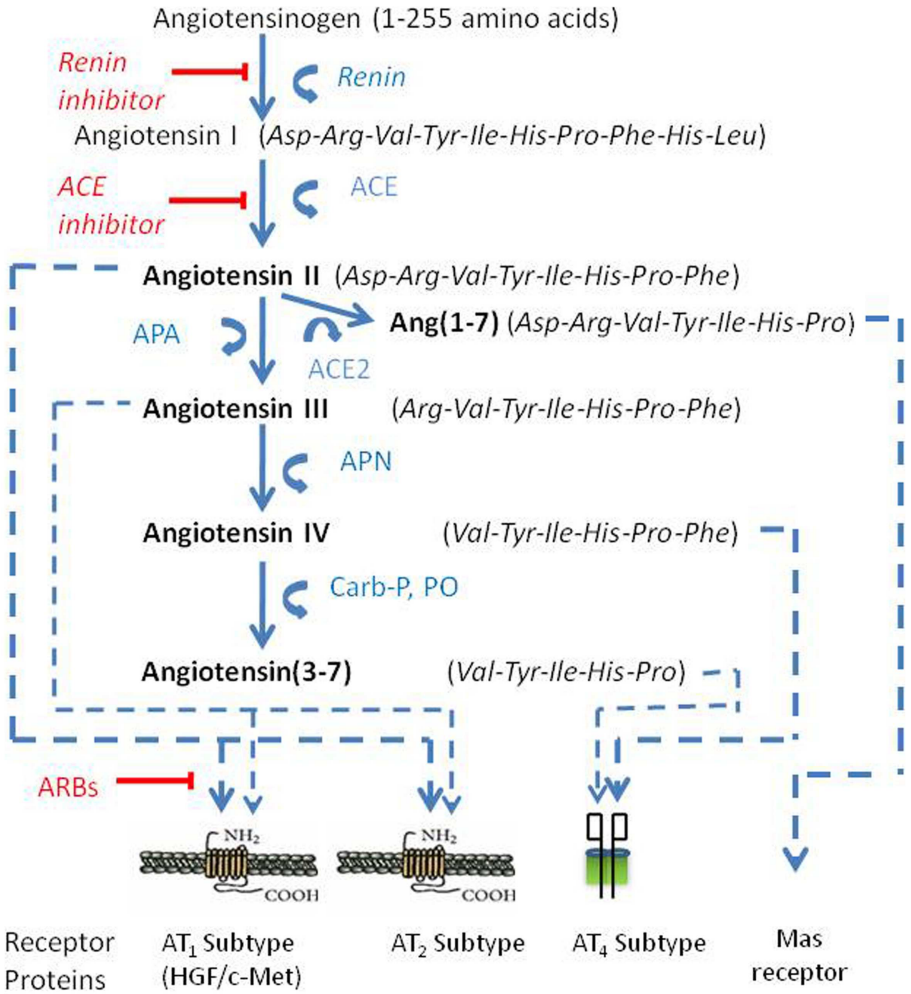

FIGURE 1 | The renin-angiotensin pathway including active ligands (bold), enzymes, receptors, and inhibitors involved in central angiotensin mediated blood pressure. Abbreviations: ACE, angiotensin converting enzyme; ACE_2, angiotensin converting enzyme 2; AP-A, aminopeptidase A; AP-N, aminopeptidase N; ARBs, angiotensin receptor blockers.

AngII (14). AngII is converted to the heptapeptide angiotensin III (AngIII) by glutamyl aminopeptidase A (AP-A; EC 3.4.11.7) that cleaves the Asp residue at the N-terminal (17–19). Membrane alanyl aminopeptidase N (AP-N; EC 3.4.11.2) cleaves Arg at the N-terminal of AngIII to form the hexapeptide angiotensin IV (AngIV). AngIV can be further converted to Ang(3–7) by carboxypeptidase P (Carb-P) and prolyl oligopeptidase (PO) cleavage of the Pro-Phe bond to form Ang(3–7).

AngII can also be converted to Ang(1–7) by Carb-P cleavage of Phe (20), by the mono-peptidase ACE_2 (21), or by ACE cleavage of the dipeptide Phe-His from Ang(1–9) (22). Note that the functional role of insertion of Alu in intron 16 of the human ACE gene has been questioned; however, Wu et al. (23) has shown this form of ACE to upregulate ACE promoter transcriptional activity by approximately 70%. Ang(1–7) is converted to Ang(2–7) by AP-A acting at the Asp-Arg bond (24). AngI is biologically inactive; while AngII and AngIII are full agonists at the AT_1 and AT_2 receptor subtypes and mediate pressor and dipsogenic functions [**Figure 2**; reviewed in Ref. (11)]. AngIV binds with low affinity to the AT_1 and AT_2 receptor subtypes (25, 26), but with high affinity and selectivity to the AT_4 receptor subtype (26–28).

Finally, AngII can be converted to Ang(1–7) by ACE_2 (29). Recent evidence indicates that this Ang(1–7)/Mas receptor system is important with regard to counteracting peripheral organ inflammation and fibrosis, increasing glucose utilization and decreasing insulin resistance (30, 31). The Mas receptor has been identified in the brain with particularly high concentrations within the dentate gyrus of the hippocampus and piriform cortex (32). In agreement with these memory-related brain distributions of Mas, Ang(1–7) has been shown to facilitate hippocampal long-term potentiation (LTP) (33) suggesting its potential importance in learning and memory. The Ang(1–7)/Mas receptor system also plays a neuroprotective role in responding to cerebral ischemia (34). The reader is referred to the following reviews for detailed characterizations of the angiotensin receptor subtypes (8, 11, 30, 35).

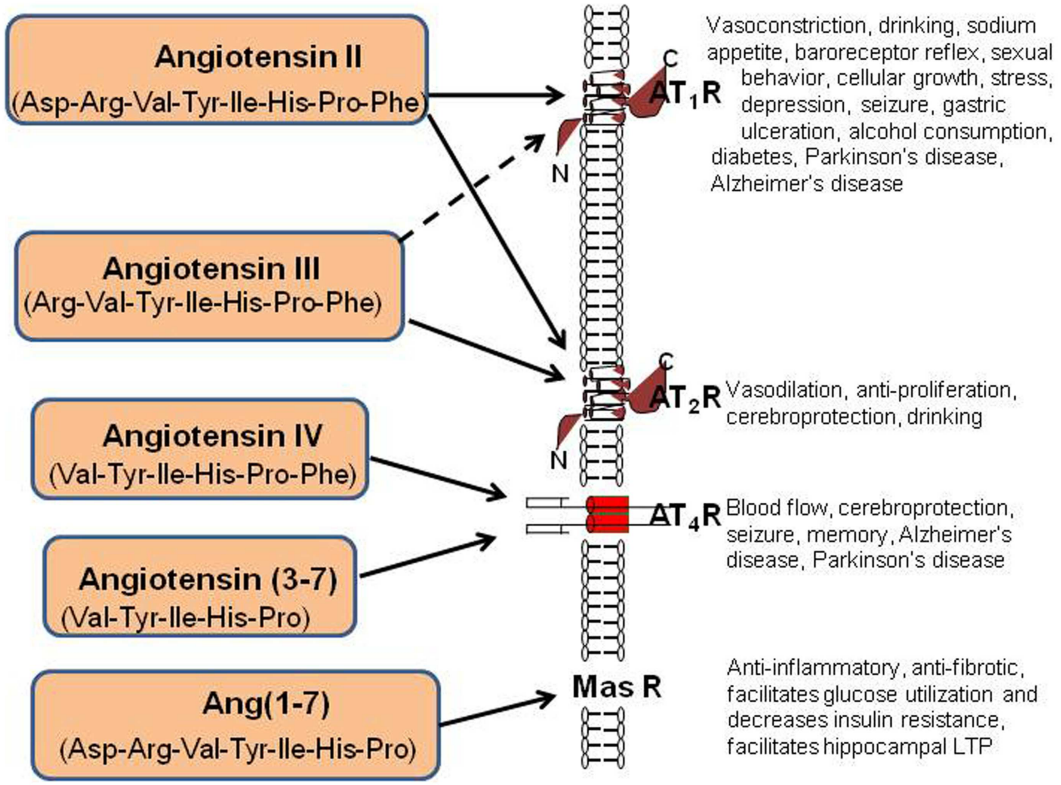

FIGURE 2 | Description of the peptide structures and enzymes involved in the conversion of angiotensinogen to angiotensin I through shorter angiotensins. The biologically active forms include angiotensin II, III, IV, angiotensin (3–7), and angiotensin (1–7). The respective receptors where these active angiotensins bind are indicated by arrows.

CURRENT HYPOTHESES OF ALZHEIMER'S DISEASE

Two prominent theories are presently offered to explain the neurochemical changes underlying AD. These are the cholinergic and amyloid cascade hypotheses. Based on the cholinergic hypothesis of memory formation it was originally proposed that drugs designed to inhibit central and peripheral acetylcholine esterase (AChE), and serve as a muscarinic M2 autoreceptor antagonist, would result in facilitated release of ACh. Further, AChE binding to the non-amyloidogenic form of β-amyloid peptide (Aβ) appears to facilitate a conformational shift to the amyloidogenic form (36–38). Treatment with an AChE inhibitor would be expected to neutralize the catalytic site of the enzyme and reduce Aβ peptide aggregation as facilitated by active AChE. To date the cholinergic hypothesis of memory formation has driven the development of the major marketed drugs in the form of AChE inhibitors (Tacrine®, Donepezil®, Rivastigmine®, and Galantamine®) which will go generic in the near future (9). These drugs are only marginally helpful in treating symptoms and do not appear to impact the underlying neuropathology of this disease (39). The FDA approved Namenda®(Memantine HCl) in 2004, an *N*-methyl-D-aspartate (NMDA) receptor antagonist designed to limit glutamate excitotoxicity and intended to treat moderate to severe AD patients (40). Namenda is also limited regarding its ability to slow disease progression and does little to stem the neuropathology. Recent research has focused on the accumulation of brain Aβ as an important target in the pathogenesis of AD (41). There may be a link between Aβ accumulation and NMDA receptor over activation in that oxidative stress, plus the elevated intracellular calcium generated due to Aβ accumulation, appear to enhance glutamate mediated neurotoxicity via increased NMDA receptor activation (42).

There are many possible reasons for the lack of an effective therapy for AD including the complexity of the disease process and the resulting inability to identify reliable biomarkers. In addition, it is now apparent that AD is multifactorial rather than a single disease (43). To further complicate drug development and diagnosis those AD criteria behaviors denoting cognitive decline can also result from a number of other clinical conditions including vascular disease (44, 45), frontotemporal dementia, PD-induced dementia, HIV infection (46, 47), as well as cumulative oxidative damage and toxicities accompanying normal aging (48). The ultimate goal of development must be a drug that prevents the progressive loss of synapses and neurons and reverses this degenerative process.

The second major hypothesis concerns amyloid peptides that range in length from 39 to 42 amino acids and are produced by the conversion of amyloid precursor protein (APP) (49). It is suggested that the cellular accumulation of Aβ(1–42) causes the neurodegenerative characteristics of AD (41). Treatment with the angiotensin receptor blocker (ARB) Valsartan has been shown to discourage amyloid β-mediated cognitive dysfunction in the Tg 2576 mouse

model of AD (50). Along these lines, intranasal injection of Losartan (also an ARB) resulted in neuroprotection, presumably via its Aβ-reducing plus anti-inflammatory effects (51).

With the recent clinical trials failure of so called "β-amyloid buster compounds" by Lilly and Pfizer Pharmaceuticals it now appears that both of these hypotheses are much too simple and new approaches must be developed and tested. One very attractive potential upstream contributor to dementia is the brain RAS. A potential role for the brain RAS in learning and memory was proposed some time ago and thus provides justification for the identification of brain RAS components that may serve as targets for the treatment of AD [reviewed in Ref. (52–56)]. Recent findings suggest that many of the memory enhancing effects initially attributed to AngII are likely due to the conversion of AngII to AngIV, and it is this peptide acting as an agonist at the AT_4 receptor subtype, that is responsible for cognitive facilitation (20, 57, 58). Taken as a whole research findings now suggest that AngII interferes with performance on most memory tasks used with animal models; while AngIV facilitates performance (59). This AngIV memory facilitation hypothesis is consistent with the finding that ARBs improve cognitive processing (60–64). It remains to be determined whether blockade of the AT_1 receptor subtype permits conversion of excess endogenous AngII to AngIV which then activates the AT_4 receptor. This notion is also supported by the observation that ACE inhibitors enhance cognitive processing in both humans (65, 66) and animal models (67). Specifically, resulting increases in AngI levels are likely converted to Ang(1–9) and then to AngIII, AngIV, and Ang(3–7). Both AngIV and Ang(3–7) act as agonists at the AT_4 receptor subtype. See below for further details concerning AngIV-induced memory facilitation. It should be noted that ACE has been shown to convert Aβ1–42 to Aβ1–40 (39). Aβ1–42 is the form that appears to be responsible for brain amyloid deposition (9). Thus, treatment with an ACE inhibitor could, over time, result in greater accumulations of amyloid plaques.

A ROLE FOR ANGIOTENSINS IN MEMORY CONSOLIDATION

A number of studies indicate that AngIV, and AngIV analogs such as Nle^1-AngIV, facilitate LTP, learning, and memory consolidation (68–72). Studies using various animal models of dementia to test the influence of Nle^1-AngIV have demonstrated reversal of deficits initiated by: (1) treatment with scopolamine (73); (2) kainic acid injections into the hippocampus (74); (3) perforant path knife-cuts (72); and (4) ischemia resulting from transient four-vessel occlusion (12). Consistent with these behavioral and electrophysiological results, brain autoradiography-determined binding sites for [^{125}I]-AngIV have been localized in structures known to mediate cognitive processing including the neocortex, hippocampus, and basal nucleus of Meynert (26, 56, 75). Denny and colleagues (76) reported that AngII blocked hippocampal LTP *in vivo* in perforant path stimulated dentate gyrus neurons. This inhibition appeared to be dependent upon AngII binding at the AT_1 receptor subtype given that co-application of Losartan with AngII significantly attenuated this inhibition; while application of the AT_2 receptor antagonist PD123, 319 failed to interfere with this AngII-induced inhibition (77). Recently it has been established that AngII, chronically perfused via subcutaneous osmotic pump in mice, resulted in hypertension and impaired spatial memory as measured using the Morris water maze task beginning during the third week of treatment (78). Such AngII-induced spatial memory impairment has also been reported in rats following acute intracerebroventricular infusion (79). Significant reductions in cerebral blood flow and brain acetylcholine levels, as well as oxidative stress, were measured 60 min following AngII injection. Taken together these results indicate that AngII generally interferes with learning and memory acquisition.

CURRENT HYPOTHESES OF PARKINSON'S DISEASE

Parkinson's disease is due to a progressive loss of dopaminergic (DA) neurons in the substantia nigra *pars compacta*. The striatum is the primary projection field of these substantia nigra neurons, thus the loss of DA results in insufficient stimulation of striatal dopaminergic D_1 and D_2 receptors (80, 81). Decreased availability of DA triggers the symptomatic triad of bradykinesia, tremors-at-rest, and rigidity. There is evidence from animal models and PD patients that neuro-inflammatory processes, triggered by reactive oxygen species (ROS), damage mitochondrial membrane permeability, enzymes, and mitochondrial genome resulting in DA cell death (82, 83). L-DOPA is efficacious at controlling motor symptoms in the majority of patients but is ineffective regarding non-motor symptoms. Current treatment strategies to relieve these symptoms include DA replacement via Levodopa (L-DOPA, the precursor of DA), DA receptor agonists, monoamine oxidase B inhibitors, and catechol-*O*-methyltransferase inhibitors, to protect the DA that is formed (84, 85). As the disease progresses periods of decreased mobility, dyskinesia, and spontaneous involuntary movements complicate treatment (86). Thus, in addition to treatment with the DA receptor agonists apomorphine and Levodopa, surgical techniques including pallidotomy and deep brain electrical stimulation may be required (87, 88). Progressive neurodegeneration also impacts additional non-dopaminergic neurotransmitter systems including noradrenergic, cholinergic, and serotonergic (89). As a result, non-motor symptoms may develop including depression, sleep disturbances, dementia, and autonomic nervous system failure (90, 91). L-DOPA is reasonably ineffective at combating non-motor symptoms (90). Current research efforts are three-pronged and directed at extending the duration of Levodopa's efficacy, controlling these additional non-motor symptoms, and developing new strategies designed to offer neuroprotection and overall disease reversal benefits. Attaining the goal of slowing or reversing the rate of DA neuron loss may also result in the protection of non-DA neurotransmitter systems.

A ROLE FOR ANGIOTENSINS IN PARKINSON'S DISEASE

Allen et al. (92) were first to suggest a potential relationship between the brain RAS and PD. These investigators measured decreased angiotensin receptor binding in the substantia nigra and striatum in post mortem brains of PD patients. A number of studies support an important role for ACE in this disease. ACE is present in the nigra-striatal pathway and basal ganglia structures (93–95). PD patients treated with the ACE inhibitor perindopril revealed improved motor responses to the DA precursor 3,4-dihydroxy-L-phenylalanine (96). Relative to this treatment with perindopril, elevated striatal DA levels have been measured

in mice (97). In addition, ACE has been shown to metabolize bradykinin and thus modulate inflammation, a contributing factor in PD. Activation of the AT_1 receptor subtype by AngII promotes nicotinamide adenine dinucleotide phosphate (NADPH)-dependent oxidases, a significant source of ROS (98, 99). Treatment with ACE inhibitors has been shown to offer protection against the loss of DA neurons in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) animal models (100, 101), as well as the 6-hydroxydopamine (6-OHDA) rat model (102). The likely mechanism underlying this ACE inhibitor-induced protection is a reduction in the synthesis of AngII acting at the AT_1 receptor subtype [reviewed in Ref. (103)]. It is known that AngII binding at the AT_1 subtype activates the NADPH oxydase complex, thus providing a major source of ROS (104–106). Further, activation of the AT_1 receptor results in the stimulation of the NF-kB signal transduction pathway facilitating the synthesis of chemokine, cytokines, and adhesion molecules, all important in the migration of inflammatory cells into regions of tissue injury (107).

If AngII activation of the AT_1 receptor subtype results in facilitation of the NADPH oxidase complex and formation of free radicals, then blockade of the AT_1 receptor should serve a protective function. This appears to be the case. Treatment with an AT_1 receptor blocker (ARB) protects DA neurons in both 6-OHDA (108–110) and MPTP animal models (105, 111, 112). ARBs have been shown to reduce the formation of NADPH oxidase-derived ROS following administration of 6-OHDA (113). While the risk of developing PD is reduced with the use of calcium channel blockers to control hypertension, the positive influences of ACE inhibitors, β-blockers, and ARBs are not clear (114). Of relevance to this issue is the PD patient who showed exacerbated motor dysfunction when treated with an ARB [Losartan; Ref. (115)]. This patient experienced severe bradykinesia while on Losartan, accompanied by frequent episodes of freezing.

The AT_2 receptor subtype is present in several fetal tissues including uterus, ovary, adrenal gland, heart, vascular endothelium, kidney, and brain (particularly neocortex and hippocampus) (11, 116–119). As the animal matures the expression of the AT_2 receptor decreases. It appears that adult mammalian brain levels of this receptor in the striatum and substantia nigra are reasonably low (56, 120). The AT_2 receptor has been linked with cell proliferation, differentiation, and tissue regeneration (121, 122). The results from a study utilizing mesencephalic precursor cells indicated that AngII, acting at the AT_2 receptor, facilitated differentiation of precursor cells into DA neurons (123). Along these lines, activation of the AT_2 receptor has been shown to inhibit NADPH oxidase activation (124). However, Rodriguez-Pallares et al. (99) found that AngII treatment of the 6-OHDA lesioned rat increased DA cell death. This could be due to the much greater numbers of brain AT_1 receptors, as compared with AT_2 receptors, such that the beneficial effects of AT_2 receptor activation was overwhelmed by AT_1 activation. Finally, the expression of AT_2 receptors in PD patients appears to be decreased in the caudate nucleus but is unchanged in the substantia nigra and putamen (125).

Basal ganglia structures possess a local RAS that evidences increased activity during dopaminergic degeneration (109, 126, 127). Villar-Cheda et al. (128) have reported that reserpine-induced decreases in DA resulted in a significant increase in the expression of AT_1 and AT_2 receptors. A similar pattern was seen with 6-OHDA-induced DA denervation in which a decrease in receptor expression was noted with L-DOPA treatment. These results indicate a direct interaction between the RAS and the dopaminergic system in basal ganglia structures. Related to this, Rodriguez-Perez and colleagues (110, 129) used intrastriatal 6-OHDA injections to produce dopaminergic degeneration and noted a significant decrease in DA neurons in ovariectomized rats. This loss of neurons was attenuated by treatment with the AT_1 receptor antagonist Candesartan, or estrogen replacement. Estrogen replacement also resulted in a down-regulation of AT_1 receptors and NADPH complex in the substantia nigra, accompanied by an up-regulation of the AT_2 receptor subtype. These results suggest an important relationship among estrogen levels, brain DA receptors, and the RAS. An increase in the expression of AT_1 receptors and decreased expression of AT_2 receptors has been reported in aged rats (130). This observation is of major importance given the potentially deleterious consequences of AT_1 receptor activation on basal ganglia structures.

Recently Rodriguez-Perez et al. (131) have reported that chronic hypoperfusion in rats resulted in a reduction in striatal DA levels accompanied by a large decline in DA neurons and striatal terminals. This DA neuron loss was countered by orally administered Candesartan. Further, AT_1 receptor expression was highest in the substantia nigra; while AT_2 expression was lower in rats that experienced chronic hypoperfusion as compared with controls. Again, Candesartan attenuated such changes in receptor expression. Taken together these findings argue that inhibition of AT_1 receptor activity serves a neuroprotective role in PD.

The involvement of AngIV in PD has been initially investigated (132). A genetic *in vitro* PD model was used consisting of the α-synuclein over-expression of the human neuroglioma H4 cell line. Results indicated a significant reduction in α-synuclein-induced toxicity with Losartan treatment combined with the AT_2 receptor antagonist PD123319, in the presence of AngII. Under these same conditions AngIV was only moderately effective. Our laboratory has recently synthesized a metabolically stable AngIV analog that acts by way of the hepatocyte growth factor (HGF)/c-Met receptor system (133–136) to overcome the motor dysfunctions that follow 6-OHDA-induced lesions of the substantia nigra *pars compacta* in the rat (unpublished results). This compound, called Dihexa, significantly improved both rope hang times and stride length over the course of a 48-day treatment period.

Taken together these findings suggest that treatment with an ARB may offer some protection against the risk of developing PD. However, much additional work employing angiotensin mimetics must be completed to better understand the relationship among brain angiotensin receptors, angiotensin ligands, inflammation, and ROS as related to PD.

AngIV, HGF, AND THE BRAIN DA SYSTEM

Aging is one of the major risk factors predisposing individuals to neurodegenerative diseases (130, 137, 138). The neurodegeneration accompanying aging is dependent in part upon oxidative stress, neuroinflammation, and microglial NADPH oxidase activity. Each is of significant importance regarding DA neuron loss (106, 139). Activation of AT_1 receptors by AngII has been shown

to facilitate DA neuron degeneration by activating microglial NADPH oxidase (109). The activation of AT_1 receptors by AngII failed to cause DA neuron degeneration when microglial cells were absent (99). Of related importance, Zawada and colleagues (140) recently reported that nigral dopaminergic neurons responded to neurotoxicity-induced superoxide in two waves. First, a spike in mitochondrial hydrogen peroxide was measured 3 h following treatment with an MPTP metabolite (MPP+). Second, by 24 h following treatment hydrogen peroxide levels were further elevated. Treatment with Losartan suppressed this nigral superoxide production suggesting a potentially important role for ARBs in the treatment of PD. Further, AngII binding at the AT_1 receptor increased DA neuron degeneration initiated by subthreshold doses of DA neurotoxins by stimulating intraneuronal levels of ROS and neuroinflammation by activation of microglial NADPH oxidase (141–144).

From the above observations it follows that AT_1 receptor blockade should have a neuroprotective effect on DA neurons in PD patients as demonstrated in animal models (112). Less obvious is the likelihood that AT_1 receptor blockade results in accumulating levels of AngII that are converted to AngIII and then to AngIV. This conversion cascade has been shown to occur intracellularly (145). In fact, this conversion of AngII appears to be necessary for DA release to occur in the striatum (146). Thus, an intriguing alternative explanation of these AT_1 receptor antagonist results is that the increased endogenous levels of AngIV facilitate activation of the HGF/c-Met receptor system and neuroprotection of DA neurons. In this way AngIV may act in combination with AT_1 receptor blockade to protect DA neurons. Our laboratory has offered evidence that AngIV, and AngIV analogs, are capable of facilitating HGF/c-Met activity (133). Support for this claim is presented in several recent reports. First we found that the action of AT_4 receptor antagonists depends on inhibiting the HGF/c-Met receptor system by binding to and blocking HGF dimerization (134, 147). In contrast, AT_4 receptor agonists facilitate cognitive processing and synaptogenesis by acting as mimics of the dimerization domain of HGF [hinge region; Ref. (135, 148)]. This work has culminated in the synthesis of a small molecule AT_4 receptor agonist capable of penetrating the blood-brain barrier and facilitating cognitive processing presumably by increasing synaptogenesis (133). This small molecule (MM-201) has a K_d for HGF ≈6.5 or 13 pM (136). This AngIV-HGF/c-Met interaction could explain earlier reports indicating that activation of the AT_4 receptor facilitates cerebral blood flow and neuroprotection (149–151).

In agreement with the above findings, HGF has been shown to positively impact ischemic-induced injuries such as cardiac (152) and hind limb ischemia (153, 154). HGF has also been shown to eliminate hippocampal neuronal cell loss in transient global cerebral ischemic gerbils (155), and transient focal ischemic rats (156). Date and colleagues (157, 158) have reported HGF-induced improvements in escape latencies by microsphere embolism-cerebral ischemic rats using a circular water maze task. These authors measured reduced damage to cerebral endothelial cells in ischemic animals treated with HGF. Shimamura et al. (159) have recently shown that over-expression of HGF following permanent middle cerebral artery occlusion resulted in significant recovery of performance in the Morris water maze and passive avoidance conditioning tasks. Treatment with HGF was also found to increase the number of arteries in the neocortex some 50 days following the onset of ischemia.

In sum, these results suggest a role for the HGF/c-Met receptor system in cerebroprotection and are consistent with the notion that AngIV increases blood flow by a NO-dependent mechanism (141). In support of this hypothesis a report by Faure et al. (160) indicated that increasing doses of AngIV via the internal carotid artery significantly decreased mortality and cerebral infarct size in rats 24 h following embolic stroke due to the intracarotid injection of calibrated microspheres. Pretreatment with the AT_4 receptor antagonist Divalinal-AngIV, or the nitric oxide synthase inhibitor Nω-nitro-L-arginine methyl ester (L-NAME), abolished this protective effect. Sequential cerebral autoradiography indicated that AngIV caused the redistribution of blood flow to ischemic areas within a few minutes. Thus, AngIV may yield its cerebral protective effect against acute cerebral ischemia via an intracerebral-hemodynamic c-Met receptor-mediated NO-dependent mechanism. Should these relationships hold then a metabolically stable blood-brain barrier penetrant small molecule that activates the HGF/c-Met system could prove highly efficacious in the treatment of PD.

FUTURE RESEARCH DIRECTIONS

The use of ACE inhibitors and AT_1 and/or AT_2 receptor blockers have shown preliminary experimental promise in the treatment of stress, depression, alcohol consumption, seizure, AD, PD, and diabetes. A number of AT_1 receptor antagonists, capable of penetrating the BBB, are now available with new ones in clinical trials (161, 162); however, the vast majority of clinical studies concerned with the use of antihypertensive agents to treat dementia have focused on ACE inhibitors and diuretics (163, 164). This is also true of studies concerned with cerebroprotection against stroke (165). Traditional antidepressant drugs for patients suffering from depression and migraine pathophysiology have taken precedence over the use of ARBs (166). Similarly, the testing of ARBs with seizure and PD patients has yet to gain momentum. The treatment of diabetic patients with ARBs is just now receiving attention (167), particularly with patients suffering diabetic related nephropathy (168, 169). The AngIV/AT_4 receptor system has been implicated in memory facilitation, cerebroprotection, seizure, Alzheimer's, and PDs. The lack of BBB penetrating AT_4 receptor agonists and antagonists has limited our understanding concerning the relative importance of brain AT_1 and AT_4 receptor subtypes in the etiology and treatment of dementias, stroke, and related memory dysfunctions. Although current drug development efforts show promise regarding small molecules that interact specifically with the AT_4 receptor, much additional effort is needed in this important research area.

There remain a number of important unanswered questions regarding whether the observed biological effects of AngIV and its analogs are mediated by the HGF/c-Met system. (1) What is the complete brain distribution of the c-Met receptor and is this receptor expressed in significant levels within cognitive mediating brain structures? (2) Can AngIV, and AngIV analogs, specifically activate the HGF/c-Met receptor system *in vivo* to induce AngIV/AT_4 receptor associated functions? (3) Are the levels of endogenous

AngIV sufficient to augment the HGF-dependent activation of brain c-Met receptors? This is a very significant issue in that the *in vivo* half-life of AngIV appears to be very short. Related to this point, what is the affinity of AngIV for HGF? (4) Does LVV-H7 bind to HGF, and if so, at what affinity? and (5) Does the activation of brain c-Met receptors produce neurogenesis, and if so can this phenomenon be utilized to replace experimentally and clinically damaged pathways? Until these questions are answered an understanding of the true mechanism of action of AngIV and its analogs will remain uncertain.

CONCLUSION

The classic RAS was originally described as a circulating hormonal system involved in cardiovascular regulation, vasopressin release, sympathetic activation, and body water/electrolyte balance. These functions appear to be primarily mediated by the AT_1 receptor subtype. With the recognition that local tissue RASs exist has come research interest in additional physiological and pharmacological functions that permit better understanding of clinical dysfunctions such as inflammation, cellular proliferation, apoptosis, and fibrosis accompanied by an increased appreciation for the role of both the AT_1 and AT_2 receptor subtypes [reviewed in Ref. (170, 171)]. It is now clear that the brain RAS is involved in a number of novel physiologies and behaviors that have important implications for the design and development of new drug treatment strategies. This review focused on the importance of the RAS with regard to two neurodegenerative diseases, Alzheimer's and PDs. The use of ACE inhibitors and ARBs with Alzheimer's patients suggests an involvement by the brain RAS in this dysfunction. Such positive results force the need to further investigate the potential roles of several angiotensins, not only the AngII/AT_1 receptor system. Clearly the AngII/AT_2 receptor and AngIV/AT_4 (c-Met) receptor systems have been shown to exert positive influences on memory acquisition and retrieval and are worthy of additional attention. The Ang(1–7)/Mas receptor system has been implicated in neuroprotection and the facilitation of LTP and also deserves further experimental evaluation.

Taken together these findings encourage new clinically relevant approaches to understanding the memory enhancing effects, especially of the angiotensin IV system, on cerebral blood flow, neuroprotection, stress and depression, alcohol consumption, seizure, Alzheimer's and PDs, and diabetes (12, 172, 173). The development of blood-brain barrier permeable AT_4 receptor agonists and antagonists presents a novel and promising new strategy for the treatment of several of these clinical dysfunctions (174–177).

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The role of tissue renin-angiotensin-aldosterone system in the development of endothelial dysfunction and arterial stiffness

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Epidemiological studies support the notion that arterial stiffness is an independent predictor of adverse cardiovascular events contributing significantly to systolic hypertension, impaired ventricular-arterial coupling and diastolic dysfunction, impairment in myocardial oxygen supply and demand, and progression of kidney disease. Although arterial stiffness is associated with aging, it is accelerated in the presence of obesity and diabetes. The prevalence of arterial stiffness parallels the increase of obesity that is occurring in epidemic proportions and is partly driven by a sedentary life style and consumption of a high fructose, high salt, and high fat western diet. Although the underlying mechanisms and mediators of arterial stiffness are not well understood, accumulating evidence supports the role of insulin resistance and endothelial dysfunction. The local tissue renin-angiotensin-aldosterone system (RAAS) in the vascular tissue and immune cells and perivascular adipose tissue is recognized as an important element involved in endothelial dysfunction which contributes significantly to arterial stiffness. Activation of vascular RAAS is seen in humans and animal models of obesity and diabetes, and associated with enhanced oxidative stress and inflammation in the vascular tissue. The cross talk between angiotensin and aldosterone underscores the importance of mineralocorticoid receptors in modulation of insulin resistance, decreased bioavailability of nitric oxide, endothelial dysfunction, and arterial stiffness. In addition, both innate and adaptive immunity are involved in this local tissue activation of RAAS. In this review we will attempt to present a unifying mechanism of how environmental and immunological factors are involved in this local tissue RAAS activation, and the role of this process in the development of endothelial dysfunction and arterial stiffness and targeting tissue RAAS activation.

Keywords: renin-angiotensin-aldosterone system, arterial stiffness, insulin resistance, endothelial dysfunction, obesity, diabetes

INTRODUCTION

Arterial stiffness is now considered an independent risk factor for the progression of cardiovascular and chronic kidney disease (CKD) (1). Arterial stiffness increases with aging and is associated with isolated systolic hypertension which occurs in most elderly persons (2). However, the process is accelerated in the presence of obesity and diabetes and occurs at earlier ages (1, 3). Given the association between arterial stiffness and obesity, it is likely that the prevalence of arterial stiffness has been increasing proportionately to the obesity epidemic, which is driven by consumption of a high fat, high fructose, and high salt western diet and further aggravated by a sedentary life style in adults and children in the Unites States and around the globe (4–7). This underscores the importance of arterial stiffness not only as a biomarker for the evaluation of progression of cardiovascular disease (CVD) and kidney disease, but also an important therapeutic target for improved cardiovascular and renal outcomes in obesity and diabetes.

ARTERIAL STIFFNESS AS A RISK FACTOR FOR CARDIOVASCULAR AND KIDNEY DISEASE

Arterial stiffness is associated with obesity, insulin resistance, and activation of the renin-angiotensin-aldosterone system (RAAS) in individuals with the cardiorenal syndrome (CRS) and even in obese children (1, 2, 5, 8). Increased arterial stiffness is also seen in normotensive subjects predisposed to develop hypertension and in pre-hypertensive subjects (9, 10). In the Atherosclerosis Risk in Communities analysis, incident hypertension was more robustly predicted when subjects were in the highest quartile of arterial stiffness. For each standard deviation decrease in elasticity, there was a 15% increase in developing hypertension

(11). Arterial stiffness increases with age, metabolic abnormalities, and increased sodium intake, all of which are associated with CVD, including heart failure (12, 13). Furthermore, arterial stiffness itself is associated with left ventricular diastolic dysfunction (14). Increased arterial stiffness is a marker of vasculopathy in CKD patients, suggesting significant cardiovascular damage (15). Arterial stiffness increases with worsening renal function (16). A significant link between aortic pulse wave velocity (PWV) and vascular calcification burden has also been described in CKD patients (17).

MEASUREMENT OF ARTERIAL STIFFNESS: *IN VIVO, EX VIVO,* AND *IN VITRO*

The evaluation of arterial stiffness *in vivo* in the clinical setting is accomplished by measurement of arterial compliance and distensibility by ultrasound, determination of PWV by measuring the velocity of the pressure wave traveling between two arterial segments, and augmentation index by measuring the augmentation pressure divided by blood pressure (1, 18). PWV closely relates to arterial wall stiffness whereas augmentation index is related to arterial wall stiffness, as well as wave reflection that is dependent on peripheral resistance and affected by heart rate variation (1, 18). The measurement of tissue and cell stiffness *ex vivo* and *in vitro* is greatly enhanced by use of atomic force microscopy (AFM) which can be performed on vascular tissues, endothelial cells, and vascular smooth muscle cells (VSMC) and complimented by confocal imaging (2, 3, 19, 20). Actin can be fluorescently labeled with Alexa 568-phalloidin and cell images, topography, and stiffness recorded with an integrated AFM-confocal microscope system. Furthermore, studies employing AFM probes that have been bio-conjugated with extracellular matrix (ECM) proteins can be used to assess the role of β1-integrin binding and cell adhesion to the ECM. These studies provided a novel concept that both β1-integrin and α-smooth muscle actin play significant role in increased stiffness of VSMCs (2, 3, 20).

ENDOTHELIAL DYSFUNCTION, ARTERIAL STIFFNESS, AND INSULIN RESISTANCE

ENDOTHELIAL DYSFUNCTION AND ARTERIAL STIFFNESS

Arterial intima consists of an endothelial cell layer and underlying layer of smooth muscle cells. It is separated from media by internal elastic lamina. In larger conduit vessels, the medial layer consists of concentric layers of elastic lamina interspersed with collagen and smooth muscle cells (18, 21). The adventitial layer is rich in fibroblasts, macrophages, lymphocytes, adipocytes, dendritic cells, and collagen (22). Arterial stiffness is regulated by a variety of factors including those from endothelial cells, VSMC alterations, cytokines, and inflammatory signals from the adventitia, and characteristic alterations in the ECM. The role of the ECM in modulation of vascular stiffness is well-recognized, and the high elastin to collagen ratio contributes to the elasticity of healthy large arteries (22). With advancing age, there is progressive thickening of arterial walls – predominantly in the intimal layer – with marked increases in the intimal to medial thickness ratio (23). There is also increased fragmentation and depletion of arterial elastin coupled with greater medial deposition of matrix metalloproteins and collagen (18, 21). Collectively, this leads to thicker and stiffer arteries, and is more predominant in the central elastic arteries compared to the peripheral, more muscular arteries. However, the relationships between stiffness in central arteries and more muscular arteries have not been clearly elucidated. The pre-diabetic state is associated with increased arterial stiffness but stiffness was unrelated to vessel wall thickness suggesting mechanisms distinct from ECM remodeling contributing to arterial stiffness (24). In this regard, accumulating evidence suggests a role for the vascular endothelium and provides new insights into the regulation of arterial stiffness (25–27). Endothelial cells regulate several arterial properties including arterial vascular tone and permeability, angiogenesis, and the vascular inflammatory response (25–28). Recently, increased intrinsic stiffness of VSMC has also been implicated in aging (2, 3, 20) and spontaneously hypertensive rats (2, 3, 20, 29). Modulation of transglutaminase 2 (TGM2) by endothelial nitric oxide (NO) (30), identification of vascular smooth muscle cytoskeletal proteins as substrates of TGM2 (31) and inhibition of smooth muscle metalloproteinase expression by NO (32) suggest the role of endothelial and smooth muscle cross talk in modulating arterial stiffness.

INSULIN AND RAAS SIGNALING AND IMBALANCE OF METABOLIC AND GROWTH SIGNALING IN THE DEVELOPMENT OF ENDOTHELIAL DYSFUNCTION AND ARTERIAL STIFFNESS

The effects of insulin in the vasculature involve metabolic signaling through the insulin receptor substrate-1 (IRS-1)/phosphatidylinositol 3-kinase (PI3 kinase) AKT/endothelial nitric oxide synthase (eNOS) pathway, as well as growth factor signaling through the ERK1/2/endothelin-1 (ET-1) pathway (28, 33–36). Regulation of endothelial function by insulin metabolic signaling is critical for normal endothelial function and vascular stiffness (1, 8, 33, 34). This insulin metabolic signaling is inhibited by both angiotensin II (Ang II) and aldosterone in vascular endothelial cells and VSMCs (**Figure 1**). The local vascular effect of insulin beyond systemic effects regulates endothelial activation of eNOS and other signaling pathways (28, 33, 35). In vascular endothelial cells, insulin stimulates production of the vasodilator NO via activation of IRS-1/PI3K signaling (**Figure 1**) (34, 35). In contrast, growth signaling pathway leads to activation of ERK1/2 and production of the vasoconstrictor ET-1. ET-1 as well as Ang II and aldosterone cause vascular stiffness (1, 8, 28, 36) and increased serum levels of ET-1 are seen in conditions associated with arterial stiffness (36). Activation of the RAAS also leads to impaired IRS-1/PI3K signaling and blunts downstream antioxidant, anti-inflammatory effects of insulin metabolic signaling (22, 34). This, in turn, further impairs insulin-induced vasodilation, capillary recruitment, and augments increases in arterial stiffness (33, 34, 37).

INSULIN RESISTANCE, ENDOTHELIAL DYSFUNCTION, AND ARTERIAL STIFFNESS AS AN EARLY EVENT IN PROGRESSION OF CVD AND CKD

Endothelial dysfunction is strongly associated with insulin resistance, arterial stiffness, and progression to CVD and CKD (24, 25, 33). Arterial stiffness may also be seen in the absence of insulin resistance in conditions such as hyperglycemia of diabetes mellitus and accumulation of advanced glycation end products (AGE) (37, 38). Individuals with obesity are likely to have an increase

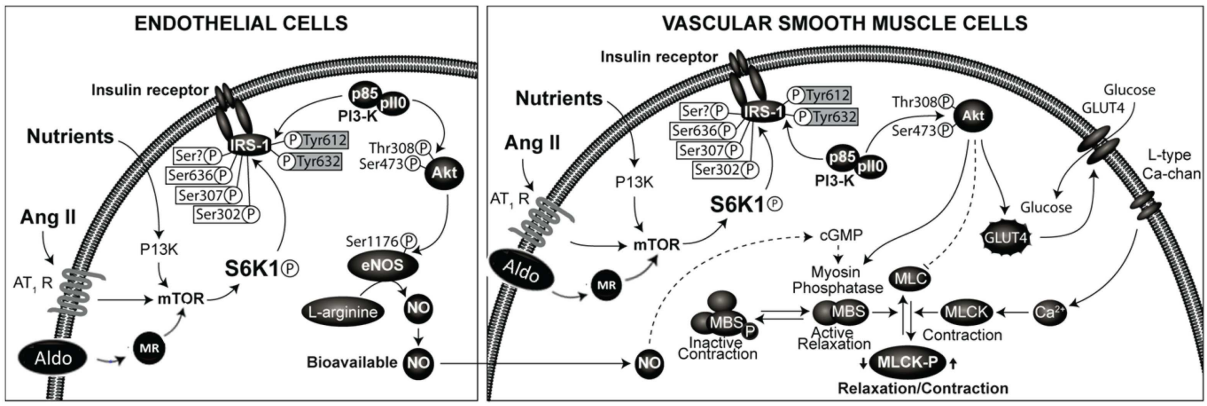

FIGURE 1 | Renin-angiotensin-aldosterone system and regulation of insulin signaling phosphorylation of docking protein insulin receptor substrate -1 (IRS-1) is the major converging point in insulin signaling. The phosphorylation of serine residues of IRS-1 by mammalian target of ribosomal p70 S6 kinase (S6K1) acts as a convergence point for the regulation of IRS-1 phosphorylation by nutrients, hormones, and cytokines. Activation of RAAS in endothelial cells and vascular smooth muscle cells leads to inhibition of insulin signaling though phosphorylation of serine residues of IRS-1. This results in impaired signaling though attenuation of phosphatidylinsositol-3 kinase (PI3- kinase)/protein kinase B (Akt) signaling pathway linked to metabolic insulin signaling. This leads to reduced production of nitric oxide and endothelial dysfunction and altered vascular smooth muscle function.

in aortic stiffness, independent of blood pressure level. Obesity and arterial stiffness are also independent factors for diastolic dysfunction (38, 39). The occurrence of arterial stiffness and diastolic dysfunction in the absence of elevated blood pressure suggest that arterial stiffness is an early event in the progression to CVD and CKD. In this regard, arterial stiffness is also associated with insulin resistance and an activated RAAS in obesity (38, 39), and insulin resistance alone in the absence of hypertension and coronary heart disease is also associated with diastolic dysfunction (obesity cardiomyopathy) (34). Insulin resistance precedes the development of vascular, cardiac, and renal complications associated with obesity (35). Reduction of aortic dilation to insulin, but not acetylcholine, prior to the onset of hypertension in the spontaneously hypertensive rats (40) and in aged rats (41) provides evidence that insulin resistance is an early event in the development of hypertension.

ROLE OF TISSUE RAAS IN VASCULAR CELLS

BEYOND CLASSICAL AND CIRCULATING RAAS

Inappropriate activation of RAAS is being increasingly recognized as a major factor in determining endothelial dysfunction, arterial stiffness, and progression to CVD and CKD (37, 38, 42–44). The RAAS is considered as an endocrine system with kidney-derived renin regulating the production of Ang II. In the blood, renin acts on liver-derived angiotensinogen to form Angiotensin I (a decapeptide). Angiotensin I is converted to biologically active Ang II (octapeptide) by the action of endothelial (mainly pulmonary endothelium) derived angiotensin converting enzyme (ACE) (45–49). Ang II acts on adrenals to stimulate the production of aldosterone and on cardiovascular and other tissues to regulate cardiovascular remodeling and blood pressure, in part by inhibiting insulin metabolic signaling in cardiovascular tissues (33, 34, 45) (**Figure 1**).

In addition to the conventional circulating RAAS, the presence of RAAS components have been detected in tissues such as heart, kidney, vasculature, adipose tissue immune cells, and brain (44–49). Recent studies have shown that VSMCs synthesize angiotensin II intracellularly. Intracellular Ang II regulates the expression of angiotensinogen and renin, generating a feedback loop. The first reaction of intracellular Ang II synthesis is catalyzed by renin or cathepsin D, depending on the cell type, and chymase, not ACE, catalyzes the second step (46, 47). The increased production of Ang II in vascular tissue in conditions of high glucose suggests this component may be of significance in diabetes (46, 47). In additional to the classical Ang II system, the role of non-classical angiotensin peptides generated by tissue ACE2 comprising Ang-(1–9) and Ang-(1–7) which generally antagonize the actions of Ang II are increasingly recognized for their bioactivity (46–49). Ang-(1–7) is also converted to Ang-(1–5) by ACE. Ang III, Ang IV, Ang-(3–7) are other peptides formed from Ang II (46–49). The role for these peptides in vascular tissue is not well understood.

Although the precise role of aldosterone-induced vascular insulin resistance has not been fully elucidated, improved endothelial function in various disease models following treatment with mineralocorticoid receptor (MR) antagonists has been reported (34, 50–53). Blockade of MR by spironolactone decreases local inflammation and vascular stiffness in rodent models of hypertension and insulin resistance (50, 52–54). The contribution of MR signaling to insulin resistance is also supported by insulin resistance in patients with primary hyperaldosteronism (55) and correlation of plasma aldosterone levels with BMI and insulin resistance in normotensive subjects (56).

CELLULAR AND MOLECULAR MECHANISMS OF VASCULAR RAAS-INDUCED INSULIN RESISTANCE, ENDOTHELIAL DYSFUNCTION, AND ARTERIAL STIFFNESS

Molecular mechanisms underlying RAAS-mediated endothelial dysfunction and arterial stiffness in aging, obesity, CRS, and diabetes is not well understood. The role of increased serine phosphorylation of IRS-1 in Ang II and aldosterone-mediated impaired

insulin signaling has been demonstrated (33, 34, 57) but the role of mammalian target of rapamycin (mTOR)/S6 kinase (S6K) mediated IRS-1 serine phosphorylation in endothelial cells are not well characterized. We have recently examined the signaling pathways mediating insulin resistance by enhanced activation of tissue RAAS in cardiovascular tissue (57). The serine phosphorylation of IRS-1 was increased and insulin-stimulated phosphorylation of eNOS was decreased by Ang II treatment. Moreover, rapamycin, an inhibitor of (mTOR) activation attenuated Ang II-stimulated phosphorylation of p70S6K and IRS-1 and blocked the ability of Ang II to impair insulin-stimulated phosphorylation of eNOS and NO-dependent arteriole vasodilation. These results suggest the role for activation of mTOR/p70S6K by Ang II in vascular endothelium in mediating impairment of insulin-stimulated vasodilation through phosphorylation of IRS-1 (57). However, MR-dependent effects on endothelial insulin signaling have not been examined.

The role of cross talk between Ang II and aldosterone signaling is increasingly recognized in the development of insulin resistance, endothelial dysfunction, and arterial stiffness (35, 50, 58–60) (**Figure 1**) and MR blockade attenuates Ang II-induced vascular damage (35, 50, 58, 59). Aldosterone activates NADPH oxidase, thereby promoting oxidative stress and decreased NO bioavailability (34, 50, 61). This is further supported by decreased reactive oxygen species production and agonist-mediated vasoconstriction by specific deletion of VSMC MR in aged mice (59). Aldosterone-induced MR activation increases expression of the intracellular cell adhesion molecule 1 (ICAM-1) (34). Moreover, aldosterone was shown to increase epithelial Na+ channel expression on the endothelial cell surface that correlated with increased cortical stiffness of the cytoskeleton in endothelial cells (62). Of potential importance is that the increase in endothelial cell stiffness was associated with a reduced release of NO (62), which in turn could impact stiffness of VSMC. These observations suggest that inhibition of MR might be a beneficial therapeutic approach for preventing vascular stiffening.

UP REGULATION OF LOCAL INTRACRINE RAAS IN OBESITY, CRS, AND DIABETES: ROLE OF MALADAPTIVE IMMUNE AND INFLAMMATORY RESPONSE

Although the significance of local RAAS may not be fully understood, the increased expression of RAAS components in vascular tissues in animal models of obesity (63, 64), and direct modulation of vascular RAAS in the vasculature *in vivo* and *in vitro* by insulin (33, 63), uric acid (65), and estrogens (66), favors the role of vascular RAAS modulating endothelial dysfunction and arterial stiffness. Importantly, these factors also cause dysregulation of immune function and a pro-inflammatory response in the vasculature that contribute to endothelial dysfunction and arterial stiffness associated with the consumption of western diet or increased cardiovascular risk in women in the setting of obesity and diabetes.

MALADAPTIVE IMMUNITY AND LOW GRADE SYSTEMIC INFLAMMATORY RESPONSE

Accumulating evidence suggests the association of inappropriate activation of RAAS and maladaptive immune and inflammatory responses in modulating endothelial dysfunction and vascular stiffness in obesity and diabetes (67–71). Increased levels of cytokines in the plasma due mainly to visceral adipocyte dysfunction, may contribute significantly to the activation of RAAS in the vascular tissue (38, 68, 69). Moreover, oxidative stress has been shown to cause increased expression of the angiotensin II type-1 (AT1) receptor (68, 69, 71). Decreased levels of interleukin (IL)-10 and impaired function of T-regulatory cells, result in activation of endothelial NADPH oxidase (68, 69, 71). Therefore, an inappropriate activation of RAAS causes cytokine imbalance in plasma and inappropriate activation of RAAS in vascular tissues by cytokines results in a feed forward loop of persistent activation of vascular RAAS in obesity and diabetes (68, 69, 72).

PERIVASCULAR ADIPOCYTE DYSFUNCTION

The role of perivascular adipose tissue contributing to inflammation, insulin resistance, endothelial dysfunction, and vascular stiffness is increasingly recognized (69, 72–74). In lean mice, perivascular fat exerts protective vasoregulatory effects, but this protective effect is lost in obese mice (74). Endothelial dysfunction in obesity is associated with a significant infiltration of macrophages and T cells in perivascular adipose tissue (72–74). Moreover, perivascular adipose tissue is also a source of Ang II and increased production of Ang II by perivascular fat may also account for impairment of vascular function (75).

HIGH FRUCTOSE DIET, URIC ACID, AND VASCULAR RAAS

Elevated serum uric acid level is a frequent finding in persons with obesity, hypertension, cardiovascular, and kidney disease. Increased consumption of a fructose-rich western diet also results in elevations in uric acid (6, 7). Elevated serum levels of uric acid appear to contribute to maladaptive immune and inflammatory responses (65, 69, 76), activation of angiotensin system in the vascular cells (65), impaired NO production/endothelial dysfunction (77), and increased vascular stiffness (78, 79).

SEX DIFFERENCES: ABROGATION OF CARDIOVASCULAR PROTECTIVE EFFECTS OF ESTRADIOL IN OBESITY AND DIABETES IN PREMENOPAUSAL WOMEN

Females of reproductive age have fewer cardiovascular events however this protection is lost after menopause, suggesting cardioprotective effects of estradiol. The cardio-protective effect of estradiol is also lost in the setting of obesity and diabetes in premenopausal women (69, 80–83). In this regard, arterial stiffness is substantially higher in women than in age-matched men, and is associated with cardiac diastolic dysfunction (82). In a community-based cohort study, increased arterial stiffness was associated with reduced left ventricular diastolic function in both men and women. However, the greater arterial stiffness observed in women was associated with higher incidence of diastolic dysfunction (83–85). Estrogen modulates both Ang II signaling and immune and inflammatory responses. Estradiol normally suppresses actions of Ang II by inhibiting the expression of AT1 (86, 87). However, under the conditions of inhibition of NO synthase and high salt, estradiol increases the expression of AT1 receptor (66, 87). Moreover, GPR-30 which also mediates estradiol effects, increases the expression of ACE2 and decreases the expression of

AT1 receptor (88, 89). Estrogen receptor alpha and GPR-30 have been shown to exert an anti-inflammatory effect via modulation of T-cell immune response (90, 91). In addition, estrogen receptor alpha-mediated signaling in macrophages contributes to enhanced insulin sensitivity (92). These findings suggest that a crosstalk between estrogen and Ang II signaling may be one of the factors contributing to sex differences in altered immune and inflammatory responses, endothelial dysfunction, and arterial stiffness, in obesity and diabetes. Furthermore, a recent study demonstrating arterial stiffness in obese pre-menopausal women underscores the role of obesity in abrogating cardiovascular protection in those women (93).

CONCLUSION

Arterial stiffness is an independent factor promoting the progression of CVD and renal disease in obesity and diabetes. Inappropriate activation of vascular RAAS in humans and animal models of obesity and diabetes is associated with endothelial dysfunction and arterial stiffness. However, accumulating evidence suggests the role of local tissue RAAS in the vascular tissue, immune cells, and perivascular adipose tissue in endothelial dysfunction contributes significantly to arterial stiffness. The cross talk between angiotensin and aldosterone underscores the importance of the MR in modulation of oxidative stress, insulin resistance, decreased bioavailability of NO, endothelial dysfunction, and arterial stiffness. In addition, both innate and adaptive immunity are involved in local tissue activation of RAAS and in turn are modulated by environmental factors such as high fat/sucrose western diet. Moreover, arterial stiffness is reported in pre-menopausal obese women and estrogen mediated cardiovascular protection is lost in obese or diabetic pre-menopausal women. Taken together, targeting endothelial function and arterial stiffness by modulating tissue RAAS system appears to be an attractive therapeutic strategy to reduce the CVD and CKD complications associated with obesity and diabetes.

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New frontiers in the intrarenal renin-angiotensin system: a critical review of classical and new paradigms

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The renin-angiotensin system (RAS) is well-recognized as one of the oldest and most important regulators of arterial blood pressure, cardiovascular, and renal function. New frontiers have recently emerged in the RAS research well beyond its classic paradigm as a potent vasoconstrictor, an aldosterone release stimulator, or a sodium-retaining hormone. First, two new members of the RAS have been uncovered, which include the renin/(Pro)renin receptor (PRR) and angiotensin-converting enzyme 2 (ACE2). Recent studies suggest that prorenin may act on the PRR independent of the classical ACE/ANG II/AT_1 receptor axis, whereas ACE2 may degrade ANG II to generate ANG (1–7), which activates the Mas receptor. Second, there is increasing evidence that ANG II may function as an intracellular peptide to activate intracellular and/or nuclear receptors. Third, currently there is a debate on the relative contribution of systemic versus intrarenal RAS to the physiological regulation of blood pressure and the development of hypertension. The objectives of this article are to review and discuss the new insights and perspectives derived from recent studies using novel transgenic mice that either overexpress or are deficient of one key enzyme, ANG peptide, or receptor of the RAS. This information may help us better understand how ANG II acts, both independently or through interactions with other members of the system, to regulate the kidney function and blood pressure in health and disease.

Keywords: angiotensin 1-converting enzyme, ACE2, angiotensin II receptor, blood pressure, hypertension, kidney, proximal tubule, signal transduction

INTRODUCTION

Although Tigerstedt and Bergman discovered the rate-limiting enzyme renin about 115 years ago (1), the renin-angiotensin system (RAS) remains to be a remarkable subject for continuous research. Our current understanding of the RAS has greatly evolved from the classical renin/angiotensin-converting enzyme (ACE)/angiotensin II (ANG II)/AT_1 receptor axis and its physiological roles in the regulation of cardiovascular and renal function, blood pressure, aldosterone biosynthesis and release, and body salt and fluid balance (2–14). However, new frontiers are continuously emerging from the RAS research in recent years, especially in uncovering new enzyme(s) and/or receptor(s) of the system, studying their novel roles, and elucidating their signaling transduction mechanisms. It is now recognized that the classical renin/ACE/ANG II/AT_1 and AT_2 axis is no longer the exclusive effector and signaling pathway for the system (15). Three new axes have been recently described to include the ACE2/ANG (1–7)/Mas receptor axis, the prorenin/PRR/MAP kinases ERK1/2 axis, and the ANG IV/AT_4/IRAP (insulin-regulated aminopeptidase, IRAP) axis (**Figure 1**) (8, 12, 15–17). The notion that ANG II is the only active peptide of the RAS appears to be outdated, since ANG II can be hydrolyzed by various angiotensinases, ACE2, and neprilysin to generate ANG (1–7), ANG III, ANG IV, and ANG A (2, 16, 18). Prorenin and smaller ANG fragments, including ANG (1–7), ANG III, and ANG IV, can bind their respective receptors or act as an agonist for ANG II receptors to induce a physiological effect (2, 8, 17, 19–21). Indeed, in addition to AT_1 and AT_2 receptors that mediate the well-recognized effects of ANG II in the kidney and other tissues, new receptors for prorenin (PRR), ANG (1–7) (Mas receptor), and ANG IV (AT_4 receptor) have been identified (21–23). Depending on the receptor activated, small ANG peptides may act as an agonist or an antagonist of ANG II. For example, appropriate concentrations of ANG (1–7), ANG III, and ANG IV may activate their respective Mas receptors (8, 9, 16), AT_2 receptors (19, 24, 25), or AT_4 receptors to oppose the known effects of ANG II (26, 27). Conversely, high concentrations of ANG (1–7), ANG III, and ANG IV may activate AT_1 receptors to induce the well-recognized effects of ANG II (16, 20, 28–30). Furthermore, the renin/prorenin receptor, PRR, not only catalyzes prorenin to generate ANG II, but also induces intracellular responses in an ANG II-independent manner (13, 31, 32). Finally, the RAS is no longer considered to act only as an endocrine system, but also acts as a paracrine, autacrine, and intracrine system (33–37). It is likely that ANG II and its smaller ANG peptides may act as both endocrine, paracrine, and intracrine peptides by stimulating cell surface, cytoplasmic and nuclear receptors to exert biological, physiological, and nuclear effects.

The major objective of this article is to review recent advances in biomedical research with a focus on the intrarenal RAS and its paracrine, autacrine, and intracrine roles. New insights, controversies, and perspectives will be discussed by reviewing recent *in vitro* and *in vivo* studies using innovative approaches or

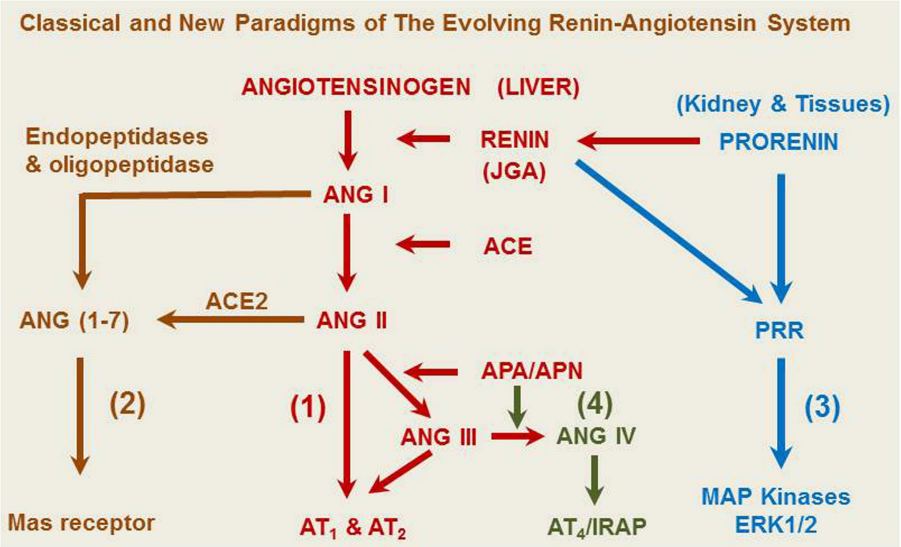

FIGURE 1 | A representative overview of the evolving renin-angiotensin system. (1) The classical angiotensinogen/renin/ACE/ANG II/AT_1 and AT_2 receptor axis. (2) The prorenin/PRR/MAP kinases ERK 1/2 axis. (3) The ACE2/ANG (1–7)/Mas receptor axis. (4) The ANG IV/AT_4/IRAP axis. ANG A, angiotensin A. ANG I, angiotensin I. ANG (1–7), angiotensin (1–7). ACE, angiotensin-converting enzyme. ACE2, angiotensin-converting enzyme 2. ANG II, angiotensin II. ANG III, angiotensin III. ANG IV, angiotensin (3–8). APA, aminopeptidase A; APN, aminopeptidase N; AT_1, type 1 ANG II receptor; AT_2, type 2 ANG II receptor; IRAP, insulin-regulated aminopeptidase or AT_4 receptor; JGA, juxtaglomerular apparatus.

animal models including global and tissue-specific RAS transgenic animals. The review article will cover the classical ACE/ANG II/AT_1 and AT_2 receptor axis, the ACE2/ANG (1–7)/Mas receptor axis, the prorenin/PRR/MAP kinases ERK1/2 axis, and the ANG IV/AT_4/IRAP axis. It is expected that this new information may further improve our understanding of physiological and pathophysiological roles of the RAS and help the development of new drugs or strategies to treat hypertension, diabetes, and cardiovascular and kidney diseases by targeting ANG II and other ANG peptides and/or their receptors.

CURRENT INSIGHTS AND FUTURE PERSPECTIVES ON THE ROLES OF THE CLASSICAL ACE/ANG II/AT_1 AND AT_2 RECEPTOR AXIS IN THE KIDNEY

It is well established that the ACE/ANG II/AT_1 and AT_2 receptor axis may function as a circulating or endocrine and paracrine system to regulate cardiovascular, neural, adrenal, and renal function, contributing to normal blood pressure homeostasis and the development of hypertension. However, the specific role of and the extent to which the intrarenal ACE/ANG II/AT_1 and AT_2 receptor axis versus the systemic counterpart plays in normal blood pressure control and the development of hypertension remain an issue of continuous debate (10, 38–42). Now, there is a general consensus that all major components of the RAS necessary for generation of ANG II are expressed or present in the kidney (**Figure 2**) (2, 18, 43–45), and that the levels of ANG II in the kidney are much higher than in plasma (2, 44, 46–49). This is especially true that high ANG II levels have been demonstrated in interstitial and proximal tubular fluid of the kidney and intracellular endosomal compartment (46–48, 50–52).

The mechanisms underlying high levels of ANG II in the kidney are not well understood. In addition to the well-documented expression of all major components of the RAS in the kidney, two major mechanisms may play a critical role under physiological conditions and during the development of ANG II-dependent hypertension. The first is that AT_1 receptors are abundantly expressed in the kidney, where AT_1 (AT_{1a}) receptor mediates the intracellular accumulation of ANG II especially in proximal tubules (48, 53–58). Classically, a receptor pharmacological dogma suggests that the purpose of G protein-coupled receptor (GPCR)-mediated internalization or endocytosis of an agonist or ligand is to desensitize the cellular responses to the agonist stimulation by moving the agonist/ligand into the cell for degradation in the lysosomal compartment (59–64). The receptor recycles back to the cell membrane to initiate a new round of biological response. However, we and others infused ANG II into rats and mice for 2 weeks, and found no desensitization of ANG II responses, because blood pressure continued to increase and hypertension persists as long as ANG II is infused (48, 53–58). Zhuo et al. reported that in ANG II-infused hypertensive rats, ANG II levels were about 10 times higher in renal cortical endosomes than in control rats via an AT_1 receptor-mediated mechanism (48). Nishiyama et al. showed that renal interstitial fluid ANG II levels were substantially increased in ANG II-infused rats, an effect also mediated by AT_1 receptors (65). In AT_{1a} receptor-deficient mice ($Agtr1a^{-/-}$), we further demonstrated that AT_1 receptor-mediated increases in ANG II uptake in the kidney were largely abolished (57, 58). These studies suggest that AT_1 (AT_{1a}) receptor-mediated uptake of ANG II at least partly contributes to the demonstrated high levels of ANG II in the kidney.

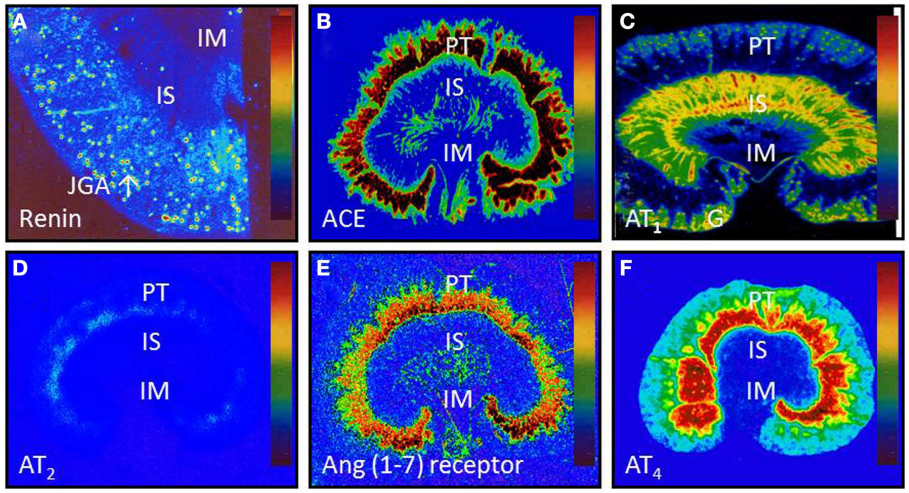

FIGURE 2 | Intrarenal localization or expression of major components of the renin-angiotensin system. **(A)** Active renin binding in juxtaglomerular apparatus in the dog kidney using the radiolabeled renin inhibitor, ^{125}I-H77. **(B)** ACE binding in the proximal tubule of the rat kidney using ^{125}I-351A **(C)** AT_1 receptor binding in the rat kidney in the presence of the AT_2 receptor blocker PD123319. **(D)** AT_2 receptor binding in the rat kidney in the presence of the AT_1 receptor blocker losartan using ^{125}I-[Sar^1,Ile^8]-Ang II. **(E)** Ang (1–7) receptor binding in the rat kidney using ^{125}I-Ang (1–7) as the radioligand. And **(F)** Ang IV receptor binding in the rat kidney using ^{125}I-Ang (3–8). The levels of binding are indicated by color calibration bars with red representing the highest, whereas blue showing the lowest levels of enzyme or receptor binding. G, glomerulus; IM, inner medulla; IS, inner stripe of the outer medulla; JGA, juxtaglomerular apparatus; P, proximal tubule. Reproduced from Li and Zhuo with permission (45).

The second classical dogma in the RAS field is that the expression and activity of the RAS is strictly regulated by a negative feedback mechanism by ANG II itself. An increase in the circulating and tissue ANG II is expected to suppress renin release from JGA cells and therefore the production of ANG II in the kidney. However, there is evidence that a positive feed-forward loop exists in the kidney during ANG II-dependent hypertension (43, 44, 66–69). Navar's group has shown that prorenin and renin (68–70), angiotensinogen (43, 67), and ACE (66) are significantly augmented in response to long-term infusion of ANG II to induce hypertension in rats or mice. Renin and prorenin expression in the collecting ducts are also stimulated during ANG II infusion, likely contributing to increased urinary levels in ANG II-infused hypertensive rats (69–72). Taken together, these studies suggest that in ANG II-infused hypertensive animals, intrarenal ANG II production may be augmented due to increased expression of prorenin and renin, AGT, and ACE.

Currently, there is a great debate on whether AGT, ACE, and AT_1 receptors in the kidney contribute to the normal blood pressure regulation and the development of hypertension (4, 10, 39–42, 73–77). The classical dogma is that the circulating RAS via the kidney derived renin, liver-derived AGT and vascular endothelial ACE, rather than the intrarenal RAS, plays an important role in the normal blood pressure control and the development of hypertension (78–82). To determine the roles of systemic/endothelial ACE versus tissue/kidney ACE in normal blood pressure and renal control, Bernstein's group first used targeted homologous recombination to create mice, ACE 2/2, expressing a form of ACE that lacks the COOH-terminal half of ACE with normal or elevated circulating ACE without tissue-bound/kidney ACE (78). Homologous ACE 2/2 mice have significantly lower blood pressure, renal vascular thickening, urine concentrating defect, and significant increase in fractional proximal tubular reabsorption (78). These studies suggests that tissue-bound ACE, rather than circulating ACE, is important for maintaining normal blood pressure (78), and that ACE in the proximal tubule may not be necessary for maintaining normal proximal fluid reabsorption (80). The same group of investigators later generated the so-called ACE 3/3 mice, which is deficient of endothelial ACE in the lung, aorta, or any vascular structure (79). ACE activity in the kidney is about 14% that of wild-type mice, but hepatic ACE expression in ACE 3/3 mice is almost 90-fold that of wild-type. Interestingly, basal blood pressure, plasma ANG II levels, response to ACE inhibitors, and renal function of ACE 3/3 mice were similar to those of wild-type mice. The underlying conclusion of this study is that endothelial ACE is not required for maintaining normal blood pressure and renal function (79). Sen's group also generated two different strains of mutant mice that express ACE either in vascular endothelial cells (Ts strain) or in renal proximal tubules (Gs strain) (81, 82). Both mutant mice show equivalent serum ACE and ANG II levels, normal kidney structure and fluid homeostasis. In contrast to Bernstein's ACE3/3 mice (79), only those mutant mice that expressed ACE in vascular endothelial cells had normal blood pressure (81). Proximal fluid reabsorption was found to be normal in the chronic absence of proximal tubule ACE (82). Thus there is still a lack of consensus with respect to the precise roles of systemic/endothelial versus tissue/kidney ACE in normal blood pressure control.

Recently, Gonzalez-Villalobos et al. further determine the role of intrarenal ACE in the normal blood pressure regulation and the development of ANG II-induced hypertension (10, 75). First, Gonzalez-Villalobos et al. also used targeted homologous

recombination to generate mice, ACE9/9, that express ACE only in the kidney tubules but not in other tissues (75), or mice with complete deficiency of the entire kidney ACE, ACE 10/10 (10). Similar to Sen's Gs strain (82), ACE 9/9 mice had lower blood pressure, associated with reduced circulating ANG II, but maintained normal kidney ANG II levels. ACE 9/9 mice responded to chronic ANG I infusion to substantially increase blood pressure (75). In ACE 10/10 mice whose basal blood pressure was similar to wild-type mice, the blood pressure responses to 2-week of ANG II infusion were substantially attenuated in the kidney ACE-KO mice (10). The later study indicates that intrarenal ACE plays a key role in the development of ANG II-induced hypertension, whereas the absence of ACE in the kidney protects against hypertension (10).

However, a careful evaluation of these studies on different strains of ACE mutant mice evokes more questions than answers in the current debate on the relative roles of circulating and intrarenal ACE and therefore ANG II in the blood pressure regulation and the development of hypertension (39, 83). For example, mice with the lack of vascular endothelial ACE may be normotensive (79) or hypotensive (75, 81). Conversely, mice with the lack of kidney/proximal tubular ACE may be normotensive (10, 81). ACE/ANG II appear not to be necessary for maintaining normal proximal tubular fluid reabsorption in mice with overexpression or deficiency of ACE in the proximal tubule (79–82) or the entire kidney (10). Furthermore, circulating or kidney ANG II levels may be normal in these ACE transgenic mice despite of the lack of systemic/endothelial or kidney/proximal tubular ACE (10, 75, 79, 82). These contradictory biochemical, blood pressure, and proximal tubular transport phenotypes, as revealed in various mutant ACE-knockout mice, are difficult to reconcile with well-recognized roles of ACE in the formation of ANG II in the circulation and the kidney, in promoting sodium reabsorption in the proximal tubule and other tubular segments, and in maintaining normal blood pressure homeostasis. However, these diverse phenotypes may provide a new insight into an important role of AT_1 (AT_{1a}) receptor-mediated uptake of circulating ANG II by the kidney, especially in the proximal tubule, in maintaining normal levels of ANG II in the kidney of ACE9/9 and/or ACE10/10 mice (10, 75). As discussed previously, AT_1 (AT_{1a}) receptor-mediated uptake of circulating ANG II at least partly contributes to higher basal ANG II levels and increased ANG II levels in the kidney during ANG II-induced hypertension (48, 54, 57, 58, 84, 85). Another new insight derived from these mutant ACE mouse models is that blood pressure and proximal tubule phenotypes of these ACE-knockout mice are likely complicated by the fact that ACE is chiefly responsible for the metabolism of bradykinin, ANG (1–7), and many other vasoactive peptides such as substance P (8, 9, 18, 86). Knockout of systemic and/or kidney ACE would lead to marked decreases in circulating and intrarenal ANG II and generation of other vasodepressor substances in the circulation and kidney, which may alter blood pressure and renal responses to ANG II or other vasoactive substances under physiological as well as pathophysiological conditions.

Recent studies using mice with kidney or proximal tubule-specific knockout of AT_1 receptors provide new insights and perspectives into the roles of the kidney or proximal tubular AT_{1a} receptors in the normal blood pressure regulation and the development of hypertension (4, 38, 40–42, 77, 87). Coffman and Crowley's group has been instrumental to use the kidney cross-transplantation approach between wild-type and global AT_{1a} receptor-knockout mice (*Agtr1a*$^{-/-}$) (4, 38, 87). These investigators transplanted the kidney of wild-type mice into *Agtr1a*$^{-/-}$ mice to generate systemic AT_{1a}-KO mice, and conversely transplanted the kidney of *Agtr1a*$^{-/-}$ mice into wild-type mice to generate the kidney-specific AT_{1a}-KO mice. Blood pressure and cardiac hypertrophic responses to ANG II infusion or high salt intake were compared in the systemic- and kidney-specific AT_{1a}-KO mice (4, 38, 87). These elegant studies confirmed that the kidney AT_1 receptors are absolutely required for the development of ANG II-dependent hypertension and cardiac hypertrophy, and systemic AT_1 receptors is not sufficient for ANG II to induce hypertension or cardiac hypertrophy (38). Using the Cre/Lox strategy, Gurley et al. (40) and Li et al. (41) generated proximal tubule-specific AT_{1a}-KO mice to determine the role of proximal tubule AT_{1a} receptors in blood pressure regulation. Both studies demonstrated that deletion of AT_{1a} receptor and its signaling in the proximal tubule alone is sufficient to significantly decrease basal blood pressure, despite intact systemic AT_{1a} receptor expression and vascular responses (40, 41). Alternatively, we have recently produced adenoviral constructs encoding GFP-tagged AT_{1a} receptor gene (AT_{1a}R/GFP) (**Figure 3**), or an enhanced cyan fluorescent protein (ECFP)-tagged ANG II fusion protein, and a proximal tubule-specific sodium and glucose cotransporter 2 (sglt2) promoter (**Figure 4**) (42). We demonstrated that intrarenal transfer of AT_{1a}R/GFP alone selectively in the proximal tubule was sufficient to increase systolic blood pressure by ∼12 mmHg 14 days after the gene transfer (42). Cotransfer of AT_{1a}R/GFP with ECFP/ANG II increased blood pressure further to 18 mmHg. The increases in blood pressure were associated with twofold increases in phosphorylated MAP kinases ERK1/2, lysate and membrane NHE3 proteins in freshly isolated proximal tubules, and a decrease in 24 h urinary sodium excretion (42). Taken together, these elegant studies strongly suggest that the proximal tubule ACE/ANG II/AT_{1a} receptor axis via promoting proximal tubular sodium and fluid reabsorption may contribute approximately 15 mmHg to basal blood pressure homeostasis in mice.

CURRENT INSIGHTS AND FUTURE PERSPECTIVES ON THE ROLES OF THE ACE2/ANG (1–7)/Mas RECEPTOR AXIS IN THE KIDNEY

ANG (1–7) is the most extensively studied smaller ANG peptide in the RAS since 1970s (8, 9, 17, 18, 88). Early studies showed that structural deletion of either phenylalanine (position 8) or the dipeptide, Pro-Phe (positions 7 and 8) from ANG II completely removed the vasoconstrictor, central pressor, or thirst-stimulating actions of ANG II (89). The structural and activity studies suggested that ANG (1–7) may be an inactive component of the RAS. However, subsequent studies primarily from Ferrario's group demonstrated that ANG (1–7) has significant vasodepressor and antihypertensive actions in hypertensive animals or humans, which may oppose the actions of ANG II either directly or indirectly by stimulation of prostaglandins and nitric oxide (8, 9, 17, 18, 88). The importance of this heptapeptide in cardiovascular, blood pressure, and renal control gains further recognition recently upon

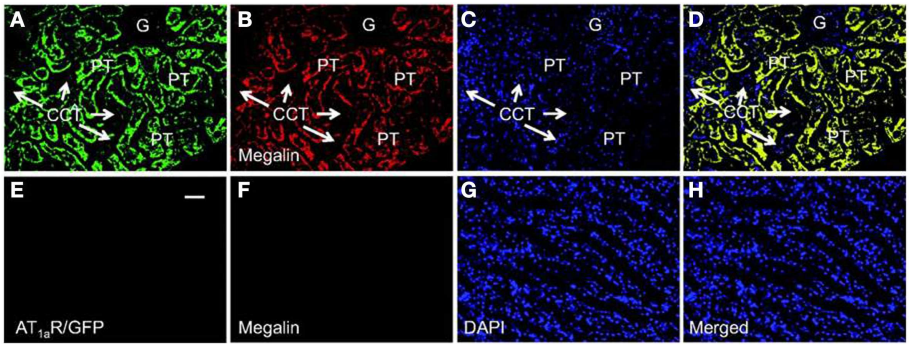

FIGURE 3 | Proximal tubule-specific expression of $AT_{1a}R$/GFP in a representative Agtr1a$^{-/-}$ mouse kidney 2 week after intrarenal adenoviral transfer. **(A)** $AT_{1a}R$/GFP expression (green) in proximal tubules (PT). **(B)** Alexa Fluor 594-labeled megalin expression (red) in proximal tubules. **(C)** DAPI-stained nuclei (blue) in the same kidney section. **(D)** Merged image of **(A–C)**, showing the colocalization of $AT_{1a}R$/GFP and megalin expression (yellow) in proximal tubules. Only very low levels of $AT_{1a}R$/GFP and megalin expression are visible in the glomerulus (G) and cortical collecting tubules (CCT). **(E)** $AT_{1a}R$/GFP expression in the outer medulla. **(F)** Alexa Fluor 594-labeled megalin expression in the outer medulla. **(G)** DAPI-stained nuclei in the outer medulla. **(H)** Merged image of **(E–G)**, showing the lack of $AT_{1a}R$/GFP and megalin expression in the outer medulla. Magnification: ×40. Reproduced from Li and Zhuo with permission (42).

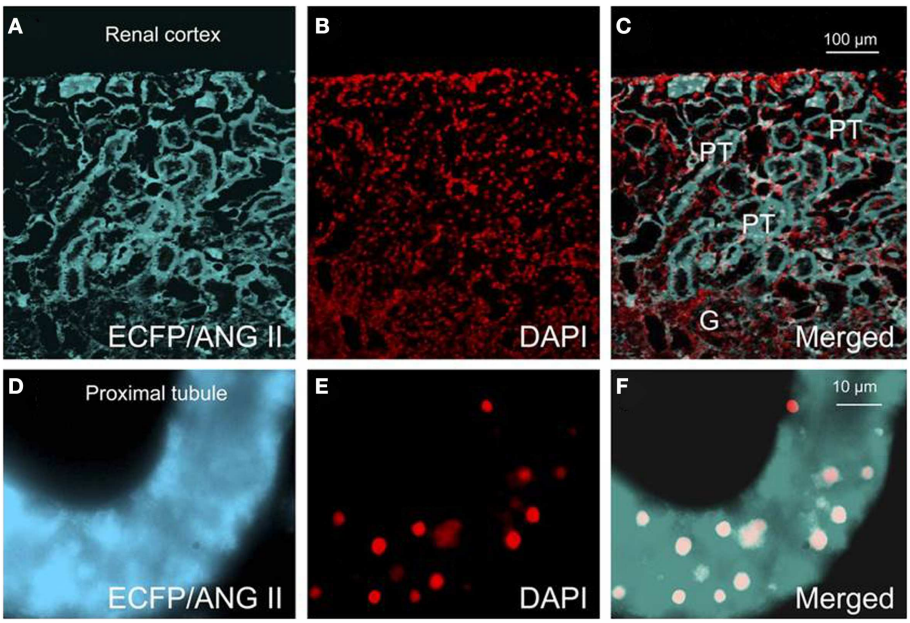

FIGURE 4 | Effects of proximal tubule-specific, adenovirus-mediated transfer of ECFP/ANG II on ECFP/ANG II expression in the renal outer cortex and freshly isolated proximal tubule of mouse kidneys 2 wk after gene transfer. **(A)** ECFP expression (blue-green). **(B)** DAPI-stained nuclei (red). **(C)** Merged image of **(A,B)**, respectively, in the outer renal cortex of a representative rat transferred with ECFP/ANG II selectively in proximal tubules. **(D–F)** Expression of ECFP/ANG II in a freshly isolated representative proximal convoluted tubule. Bars = 100 μm for the renal cortex and 10 μm for the isolated proximal tubule. G, glomerulus; PT, proximal tubule. Reproduced from Li et al. with permission (77).

the molecular characterization of a GPCR using ANG (1–7) as a ligand, the Mas receptor (23). It is increasingly recognized that the new ACE2/ANG (1–7)/Mas receptor axis acts to counteract most of the known deleterious actions of the ACE/ANG II/AT_1 receptor axis (8, 16, 17). However, recent studies on transgenic animals overexpressing ANG (1–7) have provided new insights and perspectives on whether ANG (1–7) plays beneficial cardiovascular, blood pressure, and renal hemodynamic effects (90–92).

The kidney is one of the key tissues in which ANG (1–7) is generated from the metabolism of ANG II by ACE2 with the proximal tubule exhibiting the most robust ACE2 activities (8, 49). ANG (1–7) can be easily detected in the proximal tubule and urine of rats, sheep, and humans, but it can be rapidly hydrolyzed to ANG (1–5) and ANG (1–4) by ACE and neprilysin (8, 49). Whether ANG (1–7) is primarily produced from the degradation of ANG II by ACE2 in the circulation and kidney remains an issue of continuous

debate. An early study by Yamamoto et al. showed that infusion of ANG II in WKY or SHR rats was not accompanied by significantly increased plasma ANG (1–7) levels (93). Modrall et al. reported that in tissue ACE-knockout mice, intrarenal ANG I and ANG II levels were decreased by 70–80% compared with wild-type mice, but ANG (1–7) levels were surprisingly normal in the kidney (94). Thus a more balanced view may be that ANG (1–7) is derived from both the metabolism of ANG I via the endopeptidase-dependent pathway and the metabolism of ANG II by the ACE2-dependent pathway.

Both renal hemodynamic and tubular effects have been demonstrated although the signaling mechanisms involved are not fully understood (17). However, the current insight is that ANG (1–7) acts primarily to oppose the cardiovascular and renal effects of ANG II. For example, ANG II is known to increase blood pressure, induce renal vasoconstriction to decrease renal blood flow (RBF) and glomerular filtration rate (GFR), and induce antidiuresis and antinatriuresis (43, 95–98). By contrast, ANG (1–7) infusion generally opposes and attenuates these effects of ANG II (8, 16, 17, 36, 99). The diuretic/natriuretic effects of ANG (1–7) may be partly due to the renal vasodilatation as well as inhibition of sodium and water reabsorption along the nephron segments. Previous studies demonstrated that ANG (1–7) may be a potent inhibitor of Na^+-K^+-ATPase in the proximal tubule (16, 17). ANG (1–7) may inhibit Na^+-K^+-ATPase via AT_2 receptor-mediated stimulation of the G(i/o) protein/cGMP/PKG signaling pathway (100, 101). Moreover, ANG (1–7) showed biphasic effects on the Na^+/H^+ exchanger activity in isolated proximal tubules mediated by the Mas receptor and changes in $[Ca^{2+}]_i$ (30, 102). In rat inner medullary collecting ducts (IMCD), ANG (1–7) enhanced water transport via the vasopressin V_2 receptor (103). However, some of renal effects induced by ANG (1–7) are very difficult to reconcile with the dogma on the potential roles of the ACE2/ANG (1–7)/Mas receptor axis to counteract with detrimental roles of the renin/ACE/ANG II/AT_1 receptor axis. A careful review of the above-mentioned studies reveals that ANG (1–7) may also activate the well-recognized downstream ANG II/AT_1 receptor signaling transduction to induce similar effects induced by ANG II.

New insights and perspectives into the physiological roles of ANG (1–7) acting via the Mas receptors in the cardiovascular, blood pressure, and renal regulation may be best inferred from transgenic animals with overexpression of ANG (1–7) (90, 91, 104) or ACE2 (105–107) to substantially increasing production of ANG (1–7) in the circulation or tissues or due to global or tissue-specific deletion of the Mas receptor. Santos' group has generated transgenic rats that express an ANG (1–7)-producing fusion protein, TGR(A1–7)3292, in the testis (90). Expression of ANG (1–7) in the testis acts as an ANG (1–7) biological pump to increase the plasma ANG (1–7) concentration 2.5-fold. Surprisingly, overexpression of ANG (1–7) did not alter basal blood pressure levels in TGR(A1–7)3292 rats despite of significant increases in stroke volume and cardiac index and a decrease in total peripheral resistance (90, 104). While acute intravenous infusion of ANG (1–7) induces renal vasodilatation, diuresis, and natriuresis (17, 99), GFR and 24 h urinary sodium excretion in TGR(A1–7)3292 rats are similar to those in Sprague-Dawley rats, whereas 24 h urine excretion was decreased and osmolality increased, respectively (91). The results obtained from TGR(A1–7)3292 rats appear to be contradictory to the well-known vasodepressor, diuretic and natriuretic effects of ANG (1–7). In a different study, Rentzsch et al. generated transgenic rats on a SHRSP genetic background expressing the human ACE2 in vascular smooth muscle cells by the use of the SM22 promoter, SHRSP-ACE2 (105). SHRSP-ACE2 rats have significantly elevated circulating levels of ANG (1–7), which is associated with a 15 mmHg decrease in mean arterial blood pressure and significantly attenuated responses to ANG II (105). These data suggest that vascular ACE2 overexpression may be a novel therapeutic strategy in the treatment of hypertension. Liu et al. used the adenoviral gene delivery approach to overexpress ACE2 globally and found that blood pressure was not different between control and ACE2-overexpressing Wistar rats before and after streptozotocin treatment to induce diabetic nephropathy (106). Despite of these inconsistencies, global or tissue-specific overexpression of ACE2 has been reported to reduce blood pressure or hypertension-induced injury in SHR (108, 109), and protect from ischemia-induced cardiac injury (110), and attenuate diabetic nephropathy (106).

Although the GPCR Mas was reported to be the specific receptor for ANG (1–7) more than 10 years ago (23), there is surprisingly little progress that has been made in using these Mas receptor-deficient mice (Mas-KO) to determine the physiological roles of ANG (1–7) (111–114). Too often, the reported cardiovascular, blood pressure, and renal phenotypes are sometimes contradictory between studies. Botelho-Santos reported that mean arterial pressure in anesthetized Mas-KO mice (12–16 weeks old) was not different from that of WT mice, despite of significant decreases in stroke volume and cardiac index and marked increases in vascular resistance and a decrease in blood flow in the kidney (115). Walther et al. also confirmed that neither heart rate nor blood pressure was significantly different between Mas-KO mice and controls, although salt-induced increase in blood pressure was prevented in Mas-KO mice (116, 117). Subsequent studies from the same groups of investigators showed a significantly higher basal blood pressure in Mas-KO mice (112, 118). These differences may be explained by the difference in genetic backgrounds, in that the former Mas-KO mice were generated from mixed genetic background, 129 × C57BL/6, whereas the latter were generated from the FVB/N genetic background for seven generations (16, 119). Other studies supporting the counterregulatory roles of the ACE2/ANG (1–7)/Mas receptor axis against those of the ACE/ANG II/AT_1 receptor axis in the kidney include the development of glomerular hyperfiltration and microalbuminuria in Mas-KO mice (120). However, Esteban et al. recently shown that ANG (1–7), via the Mas receptor, has proinflammatory properties at least as potent as those of ANG II and TNFα in the kidney (121). Clearly, controversies remain with respect to the specific roles of the Mas receptor in mediating the effects of ANG (1–7) in the kidney (122).

CURRENT INSIGHTS AND FUTURE PERSPECTIVES ON THE ROLES OF THE PRORENIN/PRR/MAP KINASES ERK 1/2 AXIS IN THE KIDNEY

A new frontier in the RAS research field emerges during recent years is the prorenin/PRR/MAP kinases ERK 1/2 axis. According

to the classical dogma, prorenin is primarily synthesized in the juxtaglomerular (JGA) cells and is biologically inactive (123). Prorenin becomes active renin in JGA cells and is released in response to a decrease in blood pressure (hypotension), activation of renal sympathetic nerves, and sodium depletion. Renin released from JGA cells initiates the activation of the RAS by hydrolyzing circulating and tissue AGT to generate ANG I (123). This classical dogma may be subject to significant revisions as a result of recent progresses being made in the field.

There is strong evidence that prorenin may also be constitutively secreted from the kidney, and to a less extent from extrarenal tissues including eyes and adrenal glands (11–13, 22, 124–126). Whether prorenin is physiologically or pathophysiologically relevant remains an issue of intensive debate before and after Ngyuen et al. first cloned the prorenin/renin receptor (PRR) (22, 127). PRR has a single transmembrane domain and 350-amino acid (22, 127). It has specific binding site not only for the inactive precursor prorenin, but also for active renin, which is the key initiator of the ACE/ANG II/AT_1 receptor axis. Thus it is difficult to determine whether it is prorenin or active renin that binds and activates PRR under physiological conditions and in cardiovascular, diabetic and renal diseases. However, it has been shown that prorenin has a "handle" region with higher affinity for PRR than renin, which binds to PRR to initiate the catalytic activity of prorenin, leading the activation of the prorenin/PRR/MAP kinases ERK1/2 axis (12, 22, 127). It has been further suggested that a decoy "handle" region peptide (HRP) may thus target this "handle" region by competitively inhibiting the binding of prorenin to the PRR, and produce pharmacological and therapeutical effects in treating cardiovascular, hypertensive, and diabetic diseases (31, 128, 129). Whether HRP may specifically block PRR to exert beneficial therapeutic effects remains highly controversial (13, 126, 130). Several studies have been unable to confirm the role(s) of prorenin and the effects of HRP in cultured cells and animals (131–133). Even if HRP is indeed effective in blocking prorenin and PRR interactions, its clinical relevance remains unknown due to its peptide properties. The renin-specific inhibitors have been developed to treat hypertension and cardiovascular and kidney diseases. Whether the renin inhibitors are therapeutically superior to classical ACE inhibitors or ARBs remains to be determined. If prorenin and PRR indeed play important physiological and pathophysiological roles in blood pressure regulation and pathologies of cardiovascular, renal, and diabetic diseases, the development of orally active PRR-specific inhibitors to block prorenin-induced activation of PRR will be highly necessary.

While prorenin and renin are present primarily in JGAs of the renal cortex under physiological conditions, PRR is reportedly expressed in glomerular mesangial cells and the subendothelium of renal arteries (22), and in the apical membrane of intercalated cells in collecting ducts (134). Activation of PRR by the rat recombinant prorenin has been shown to stimulate cyclooxygenase-2 (COX-2)-derived prostaglandins via MAP kinases 1/2 in rat renal inner medullary collecting duct cells (IMCD) (135). Furthermore, prorenin appears to activate the prorenin/PRR/MAP kinases ERK 1/2 axis to increase V-ATPase activity (vacuolar-type H^+-ATPase) at nanomolar concentrations in intercalated cells, MDCK.C11 (136). PRR has been described as an accessory subunit for V-ATPase, and may function as a H^+-ATPase subunit in distal nephron segments of the kidney (137). However, Oshima et al. reported that PRR may be necessary for the maintenance of normal podocyte structure and function (138).

Activation of PRR by prorenin may be implicated in the development and progression of renal diseases in animal models. Kaneshiro et al. generated transgenic rats with overexpression of human prorenin/renin, and showed that these rats slowly developed nephropathy via MAP Kinases ERK1/2 signaling through an ANG II-independent mechanism (139). Ichihara et al. showed that the prorenin/PRR/MAP kinases ERK1/2 axis plays a pivotal role in the development of diabetic nephropathy in ANG II AT_{1a} receptor-deficient mice (129) and in diabetic rats (128). Furthermore, Prieto and Navar' group has shown that prorenin and PRR expression are markedly increased in the collecting ducts of distal nephron in ANG II-induced and 2K1C renal hypertension, although the precise roles of prorenin and PRR as a byproduct or mediator of ANG II-dependent hypertension remain unknown (69, 72).

Overall, prorenin and PRR have been studied extensively during last several years and appear to play important roles under certain biological, physiological, and pathophysiological conditions or animal models (12, 140, 141). However, their specific roles in the physiological regulation of cardiovascular, blood pressure, and renal function and the development of cardiovascular, hypertensive, and renal diseases in humans remain to be confirmed (13, 126). Recently, Reudelhuber (13) and Campbell (126) have provided excellent critical reviews in these issues. One key issue is that mice is known to express abundant prorenin and PRR than rats and humans, but they do not develop hypertension or cardiovascular and renal diseases. Another issue is that it is difficult to prove the activation of PRR by prorenin independent from renin without genetic deletion of PRR in mice, which is lethal at present (142, 143). The third issue is that prorenin may be overexpressed in transgenic rats or mice with hundreds or even thousands of time higher than those in humans to manifest cardiovascular, blood pressure, and renal phenotypes, which is unlikely replicated in normal and diseased humans (125, 144, 145). Finally, some, if not all, prorenin-induced blood pressure and cardiovascular and renal responses remain to be ANG II/AT_1 receptor-dependent (13, 32, 126).

CURRENT INSIGHTS AND FUTURE PERSPECTIVES ON THE ROLES OF INTRACRINE OR INTRACELLULAR ANG II IN THE KIDNEY

A new frontier in the RAS research field has recently gained increasing attention (33–37, 146). This is now recognized as an intracrine or intracellular RAS. Many tissues or cells may synthesize ANG II within the cells, wherein ANG II bind to intracellular and/or nuclear receptors, activate downstream signaling pathways, and induce cellular and/or nuclear responses independent of cell surface receptors (33, 147–150). Alternatively, we and others have shown that circulating, paracrine, and autacrine ANG II may enter cells via AT_1 (AT_{1a}) receptor-mediated uptake or internalization in the kidney, primarily in the proximal tubule (48, 52, 57, 58, 151, 152). There is substantial evidence that not all internalized ANG II are degraded in lysosomes as the classical

receptor pharmacology dogma suggests, and ANG II may escape from degradation by lysosomes. For example, systemically infused ^{125}I-labeled ANG I or ^{125}I-ANG II have been identified and quantified in pig kidney cells (55, 56, 85) and rat kidney cells (153, 154). Imig et al. demonstrated ACE, ANG II and AT_{1a} receptors in cortical endosomes of the rat kidney (52). In ANG II-infused rat kidney, we found that ANG II levels in the renal cortical light and heavy endosomes were up to 10-fold higher compared with control rats (48). Intracellular accumulation of ANG II in the proximal tubule of the kidney may be blocked by the AT_1 receptor blockers candesartan (48), losartan or in AT_{1a}-KO mice (57, 58). To further support the new intracellular ANG II paradigm, specific and functional AT_1 (AT_{1a}) and AT_2 receptors have been demonstrated in rat renal cortical endosomes (48, 52), mouse kidney proximal tubule mitochondria (155), and rat or sheep renal cortical nuclei (156–159). Thus the localization of intracellular and/or nuclear ANG II and AT_1/AT_2 receptors provides evidence that ANG II may interact with AT_1/AT_2 receptors within the kidney cells to induce biological and physiological effects.

In the kidney, previous studies demonstrated that AT_{1a} receptor-mediated endocytosis of ANG II is required for ANG II-stimulated proximal tubular sodium transport or uptake of $^{22}Na^+$ (160–163). We also showed that AT_{1a} receptor-mediated ANG II uptake was associated the inhibition of cAMP signaling (151), activation of NF-κB signaling (163), and increases in lysate and membrane phosphorylated NHE3 proteins in proximal tubule cells (164). However these studies by no means provide direct evidence to support the role of intracellular and/or nuclear ANG II in the regulation of renal function and blood pressure responses. Several recent proof-of-concept studies have provided some new insights and perspectives into the potential roles of intracellular ANG II in the kidney. First, we used the single cell microinjection approach as described by Haller et al. (149) to determine the role of intracellular ANG II and its receptors in mobilizing intracellular calcium responses in rabbit proximal tubule cells (150). While the cell surface AT_1 receptors were blocked by losartan in the medium, ANG II was directly microinjected into single monolayer proximal tubule cells subcultured on glass coverslips with or without the AT_1 receptor blocker losartan or the AT_2 receptor blocker PD123319. Microinjection of ANG II evoked marked increases in intracellular calcium responses, which were largely blocked by concurrent microinjection of losartan, but not by PD123319, indicating an AT_1 receptor-mediated response (150). In a subsequent proof-of-concept study, we isolated fresh nuclei from the renal cortex of the rat kidney and incubated the nuclei with ANG II in an *in vitro* transcriptional system to determine the transcriptional effects of ANG II (156). We demonstrated that ANG II directly stimulated nuclear AT_{1a} receptors to increase *in vitro* transcription of mRNAs for TGF-β1, MCP-1, and NHE3, which are known to play important roles in cell proliferation and hypertrophy, tissue fibrosis, and sodium transport in the kidney. Again, these nuclear transcriptional responses to ANG II were blocked by losartan but not by PD123319, further underlying an important role of AT_1 (AT_{1a}) receptors in proximal tubule cells. In alternative proof-of-concept studies, Chappell's group showed that ANG II and ANG (1–7) directly stimulated nuclear AT_2 or ANG (1–7) receptors to increase NO production, and activated AT_1 receptors to increase super oxide production in freshly isolated sheep kidney nuclei (157, 158, 165).

Although it has been hypothesized that intracellular ANG II may play a physiological role in the cardiovascular and renal systems and blood pressure regulation, there was no direct evidence supporting this role until recently. Cook's group was instrumental in generating transgenic mice that globally express an ANG II fused downstream of ECFP in all tissues, and its expression was driven by the mouse metallothionein promoter (146). The fusion protein, ECFP/ANG II, lacks a secretory signal, so its expression is retained intracellularly. Although plasma ANG II was not altered in these transgenic mice, basal blood pressure was significantly increased by approximately 16 mmHg, and thrombotic microangiopathy or microthrombosis was developed within the glomerular capillaries and small vessels (146). This study demonstrated for the first time that overexpression of an intracellular ANG II fusion protein is sufficient to elevate basal blood pressure and induce renal pathology. To determine the role of intracellular ANG II in the regulation of proximal tubular reabsorption and blood pressure, we performed intrarenal transfer of the same ECFP/ANG II selectively in the proximal tubule of rats and mice (**Figures 3** and **4**) (42, 77, 166). We showed that proximal tubule-specific overexpression of intracellular ECFP/ANG II significantly increased blood pressure by approximately 15–20 mmHg in rats and C57BL/6J mice 7 days after the gene transfer, and the blood pressure responses were blocked by losartan treatment or in AT_{1a}-KO mice (42, 166, 167). Furthermore, the hypertensive effects of proximal tubule-specific ECFP/ANG II expression were associated with decreases in 24 h urinary sodium excretion, increases in phosphorylated ERK1/2, lysate, and membrane NHE3 proteins in freshly isolated proximal tubules and decrease in fractional lithium excretion (42, 166, 167). These responses are consistently with the concept that intracellular ANG II may stimulate AT_1 receptor to increase proximal tubular sodium and fluid reabsorption, which in turn contributes to the regulation of blood pressure.

CURRENT INSIGHTS AND FUTURE PERSPECTIVES ON THE ROLES OF ANG III, ANG IV, OR ANG A IN THE KIDNEY

Two other smaller ANG peptides, ANG III and ANG IV, have been reported to have significant effects on blood pressure and renal function (2, 18, 19, 24, 28, 168). ANG III, ANG (2–8), is derived from the metabolism of ANG II by aminopeptidase A. To date, there is no evidence for a specific ANG III receptor. In the kidney, ANG III normally binds to the AT_1 receptor and AT_2 receptors, and the reported natriuretic and antinatriuretic effects of ANG III may be dose-dependent on whether the AT_1 or AT_2 receptor is activated (2, 18, 28, 168). When centrally administrated, ANG III appears to enhance vasopressin release, thirst, and blood pressure (169). Most recently, Carey's group has shown that intrarenal interstitial ANG III infusion induced natriuresis via the AT_2 receptor/nitric oxide/cGMP-dependent mechanism (19, 24, 170).

In the kidney, ANG III can be further hydrolyzed by aminopeptidase N to generate ANG IV or ANG (3–8) (2, 18, 171, 172). The

receptor for ANG IV, AT_4, has been identified as an IRAP, associated with the M1 family of aminopeptidases and GLUT4 vesicles in insulin-responsive cells (21, 173). The AT_4 receptor has been localized in different tissues in the brain, heart, blood vessels, and kidney (20, 26, 174, 175). It is worth mentioning that other peptides such as LVV-hemorphin 7 also bind the AT_4 receptor (21, 175, 176), and ANG IV also stimulates the AT_1 receptor (20, 177–179). ANG IV is implicated in the regulation of learning and memory in rodents and improves memory in animal models of amnesia, and has been suggested to be used to treat Alzheimer's disease (21, 175, 176). Aminopeptidases A and N are particularly abundant in the kidney, especially in the glomeruli and proximal nephron segment (2, 18, 171, 172). We have previously shown that nanomolar concentrations of ANG IV may increase blood pressure and induce renal vasoconstriction via the AT_1 receptor-activated signaling in anesthetized rats (20), but others showed increased renal cortical blood flow and decreases Na^+ transport in isolated renal proximal tubules (26, 27). Furthermore, Ang IV infusion into the renal artery decreased RBF, without any change in blood pressure, suggesting an AT_1-mediated constriction in renal vascular bed (180). Other Ang IV responses in different kidney cells appear to occur via AT_1 receptor activation as well, such as Ca^{2+} mobilization in glomerular mesangial cells (20, 178), and in human proximal tubules cells (181). In wild-type and AT_{1a}, AT_{1b}, AT_2 receptor and IRAP knockout mice, Ang IV was found to mediate blood pressure and renal vasoconstrictor effects via AT_{1a} receptors (182, 183). Thus, the physiological roles of ANG IV/AT_4 receptors in blood pressure and renal regulation remain uncertain, given that circulating and tissue ANG IV levels are unlikely to be higher than those of ANG II in health and disease and that ANG IV also binds and stimulates AT_1 receptors.

Recently, an ANG peptide-derived fragment called ANG A (Ala-Arg-Val-Tyr-Ile-His-Pro-Phe) has been described in the plasma of healthy humans and with increased concentrations in end-stage renal failure patients (184–186). ANG A may be generated from ANG II by decarboxylation of Asp^1 and have the same affinity for AT_1 receptor as ANG II, and higher affinity for AT_2 receptor (186, 187). In rats, ANG A and ANG II have similar hypertensive effects, but ANG A possesses a greater proliferative effect on vascular smooth muscle cells than ANG II (186, 187). In genetically modified mice and in normotensive and hypertensive rats, ANG A induces pressor and renal vasoconstrictor responses also in the AT_1 receptor-dependent manner (186). The role(s) of ANG A and its receptor-mediated downstream signaling mechanisms remain incompletely understood. However, since the ANG II/AT_1 receptor-dependent pathways are involved, the translational impact of the ANG A research may likely be limited because the available ARBs are expected to block the actions of ANG A in tissues.

CONCLUDING REMARKS

In summary, the RAS has evolved from a circulating and endocrine system to multiple endocrine, paracrine, and intracrine systems. At least four axes for the RAS have been identified in the kidney and other tissues (**Figure 1**) and their physiological and pathophysiological roles explored. These include the most-studied and recognized classical renin/ACE/ANG II/AT_1 and AT_2 receptor axis, and three new axes including the ACE2/ANG (1–7)/Mas receptor, the prorenin/PRR/MAP kinases ERK1/2, and the ANG IV/AT_4/IRAP axis. Each of these axes has its own enzyme(s), substrate(s), agonist(s), or ligand(s), respective receptor and downstream signaling mechanisms. Thus the roles of the RAS have been extended far beyond the regulation of blood pressure, aldosterone synthesis, and body salt and fluid homeostasis by the AT_1 and AT_2 receptors. Indeed, novel actions have been described for each axis of the entire RAS, interactions of which undoubtedly contribute to the overall regulation of cardiovascular, neural, and renal function and blood pressure. It is now well understood that imbalance of actions induced by ANG II and its smaller metabolites, ANG (1–7), ANG III, and ANG IV in favoring increases in tissue ANG II formation and the activation of the ACE/ANG II/AT_1 receptor axis may lead to the development of hypertension and ANG II-induced target organ injury and diseases. Conversely, genetic and pharmacological approaches to increase the production of ANG (1–7) via overexpression of ACE2 or ANG (1–7) fusion protein may partially oppose the well-recognized actions of ANG II through activation of the Mas receptor. However, despite of the great progress new challenges still remain in the RAS research field. For example, the challenges for studying the classical ACE/ANG II/AT_1 receptor axis may include determining the roles of intracellular and nuclear ANG II and its receptors play in the nuclear and/or transcriptional responses to ANG II in various diseases, and developing novel molecular and pharmacological approaches or drugs to block the transcriptional actions of intracellular ANG II. Since ANG III, ANG IV, and ANG A may also function as potent agonists of the AT_1 and/or AT_2 receptor to alter blood pressure and renal function, their physiological and pathophysiological roles remain to be determined. Similarly, the challenges for studying the roles of the prorenin/PRR/MAP kinases ERK1/2 axis is how to better differentiate the ANG II/AT_1 receptor-dependent and independent effects of prorenin/PRR activation, and whether blockade of prorenin activation provides additional and beneficial effects beyond renin and ACE inhibitors or AT_1 receptor blockers. Finally, although the ACE/ANG (1–7)/Mas receptor axis may play a counterregulatory role to oppose the effects of the renin/ACE/ANG II/AT_1 receptor axis, the development and clinical relevance of the orally active agonists or compounds that promote metabolism of ANG II to increase ANG (1–7) production or to activate the Mas receptor still await clinical trials.

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Update on the angiotensin converting enzyme 2-angiotensin (1–7)-Mas receptor axis: fetal programing, sex differences, and intracellular pathways

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The renin-angiotensin-system (RAS) constitutes an important hormonal system in the physiological regulation of blood pressure. Indeed, dysregulation of the RAS may lead to the development of cardiovascular pathologies including kidney injury. Moreover, the blockade of this system by the inhibition of angiotensin converting enzyme (ACE) or antagonism of the angiotensin type 1 receptor (AT_1R) constitutes an effective therapeutic regimen. It is now apparent with the identification of multiple components of the RAS that the system is comprised of different angiotensin peptides with diverse biological actions mediated by distinct receptor subtypes. The classic RAS can be defined as the ACE-Ang II-AT_1R axis that promotes vasoconstriction, sodium retention, and other mechanisms to maintain blood pressure, as well as increased oxidative stress, fibrosis, cellular growth, and inflammation in pathological conditions. In contrast, the non-classical RAS composed of the ACE2-Ang-(1–7)-Mas receptor axis generally opposes the actions of a stimulated Ang II-AT_1R axis through an increase in nitric oxide and prostaglandins and mediates vasodilation, natriuresis, diuresis, and oxidative stress. Thus, a reduced tone of the Ang-(1–7) system may contribute to these pathologies as well. Moreover, the non-classical RAS components may contribute to the effects of therapeutic blockade of the classical system to reduce blood pressure and attenuate various indices of renal injury. The review considers recent studies on the ACE2-Ang-(1–7)-Mas receptor axis regarding the precursor for Ang-(1–7), the intracellular expression and sex differences of this system, as well as an emerging role of the Ang1-(1–7) pathway in fetal programing events and cardiovascular dysfunction.

Keywords: Ang-(1–7), Ala[1]-Ang-(1–7), ACE2, ACE, Mas receptor, Mas-related receptor D, fetal programing

INTRODUCTION

Over the past 20 years the concept of the renin-angiotensin-system (RAS) as a monolithic endocrine system reflected primarily by the interaction of the peptide Angiotensin II (Ang II) with the AT_1-receptor subtype has undergone extensive revision. The emerging view of alternative pathways within the RAS that may functionally antagonize the Ang II-AT_1-receptor axis may be traced back to both the characterization of the AT_2 receptor subtype and the identification of the heptapeptide des-[Phe^8]-Angiotensin II or Angiotensin-(1–7) [Ang-(1–7)] in the circulation and various tissues (1–4). Since that time, the elaboration of distinct biochemical components that comprise the "Ang-(1–7) axis" is now firmly established with the identification of a unique receptor for Ang-(1–7) – the G-protein coupled Mas receptor, selective antagonists and agonists for the receptor, and an angiotensin II converting enzyme (ACE2) that catalyzes the processing of Ang II to Ang-(1–7) (5–9). In addition to the identification of the components of the Ang-(1–7) system, there is the recognition of various signaling pathways including nitric oxide (NO), prostaglandins, and cellular phosphatases that are stimulated by the peptide (10, 11). Although the early studies of Ang-(1–7) primarily sought to establish a role for Ang-(1–7) in the regulation of blood pressure, particularly as endogenous levels of the peptide increase markedly following angiotensin converting enzyme (ACE) or AT_1-receptor blockade; the pressure-independent actions of the Ang-(1–7) axis should be considered with perhaps equal importance (6, 12, 13). Indeed, the beneficial actions of Ang-(1–7) system encompass various pathologies from cancer and the anti-proliferative actions of the peptide to diabetes and the cellular effects on stem cells (8, 9, 14). In turn, deficiencies in Ang-(1–7) that contribute to autonomic dysfunction were apparent in hypertension (15) and aging (16); Ang-(1–7) deficiency in hypertension was restored by ACE inhibitor treatment (17) or chronic Ang-(1–7) replacement (18). The breadth of these effects is not surprising as the RAS is a tissue system whose protein and peptide components are expressed in essentially every organ and whose actions are implicated in numerous physiological events that influence renal, neuronal, cardiac, pancreatic, vascular, adrenal, pituitary, cognitive, aging, inflammatory, and reproductive functions (19). As the Ang II-AT_1-receptor axis of the RAS is increasingly recognized as a key regulatory pathway in various tissues and cells, the counter-balancing Ang-(1–7) axis should be evident as well. In this review, we consider the current

literature on the ACE2-Ang-(1–7)-Mas receptor axis regarding the sources for Ang-(1–7), the intracellular expression of this system, the emerging role of Ang1-(1–7) pathway in fetal programing events and cardiovascular dysfunction, and finally, the evidence for sex-dependent regulation and function of the Ang-(1–7) axis.

SOURCES OF ANGIOTENSIN-(1–7)

ENDOPEPTIDASES

Angiotensinogen, a glycosylated protein that is primarily synthesized and secreted by the liver as well as other tissues is the sole precursor for angiotensin peptides (20). The only known substrate for the aspartyl protease renin is angiotensinogen which releases the decapeptide Ang I from the amino-terminal portion of the protein (**Figure 1**). Ang I is then cleaved by ACE to form the bioactive peptide Ang II. Early studies revealed that endogenous levels of both Ang I and Ang-(1–7) were markedly increased following the administration of ACE inhibitors (21, 22). The augmented response in Ang-(1–7) suggested that the circulating peptide may contribute to the beneficial actions of the inhibition of ACE pathway in addition to that of reducing endogenous levels of Ang II. The increase in Ang-(1–7) in the presence of ACE blockade necessitates a processing pathway independent of the formation of Ang II. Several studies subsequently showed that the endopeptidase 3.4.24.11 (neprilysin) contributed to the circulating levels of Ang-(1–7) in animals chronically treated with various ACE inhibitors (23–26). Ang I infusion in normotensive WKY and hypertensive spontaneously hypertensive rat (SHR) treated with the ACE inhibitor lisinopril resulted in higher plasma levels of Ang-(1–7) and co-administration of the neprilysin inhibitor SCH39370 but not the prolyl oligopeptidase (POP) inhibitor *z*-prolyl prolinal abolished the circulating levels of the peptide (27). Moreover, acute infusion of a similar dose of Ang II did not increase circulating Ang-(1–7) in either control or lisinopril-treated WKY and SHR. The increase in Ang-(1–7) following ACE blockade reflects both a reduction in Ang-(1–7) metabolism and alternative processing of Ang I through tissue-specific endopeptidases (23). In this regard, Pereira et al. recently demonstrated that the endopeptidase EC3.4.24.15 (thimet oligopeptidase) may contribute to formation of Ang-(1–7) in the rat hippocampus (28). Interestingly, these investigators reported higher expression of this peptidase and the Mas receptor in a rat model of temporal lobe epilepsy suggesting a possible role of the Ang-(1–7)-Mas axis in this central pathology (28). Indeed, the study supports earlier reports of the direct processing of Ang I to Ang-(1–7) by thimet oligopeptidase in vascular smooth muscle cells and a rat hindlimb perfusion system (29, 30).

ACE2

Apart from endopeptidases that process Ang I or Ang-(1–12) to Ang-(1–7), various mono-carboxypeptidases including prolyl carboxypeptidase (PCP), POP, and the ACE homolog ACE2 generate Ang-(1–7) directly from Ang II. It should be emphasized that PCP requires an acidic pH optimum for activity, but may contribute to lysosomal pathways for metabolism of internalized Ang II or to the processing of Ang II to Ang-(1–7) in urine (31). ACE2 continues to be of primary focus given its ability to effectively metabolize Ang II and generate Ang-(1–7) (32, 33). ACE2 exhibits an efficiency constant (V_{max}/k_M or k_{cat}) for Ang II that is 10- to 100-fold higher than that of PC or POP. In this regard, the soluble form of ACE2 has been utilized as a therapeutic agent to reduce blood pressure and attenuate target organ damage in hypertensive and diabetic animal models (34–37). ACE2 mRNA expression was increased in the brain medulla following long-term AT1-receptor blockade (38). It is unclear whether the beneficial effects of ACE2 administration reflect the reduction in Ang II, the enhanced formation of Ang-(1–7) or the increased ratio of Ang-(1–7) to Ang II. Moreover, Turner and colleagues report that soluble ACE2 attenuated the integrin-dependent stimulation of focal adhesion kinase (FAK) and increased the expression of the Akt kinase suggesting the peptidase may have direct cellular effects apart from its peptidase activity (39).

In addition to the functional role of ACE2 that catalyzes the conversion of Ang II to Ang-(1–7), the peptidase may serve as a biomarker of renal and cardiac pathologies. Two studies in type I (streptozotocin-induced) and type II (db/db mice) diabetic models reported an early increase in the urinary excretion of ACE2 (40, 41). The enhanced excretion of ACE2 in db/db mice closely correlated to the increase in albuminuria or proteinuria. Moreover, chronic treatment with insulin-sensitizing agent rosiglitazone improved the metabolic balance in the db/db mice and reduced the excretion of both ACE2 and albumin (40). In contrast to the reduction in urinary levels of ACE2, the increased renal expression of ACE2 in the db/db mouse was not altered by rosiglitazone which may reflect an added therapeutic benefit to maintain the peptidase in the diabetic kidney (40). An important aspect of the two latter studies suggests that in the diabetic kidney, the development of tissue injury should not necessarily be interpreted as arising from a deficit in ACE2 expression. Indeed, the increase in tissue and urinary levels of ACE2 in pathological conditions may reflect a compensatory response to alter the balance of Ang II and Ang-(1–7) pathways within a particular tissue or cell type (7). In this regard, the deleterious effects of an

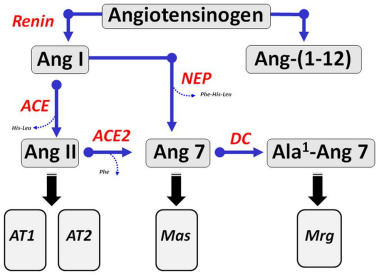

FIGURE 1 | Enzymatic cascade of angiotensin peptide formation and metabolism. Renin cleaves the precursor protein angiotensinogen to angiotensin-(1–10) (Ang I) which is further processed to the biologically active peptides Ang-(1–8) (Ang II) by angiotensin converting enzyme (ACE) and Ang-(1–7) by endopeptidases such as neprilysin (NEP). Ang II undergoes further processing at the carboxyl terminus by the carboxypeptidase ACE2 to yield Ang-(1–7) (Ang 7). Ang-(1–7) undergoes decarboxylation (DC) of the aspartic acid residue to form Ala^1-Ang-(1–7) (Ala^1-Ang 7). The dodecapeptide Ang-(1–12) is derived from the hydrolysis of the Tyr^{12}-Tyr^{13} bond of rat angiotensinogen by an unknown enzymatic pathway. Ang II recognizes both AT_1 and AT_2 receptors. Ang-(1–7) activates the Mas receptor and Ala^1-Ang-(1–7) recognizes the Mas-D related receptor (Mrg).

ACE2 inhibitor or knockdown of the enzyme may be particularly evident under conditions of enhanced ACE2 expression. The circulating levels of ACE2, which are typically low to not detectable, are also increased in experimental conditions of diabetes. We show in a model of diabetic hypertension that circulating ACE2 activity increased over fivefold in female mRen2.Lewis rats (42). However, serum ACE activity also increased suggesting that the potential beneficial effects of higher ACE2 may be offset by ACE acting to increase Ang II and metabolize Ang-(1–7). Indeed, plasma levels of Ang-(1–7) were not changed in the diabetic mRen2.Lewis despite the marked increase in ACE2 activity. Moreover, circulating ACE activity was substantially higher than that of ACE2 when assessed under similar incubation and substrate conditions for each enzyme (42).

In the db/db mice, infusion of exogenous ACE2 that markedly increased serum levels of the enzyme did not alter urinary ACE2 suggesting that the enzyme is not readily filtered by the glomerulus (41). One mechanism for the increase in urinary excretion of ACE2 is the regulated shedding of the enzyme from the apical face of the proximal tubules (**Figure 2**). Studies by Lambert and colleagues originally reported that the disintegrin and metalloproteinase (ADAM17) secretase was responsible for the release of ACE2 (43). A subsequent report identified a specific sequence of the juxtamembrane stalk of ACE2 hydrolyzed that was by ADAM17 to release the peptidase from human pulmonary epithelial cells (44). In proximal epithelial cells of the db/db mouse kidney, there was extensive overlap of ACE2 and ADAM17 immunostaining (40). Moreover, rosiglitazone treatment attenuated ADAM17 expression which may contribute to the reduced shedding of ACE2 into the tubular fluid and subsequent excretion in the urine. In addition to the shedding of ACE2, ADAM17 may influence tissue damage by the release of the tethered inflammatory factors TNFα, EGF, and TGFα that subsequently activate their respective receptors in an autocrine or paracrine manner (45). If expression of ACE2 on the apical membrane of the tubules contributes to the regulation of the local concentrations of Ang II, an increase in ADAM17 may lead to inflammatory and fibrotic events through enhanced Ang II-AT_1-receptor signaling, as well as increased cytokine and growth factor activation (**Figure 2**). Lazartigues and colleagues report that knockdown of ADAM17 in the brain of DOCA-salt mice reduced blood pressure, and increased the tissue expression of ACE2 (46). In this model of neurogenic hypertension, the benefit of ADAM17 knockdown may reflect a reduction of Ang II in brain; however, the direct effects on the release of EGF and other cytokines cannot be discounted. Indeed, the transactivation of the EGF receptor (EGFR) and signaling pathways is a key signaling event of the Ang II-AT_1-receptor pathway (47). The increased shedding of ACE2 may also reduce levels of Ang-(1–7) and attenuate the inhibitory actions on the Ang II-AT_1-receptor axis and other pro-inflammatory and pro-fibrotic pathways. Akhtar et al. recently reported that Ang-(1–7) attenuated EGFR activation in response to Ang II, as well as reduced the extent of renal injury in the diabetic SHR (48). Moreover, increasing evidence suggests that one of the primary pathways activated by Ang-(1–7) is the stimulation of various cellular phosphatases (PTP) including SHP-1 and DUSP-1 that may attenuate activated kinase-dependent pathways (49–53) (**Figure 2**).

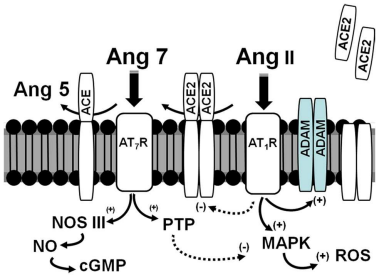

FIGURE 2 | Scheme for the interaction of ACE2 and ADAMs on the apical surface of the proximal tubules in the diabetic kidney. Ang II binds to the AT_1 receptor (AT_1R) and stimulates MAP kinase (MAPK) pathways and production of reactive oxygen species (ROS). The Ang II-AT_1-receptor axis may attenuate ACE2 expression but increase ADAM levels. ACE2 is anchored to the apical membrane and directly converts Ang II to Ang-(1–7) (Ang 7); ACE also anchored to the membrane metabolizes Ang-(1–7) to Ang-(1–5) (Ang 5). Ang-(1–7) recognizes the AT_7/Mas receptor (AT_7R) to antagonize the actions of the Ang II-AT_1R by stimulation of protein phosphatases (PTP) and nitric oxide synthase (NOS) to form nitric oxide (NO) and cGMP. In pathological conditions, increased expression of ADAMs may hydrolyze ACE2 away from the apical surface to increase local concentrations of Ang II and reduce the levels of Ang-(1–7).

ANGIOTENSIN-(1–12)

Nagata and colleagues identified a novel endogenous angiotensin peptide termed Ang-(1–12) that contains the first 12 amino acids of the N-terminal sequence of rat angiotensinogen (Asp^1-Arg^2-Val^3-Tyr^4-Ile^5-His^6-Pro^7-Phe^8-His^9-Leu^{10}-Leu^{11}-Tyr^{12}) (54) (**Figure 1**). These investigators developed antibodies directed to the amino- and carboxyl-terminal sequences of Ang-(1–12) and demonstrated expression of Ang-(1–12) in essentially all tissues that contain Ang II with the highest levels in the intestine, brain, heart, plasma, and kidney of rat. Differential expression of Ang-(1–12) was evident in the heart and kidney of the SHR and the normotensive control Wistar Kyoto strain (WKY) (55). An antibody specific to the C-terminal sequence of rat Ang-(1–12) including Leu^{11}-Tyr^{12} revealed selective staining in cardiac myocytes and proximal tubule cells of the kidney. The site of hydrolysis for formation of Ang-(1–12) from rat angiotensinogen occurs at residues Tyr^{12}-Tyr^{13} which is distinct from the Leu^{10}-Leu^{11} sequence cleaved by renin to form Ang I. Thus, the generation of Ang-(1–12) is likely through a non-renin dependent pathway and may be apparent in conditions of low or suppressed renin activity, particularly with the use of selective renin inhibitors. Similar to Ang I, Ang-(1–12) can be hydrolyzed at the Phe^8-His^9 bond by ACE or chymase to form Ang II (54, 56, 57). The conversion of Ang-(1–12) to Ang II by ACE in the circulation is consistent with the acute increase in blood pressure following an infusion of Ang-(1–12) in normotensive rats, as well as the blockade of the pressor response by either an ACE inhibitor or AT_1-receptor antagonist. Arnold et al. also find that central Ang-(1–12) administration attenuated baroreflex sensitivity and the response was blocked by either an ACE inhibitor or AT_1-receptor antagonist (57). Moreover, neutralization of Ang-(1–12) by intracerebroventricular (ICV) infusion of an affinity-purified antibody reduced blood pressure in the (mRen27)2 hypertensive rats consistent with

the biochemical and immunocytochemical evidence for Ang-(1–12) in the rat brain (58). To our knowledge, the latter study by Isa and colleagues is the only report to date that demonstrates an endogenous role for Ang-(1–12).

As to the Ang-(1–7) axis, we recently demonstrated that Ang-(1–12) may be an alternative substrate for the generation Ang-(1–7) in the kidney (59). Isolated cortical membranes from the kidney of the hypertensive mRen2.Lewis rat processed Ang-(1–12) to Ang-(1–7) and Ang-(1–4). We observed a similar pattern of metabolism using the recombinant forms of mouse and human neprilysin. The selective neprilysin inhibitor SCH39370 abolished the formation of Ang-(1–7). We noted a peak corresponding to Ang I in the processing of Ang-(1–12) by the cortical membranes that was also abolished by the neprilysin inhibitor suggesting the peptide may be an intermediate in the processing of Ang-(1–12) to Ang-(1–7) (59). In these studies, we also show that circulating or renal renin did not metabolize Ang-(1–12) particularly in the presence of the ACE inhibitor lisinopril which implies that the peptide lacks the minimal sequence for recognition by renin (59). Bujak-Gizycka and colleagues demonstrated the generation of Ang-(1–12) in rat aorta homogenates by a serine peptidase using Ang-(1–14) as the substrate; however, the extent that this activity will process the angiotensinogen protein to Ang-(1–12) is not currently known (60). We did not detect the conversion of Ang-(1–12) to Ang-(1–7) in serum which would be consistent with the lack of soluble forms of neprilysin in the circulation, nor were there significant levels of Ang-(1–11) suggesting the absence of processing by ACE2 or other carboxypeptidases (59). It is feasible that Ang-(1–12) may be a potential substrate for Ang-(1–7) through the initial conversion to Ang-(1–11) by ACE2 and subsequent processing to Ang-(1–9) and Ang-(1–7) by ACE. However, ACE activity is far higher in the circulation than ACE2 and Ang-(1–7) formation from Ang-(1–12) or Ang I more likely reflects endopeptidase activity. Although further studies are required to discern the endogenous pathways for the formation and processing of Ang-(1–12), the peptide constitutes a potential substrate for the conversion to either the active products Ang II or Ang-(1–7).

Ala^1-ANGIOTENSIN-(1–7) AND Pro^1-Glu^2-Ang II

In addition to the precursors to Ang-(1–7), the peptide itself may serve as a precursor to other active forms. Santos and colleagues recently identified an endogenous analog of Ang-(1–7) in which the aspartic acid residue was decarboxylated to alanine (Ala) forming Ala^1-Ang-(1–7) (**Figure 1**) (61). The Ala^1-Ang-(1–7) analog (also termed almandine) may also potentially arise from the proteolytic processing of endogenous Ala^1-Ang II (Ang A) by ACE2 (62). Similar to Ang-(1–7), Ala^1-Ang-(1–7) induced the relaxation of isolated aortic vessels and chronic infusion of the analog lowered blood pressure. Interestingly, the vascular effects of Ala^1-Ang-(1–7) were not blocked by the typical receptor antagonist D-Ala7-Ang-(1–7) (A779) against the Mas receptor, but were attenuated by D-Pro^7-Ang-(1–7) and the AT_2 receptor antagonist PD123319. This study further showed that Ala^7-Ang-(1–7) stimulated the Mas-related receptor (MrgD) and did not interact with the Mas receptor. Identification of Ala^1-Ang-(1–7) in the human circulation and in an isolated heart perfusion system was achieved by a HPLC-Mass spectrometry approach. It is worth noting that the available direct RIA or ELISA assays will not distinguish between the Asp^1- and Ala^1- forms of Ang-(1–7) since both isoforms share the identical C-terminal sequence that is typically recognized by the immunoreactive antibodies. Thus, an initial separation step such as HPLC combined with conventional immunoreactive assays will be required to routinely detect and quantify the different forms of Ang-(1–7) in the circulation and tissues. The potential importance of these findings may reflect the greater diversity of the Ang-(1–7) axis regarding the identification of both a novel ligand and receptor that contributes to vascular tone. Moreover, that the AT_2 antagonist PD12319 antagonized the actions of Ala^1-Ang-(1–7) at the MrgD receptor may explain the apparent interaction of Ang-(1–7) with the AT_2 receptor noted in several studies (63–65).

Although distinct from either Ang-(1–7) or its Ala analog, Jankowski et al. identified another endogenous ligand to the AT_7/Mas receptor in human serum termed angioprotectin (66). This peptide resembles the octapeptide Ang II but has substitutions of Pro and Glu at the first two N-terminal residues to form Pro^1-Glu^2-Ang II. Despite the fact that the angioprotectin contains both the Tyr^4 and Phe^8 residues considered to be essential to the actions of Ang II, the peptide lacked any vasoconstrictor activity in isolated aortic rings. However, the peptide induced a dose-dependent vasorelaxation of isolated vessels that was absent in vessels from the Mas-knockout mice, as well as acutely reduced blood pressure in the SHR. Moreover, Pro^1-Glu^2-Ang II stimulated NO formation in Mas-transfected CHO cells but not in the control cells. Finally, the study presented evidence for local formation of Pro^1-Glu^2-Ang II from Ang II in human endothelial cells that was enhanced by addition of exogenous proline and glutamic acid suggesting a post-transcriptional modification of Ang II. It is not known to what degree Pro^1-Glu^2-Ang II is processed by ACE2 or other carboxypeptidases to the Ang-(1–7) analog and whether Pro^1-Glu^2-Ang-(1–7) is functionally active at the either the Mas or MrgD receptors. It is also unclear the extent conventional immunoreactive assays for Ang II will detect endogenous Pro^1-Glu^2-Ang II in plasma or tissues given their identical C-terminal sequence. The circulating levels of Pro^1-Glu^2-Ang II were 15% of Ang II in humans, but the Ang II analog increased fivefold in patients with end-stage renal disease that may perhaps reflect a compensatory response in pathological conditions (66).

Ang-(1–7) METABOLISM

The endogenous levels of Ang-(1–7) are influenced by access to processing enzymes such as the carboxypeptidase ACE2 or the endopeptidases neprilysin, thimet oligopeptidase, and prolyl endopeptidase (oligopeptidase). The levels of Ang-(1–7) are also dependent on peptidases that metabolize the peptide. Similar to bradykinin and substance P, ACE plays a significant role in the hydrolysis of Ang-(1–7) to the pentapeptide Ang-(1–5) in the circulation and the proximal tubules of the kidney cortex (**Figure 2**) (22, 67). ACE inhibition increased the half-life of Ang-(1–7) sixfold in the circulation and is necessary to demonstrate the accumulation of Ang-(1–7) from both Ang I- and Ang II-dependent pathways in the renal proximal tubules (67, 68). Thus, the mechanism for the increased levels of

Ang-(1–7) following ACE inhibitor treatment reflects both protection of the peptide from ACE hydrolysis to Ang-(1–5) and shunting of Ang I to Ang-(1–7) through endopeptidase pathways such as neprilysin or thimet oligopeptidase (23). There is relatively little information on other peptidases that participate in the metabolism of Ang-(1–7) other than ACE. We recently detected an endopeptidase activity in the cerebrospinal fluid (CSF) of sheep that metabolized Ang-(1–7) at the Tyr^4-Ile^5 bond to yield Ang-(1–4) and constituted the majority of Ang-(1–7) degrading activity in CSF (69, 70). Although the identity of the peptidase is currently unknown, the activity was insensitive to inhibitors against neprilysin, thimet oligopeptidase, or neurolysin (EC3.4.24.26) (70). The Ang-(1–7) peptidase activity was abolished by the mercury-compounds *p*-chloromercuribenzoate (PCMB) and aminophenyl-mercuriacetate (APMA), as well as the chelating agents *o*-phenanthroline and EDTA, but not the cysteine epoxide inhibitor E-64 suggesting a metallopeptidase-like activity in CSF (70). The regulation of the CSF peptidase is described in the proceeding section on fetal programing.

INTRACELLULAR Ang-(1–7)-Mas RECEPTOR SYSTEM

The RAS was traditionally viewed as an endocrine system whereby circulating renin catalyzes an enzymatic cascade to form active peptide products; however, it is apparent that multiple tissues contain the necessary components for the local generation of angiotensin peptides (71, 72). These tissue systems may release the precursor angiotensinogen, the intermediate products Ang I and Ang-(1–12), or the active peptides Ang II and Ang-(1–7) to bind directly to cell surface receptors in an autocrine or paracrine manner. Robertson and Khairallah reported over 40 years ago the localization of Ang II binding sites on the chromatin fraction of vascular smooth muscle cells and cardiomyocytes suggesting an intracellular site of action for Ang II (73). Several laboratories subsequently identified Ang II receptors using classical receptor binding techniques on nuclei isolated from liver (74–76). Eggena and colleagues demonstrated that Ang II stimulated mRNA transcripts for angiotensinogen, renin, and PDGF from isolated liver nuclei suggesting that the nuclear binding sites were functional and capable of directly mediating gene expression (77, 78). Moreover, AT_1 receptors were also evident on nuclei isolated from cortical and medullary areas of the rat kidney (79–81). Ang II-AT_1-receptor stimulation on isolated renal nuclei increased mRNA expression of angiotensinogen, the sodium-hydrogen exchanger (NHE3) and the cytokine monocyte chemoattractant protein (MCP-1) (79). Ang II also elicited an immediate increase in calcium by isolated cortical nuclei or via microinjection of the peptide in intact epithelial cells (82). We find that Ang II directly stimulates reactive oxygen species (ROS) as demonstrated by the enhanced fluorescent signature of dichlorofluorescein (DCF); ROS formation was sensitive to the NAD(P)H oxidase inhibitor diphenyleneiodonium (DPI) and the AT_1 antagonist losartan (83). Blockade of phosphoinositol 3-kinase (PI3K) and protein kinase C (PKC) abolished the Ang II-AT_1-receptor-dependent stimulation of ROS in renal nuclei. In lieu of the nuclear localization of the NAD(P)H oxidase isoform NOX4, activation of AT_1 receptors may acutely stimulate ROS by a PI3K-PKC pathway and subsequent phosphorylation of NOX4 (**Figure 4**) (83–87).

The studies demonstrating nuclear AT_1 receptors within the kidney and other tissues clearly support an emerging view for the localization of various G-protein coupled receptors (GPRCs) to the nucleus (88–94). In regards to the Ang-(1–7)-Mas receptor system, O'Dowd and colleagues noted a canonical nuclear localization sequence on the Mas protein in their studies on AT_1-receptor trafficking and localization in vascular smooth muscle cells (95). We undertook a series of studies to establish an intracellular role for Ang-(1–7) in the cortical tissue and proximal tubules isolated from the sheep kidney. Immunoblot analysis of nuclei isolated from sheep proximal tubules demonstrated a single immunoreactive band of 35 kDa utilizing an affinity-purified antibody against the human Mas protein (96). Receptor binding studies with the non-selective antagonist ^{125}I-(Sarcosine^1, Threonine^8)-Ang II (Sarthran) revealed significant competition by the AT_7/Mas receptor antagonist D-Ala^7-Ang-(1–7) in nuclei isolated from the renal cortex. Functional assessment of the nuclear AT_7 receptor was then assessed with the sensitive NO fluorophore DAF in the presence or absence of the NO synthase inhibitor L-NAME. Ang-(1–7) dose-dependently increased the fluorescent signature for NO which was abolished by prior treatment with L-NAME or the Ang-(1–7) antagonist, but not antagonists to the AT_1 or AT_2 receptors. Consistent with the stimulation of NO by Ang-(1–7), protein expression for endothelial nitric oxide synthase (eNOS) and soluble guanylate cyclase (sGC) was evident in the isolated nuclei of sheep proximal tubules (96). These data further support previous studies that localized eNOS and sGC to liver nuclei, as well as the stimulation of NO and cGMP by activation of the bradykinin B2 receptor (90, 97). The exact function of the Ang-(1–7) axis of the RAS within the nucleus is not known; however, we hypothesize this system may antagonize the intracellular actions of the Ang II-AT_1-receptor pathway. To address this possibility, we assessed the influence of the selective ACE2 inhibitor MLN4760 and the Mas receptor antagonist on the activation of ROS by Ang II in renal cortical nuclei. The Ang II-AT_1-receptor dependent increase in ROS was significantly augmented to a similar extent by treatment of nuclei with either the ACE2 inhibitor or the AT_7 receptor antagonist (98). That both MLN4760 and D-Ala^7-Ang-(1–7) increased the stimulation of ROS suggests that the conversion to Ang-(1–7) by ACE2 antagonizes the actions of the Ang II-AT_1-receptor axis on the nucleus. It is possible that that simply blocking the degradation of Ang II with the ACE2 inhibitor may augment the actions of Ang II; however, the comparable effects of the AT_7 receptor antagonist D-Ala^7-Ang-(1–7) suggests a distinct role for Ang-(1–7). Since the Ang-(1–7) antagonist is a peptide and may potentially interact with ACE2, we further demonstrated that D-Ala^7-Ang-(1–7) does not inhibit nuclear ACE2 activity as assessed by the HPLC-based conversion of Ang II to Ang-(1–7). Moreover, our studies suggest that the processing of Ang II to Ang-(1–7) by ACE2 on the nuclear membrane leads to the activation of signaling pathways distinct from that of Ang II (98). We do not know, however, whether the attenuation of ROS production by Ang-(1–7) involves the stimulation of NO or other signaling pathways. As previously discussed, Ang-(1–7) may attenuate the actions of Ang II and other growth hormones by the activation of intracellular phosphatases such as the dual specificity phosphatases MKP-1 and SHP-1 (50, 51). Several classes of phosphatases including MKP-1 traffic to the

nucleus; however, it is unknown whether Ang-(1–7) can influence these enzymes to attenuate the actions of Ang II (99).

Clearly, one issue regarding the intracellular RAS and other peptidergic systems is the localization of the components within the cell. The nucleus is composed of two distinct bilayers termed the outer (OMN) and inner (INM) nuclear membranes. Nuclear pore proteins traverse both membrane domains and facilitate transport between the cytosol and the nuclear matrix which contains the chromatin-DNA complex. Portions of the ONM are continuous with the endoplasmic reticulum (ER) such that perinuclear space is shared with the ER. The nuclear envelope comprising both OMN and INM invaginates into the nuclear matrix creating a nuclear reticulum that is key in the regulated release of nuclear Ca^{2+} (100–102). Although various studies have localized GPRCs primarily to the nuclear envelope and matrix, it is currently unclear how the peptide ligands target the nuclear GPRCs, as well as the precise coupling of the receptors to their signaling pathways within the nucleus. Moreover, elucidation of the pathways that deliver peptide ligands to their respective intracellular receptors, as well as the intracellular regulation under normal and pathological conditions has not been established. As to the intracellular expression of angiotensins in the kidney, there is evidence for expression and uptake of angiotensinogen, as well as the uptake of Ang II and Ang-(1–7) by protein transporters such as megalin (71, 103–106). In addition, AT_1-receptor mediated internalization of Ang II may contribute to the intracellular content of the peptide (72, 89). In this regard, intracellular peptidases such as ACE2 may potentially process the internalized Ang II to Ang-(1–7) as alternative pathway to attenuate AT_1-receptor activity and stimulate the cellular actions of Ang-(1–7). Utilizing the renal epithelial NRK-52E cell line, we find evidence for the nuclear localization of angiotensinogen (**Figure 3**, left panels) consistent with earlier findings by Sherrod and colleagues regarding nuclear angiotensinogen in brain astrocytes and isolated nuclei of sheep proximal tubules (96, 107, 108). Interestingly, a second antibody directed to the Ang I sequence of angiotensinogen failed to detect the protein in the nucleus of the NRK-52E cells suggesting that enzymatic processing of the precursor may occur in this compartment (107). In support of an intracellular processing pathway, renin expression was also evident in the nucleus of the NRK cells (**Figure 3**, right panels). Isolated nuclei exhibited both renin and prorenin activity (following activation by trypsin) that was sensitive to the specific renin inhibitor aliskiren (**Figure 3**, bottom left panel), as well as immunoreactive levels of Ang II and Ang-(1–7) (107). In addition, peptide metabolism studies in isolated nuclei revealed the direct conversion of Ang I to Ang-(1–7) that was essentially abolished by a selective inhibitor (CPP) of the metalloendopeptidase thimet oligopeptidase (**Figure 3**, bottom right panel). Others have reported the nuclear expression of thimet oligopeptidase in brain, as well as the identification of a nuclear localization sequence for the human peptidase (109, 110). The NRK-52E cells may constitute a relevant cell model to establish the pathways that contribute to the intracellular generation and actions of Ang II and Ang-(1–7) within renal epithelial cells. As an alternative concept to intracellular formation, Ibarra and colleagues presented evidence for another model of nuclear signaling whereby the plasma membrane invaginates to the perinuclear area that facilitates presentation of intracellular signals (IGF receptor coupled to IP_3 formation) discretely to the nucleus in cardiomyocytes (111). The apparent advantages of this system may reflect a more selective activation of the signaling cascade and independence from the intracellular generation of the peptide ligands (112). The latter study adds another potential mechanism to the complex pathways of the intracellular receptor system for angiotensins and other peptides, as well as emphasize the need for additional studies to elucidate their organization and function.

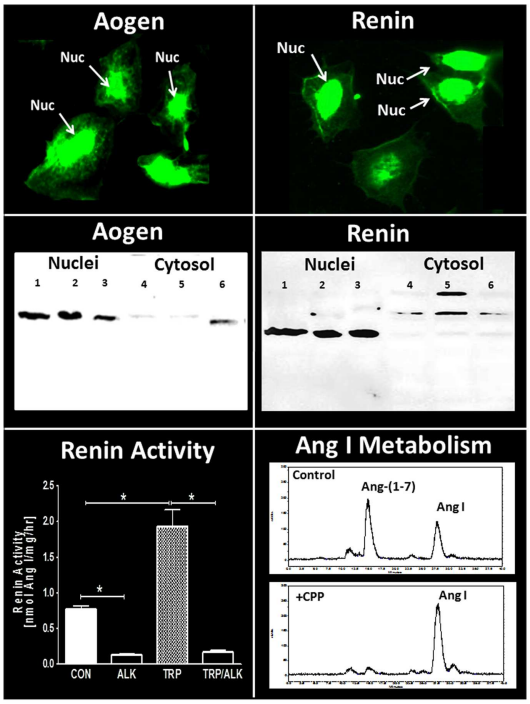

FIGURE 3 | Expression of intracellular components of the renin-angiotensin system in NRK-52E renal epithelial cells. Immunofluorescent (IMF) staining and protein immunoblot for rat angiotensinogen (Aogen) and renin. Immunoblots of Aogen and renin in nuclei (lanes 1–3) and cytosol (lanes 4–6) were from three separate passages of NRK-52E cells. Major bands for Aogen and renin were identified at approximately 55 kDa. Renin activity (conversion of Aogen to Ang I) in isolated nuclear fractions was increased threefold following activation by trypsin (TRP) and was essentially abolished by the renin inhibitor aliskiren (ALK). Conversion of ^{125}I-Ang I to ^{125}I-Ang-(1–7) in the isolated nuclear fraction was predominantly blocked by the thimet oligopeptidase inhibitor CPP. Renin activity data are mean ± SEM; $n = 4$; $^*P < 0.05$. Ang I metabolism representative of data from $n = 4$ separate cell passages. Adapted from Alzayadneh and Chappell (107).

In the endeavor to elucidate the intracellular pathways, the importance of robust biochemical and molecular techniques to characterize the RAS cannot be overly emphasized. Several reports have raised concerns regarding the specificity of commercial AT_1 and AT_2 antibodies widely utilized for western immunoblot and immunocytochemical distribution studies (113–115). Importantly, these studies find that receptor protein bands at the appropriate molecular weights were not abolished in AT_1- or AT_2-deleted cell and tissue samples. We have utilized antibodies to

both AT_1 and AT_2 receptors to establish their molecular weight in the nuclear fraction as this pertains to the maturation or processing of the receptor protein. However, studies by our laboratory and others also incorporate peptide binding assays to quantitate receptor density and affinity, as well as various antagonists to identify the receptor subtype. The receptor binding assays also parallel the demonstration of functional signaling pathways (ROS, NO) on nuclei and the sensitivity to receptor antagonists. Reliance on the assessment of mRNA for the receptor may not equate to protein expression and certainly does not reveal the discrete intracellular distribution of the receptor. Antibodies to angiotensin receptors or other RAS components are useful and convenient tools to characterize this system; however, parallel approaches to establish the expression and regulation of the RAS particularly within the cell are clearly warranted.

FETAL PROGRAMING

Increasing evidence for the influence of early prenatal events in the fetus to induce a greater susceptibility to cardiovascular and metabolic pathologies is evident in both experimental models and in humans (116–119). Although the precise nature of fetal programing events is not known, alterations in the biochemical components and functional aspects of the RAS may constitute an important underlying mechanism (69, 120–128). Our recent studies utilize a sheep model of fetal programing in which pregnant ewes are administrated the glucocorticoid betamethasone at day 80 of gestation. This regimen parallels the dose and time that pregnant women are typically treated with glucocorticoids to enhance pulmonary function and reduce mortality of the fetus delivered preterm. Fetal exposure to glucocorticoids in sheep results in a significant reduction in the nephron number within the kidney, an increase in mean blood pressure, attenuation of the baroreflex response (BRS) in the control of heart rate and increased indices of metabolic dysfunction in adult animals (129) (**Figure 4**). In regards to the function of the RAS following glucocorticoid exposure, acute treatment with the AT_1-receptor antagonist candesartan normalized blood pressure in the exposed sheep and improved the impaired BRS, but had no overall effect on pressure in the control or unexposed adult sheep (129). In contrast, administration of D-Ala^7-Ang-(1–7) increased blood pressure and attenuated BRS in the control but not the betamethasone-exposed (BMS) sheep suggesting that the loss of Ang-(1–7) tone may be an additional consequence of fetal programing events (126). The protein expression of the AT_7/Mas receptor was significantly lower in the brain medulla in both 6-month- and 1.8-year-old BMS sheep as compared to age-matched control sheep; however, the AT_1-receptor protein expression was unchanged (122). We also find reduced CSF levels of Ang-(1–7) in the exposed sheep, as well as higher activities of the Ang-(1–7) peptidase (**Figure 4**) (69, 70). Indeed, the CSF content of Ang-(1–7) inversely correlated to Ang-(1–7) peptidase activity in the control and BMS sheep (**Figure 4**, lower right panel). Thus, the reduced expression of the AT_7/Mas receptor and increased metabolism of Ang-(1–7) in brain may contribute to the loss of Ang-(1–7) tone in BMS sheep, as well as the enhanced responsiveness of the Ang II-AT_1-receptor pathway in glucocorticoid-dependent programing without significant changes in the AT_1-receptor levels.

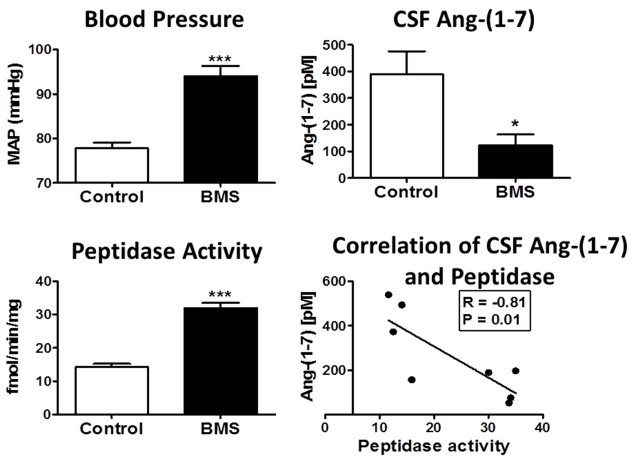

FIGURE 4 | Betamethasone-exposed (BMS) offspring exhibit higher mean arterial pressure (MAP) and CSF endopeptidase activity than non-exposed sheep. Blood pressure (MAP) was higher in BMS animals at 6 months of age. CSF peptidase activity was twofold higher in BMS animals as compared to controls. CSF Ang-(1–7) peptide levels were lower in BMS animals. Ang-(1–7) peptide levels negatively correlate with peptidase activity in the CSF ($r = -0.81$, $P = 0.01$). Data are mean ± SEM; 4–5 per group; $^*P < 0.05$ or $^{***}P < 0.001$ vs. controls. Adapted from Marshall et al. (70).

In regards to the renal RAS in fetal programing, Ang-(1–7)-dependent stimulation of sodium excretion was abrogated in the BMS sheep. Moreover, the anti-natriuretic response to Ang II was enhanced in the BMS sheep, as well as the reduction in renal plasma flow (130, 131). Consistent with the altered renal responses to Ang II and Ang-(1–7), expression of ACE2, the peptidase that contributes to the balance of Ang II to Ang-(1–7), was significantly reduced in the circulation, the proximal tubules and the urine of the BMS adult sheep (125). That both tubular and urinary forms of ACE2 were reduced in the BMS sheep suggests down regulation or reduced synthesis of the peptidase in the proximal tubules that may lead to the lower release or shedding of the enzyme from the apical membrane. Both ACE and neprilysin activities were readily detected in the proximal tubules and urine of adult sheep; however, their activities were not changed following betamethasone exposure. Moreover, circulating ACE activity increased while ACE2 activity decreased in the serum of BMS adult sheep (125). A kinetic analysis of ACE2 activity revealed a reduction in the maximal velocity (V_{max}) of the enzyme rather than a change in substrate affinity (Km) suggesting reduced protein content in the circulation. These data further suggest that the soluble forms of the enzyme in serum exhibits similar kinetic characteristics as the native form, at least regarding the metabolism of Ang II to Ang-(1–7). The ratio of ACE to ACE2 also closely correlated with the mean blood pressure values in the control and BMS sheep (125).

In addition to the altered expression of ACE2, the balance of angiotensin receptor subtypes was changed as well (132). Moreover, the proportion of both AT_2 and Mas receptor subtypes were lower in the renal cortex of the exposed group. However, the AT_1 subtype was the predominant angiotensin receptor in the renal medulla and the receptor subtypes were unchanged between

the control and exposed sheep. These data again emphasize the selective effects of fetal glucocorticoid exposure on expression of the RAS components within different tissue compartments. The intracellular studies on the Ang II and Ang-(1–7) signaling pathways further support the overall findings that the expression of angiotensin receptor subtypes were altered following glucocorticoid exposure. In isolated nuclei from the real cortex, the generation of NO by Ang-(1–7) was markedly reduced in the exposed sheep. In contrast, the Ang II-dependent stimulation of ROS via the AT_1 receptor was augmented in renal nuclei from the BMS sheep. Furthermore, addition of the AT_2 antagonist PD122319 exacerbated the production of ROS by Ang II, and this augmented response was particularly evident in the glucocorticoid-exposed group (132). It is not clear whether the AT_2 receptor exhibits functional antagonism of the AT_1 receptor to influence ROS formation or that the AT_2 subtype sequesters Ang II from the AT_1 receptor given the higher ratio of AT_2 to AT_1 sites in the sheep cortex.

Finally, fetal programing events may convey greater sensitivity to an additional stressor or insult, particularly as these animals age (119). Therefore, recent studies ascertained the renal responses to Ang-(1–7) in control and glucocorticoid-exposed adult sheep following removal of one kidney. In contrast to the intact, non-exposed sheep, Ang-(1–7) infusion reduced sodium excretion in uni-nephrectomized animals (133). The anti-natriuretic response to Ang-(1–7) was enhanced by the AT_7 receptor antagonist D-Ala^7-Ang-(1–7) and was subsequently blocked by the AT_1 antagonist candesartan. Moreover, the exposed animals still exhibited an attenuated natriuretic response to the combination of Ang-(1–7) and candesartan in comparison to the unexposed control group. Similar hemodynamic responses were also observed for Ang-(1–7) in the uni-nephrectomized animals whereby the peptide alone reduced blood flow and the combination of Ang-(1–7)/candesartan increased flow; however, the overall vascular responses were similar between the control and exposed animals (133). Certainly, differences in species and sex, the primary or immortalized status of the cells, the dose of both peptide and antagonists, and the duration of treatment may influence the functional actions of Ang-(1–7) and the receptor(s) mediating these effects (134). In regards to the studies in the uni-nephrectomized sheep, the differential response in sodium handling to Ang-(1–7) likely reflects the compensatory mechanisms of the remaining kidney to accommodate the marked increase in cardiac output and fluid handling. The fascinating aspect of the renal studies is the plasticity in the Ang-(1–7) response that apparently encompasses an AT_1 receptor interaction to reduce sodium excretion in the uni-nephrectomized animal, as well as the Mas receptor interaction to stimulate sodium excretion in the intact sheep. Moreover, the natriuretic response of Ang-(1–7) potentially mediated by the Mas receptor remains intact in the uni-nephrectomized sheep since it was unmasked by blockade with the AT_1-receptor antagonist. However, the mechanism underlying the functional interaction of Ang-(1–7) with AT_1 receptors in the single kidney is not known, as well as whether Ang-(1–7) stimulates signaling pathways identical to that of the Ang II-AT_1-receptor axis to reduce sodium reabsorption and renal blood flow.

SEX DIFFERENCES IN THE Ang-(1–7)-Mas RECEPTOR AXIS

Both experimental and clinical evidence suggest an important influence of sex on the development of cardiovascular disease that may reflect the regulation of the RAS by gonadal hormones including testosterone and estrogen. Women are generally thought to be protected from cardiovascular pathologies up to the time of menopause suggesting a beneficial effect of estradiol; however, several large clinical trials utilizing estrogen or combined estrogen/progesterone replacement in older women with underlying cardiovascular disease revealed adverse effects of either treatment. Experimental studies have largely focused on the role of estrogen to influence the ACE-Ang II-AT_1-receptor axis of the RAS and generally reveal an inhibitory effect on the expression of ACE and the AT_1 receptor (135–138). Estrogen depletion by ovariectomy in young mRen2.Lewis rats markedly exacerbated the hypertension and essentially abolished sex differences in blood pressure between the male and female congenics (139). In this model, estradiol replacement or treatment with the AT_1-receptor antagonist olmesartan normalized blood pressure suggesting the loss of estrogen may lead to the dysregulation of the RAS. Indeed, circulating levels of Ang II and ACE activity were higher in the estrogen-depleted mRen2.Lewis while plasma levels of Ang-(1–7) were reduced, and the overall ratio of Ang II to Ang-(1–7) increased (139, 140). Brosnihan and colleagues originally proposed that the protective effects of estrogen may, in part, reflect a shift in the balance between circulating Ang II and Ang-(1–7) that may arise from the inhibitory effects of the steroid on ACE to promote Ang-(1–7) expression via increased synthesis and/or reduced metabolism of the peptide (141).

There are relatively few studies that have assessed tissue differences in Ang II and Ang-(1–7) in males and females. In the kidney of the mRen2.Lewis hypertensive rats, the tissue content of Ang II was twofold higher in the males (**Figure 5**). Conversely, renal levels of Ang-(1–7) were threefold lower in the males as compared to females. Interestingly, cortical ACE2 activity was 70% higher in the males perhaps suggesting a compensatory effect to buffer the higher Ang II content and blood pressure evident in the male mRen2.Lewis (**Figure 5**). In contrast, cortical neprilysin activity and protein expression were significantly higher in the female congenics as compared to males. The higher content of the endopeptidase may contribute to the differential expression of angiotensins in the female kidney to favor the enhanced conversion of Ang I to Ang-(1–7), as well as the metabolism of Ang II to Ang-(1–4). Cardiac ACE2 activity was also significantly higher in the male congenics; however, tissue levels of Ang II and Ang-(1–7) were not different between males and females. The renal content of Ang-(1–7) was also higher in female SHRs as compared to the males; however, tissue levels of Ang II were not different (142). Although this study did not determine peptidase expression in the SHR kidney as a potential mechanism for the higher Ang-(1–7) content, tissue levels of Ang-(1–7) were higher in females following Ang II treatment perhaps suggesting greater processing of Ang II to Ang-(1–7). Sandberg and colleagues assessed sex differences in ACE2 expression in the mouse kidney using the "four core" approach to distinguish the effect of sex chromosomes and ovarian steroids (143). Consistent with the results in the mRen2.Lewis

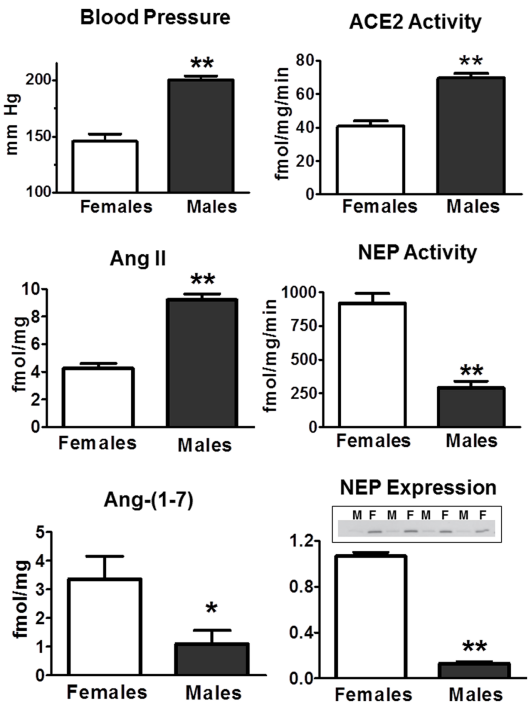

FIGURE 5 | Sex differences in systolic blood pressure and RAS components in the renal cortex of 15-week-old hemizygous mRen2.Lewis congenic rats. Systolic blood pressure (mmHg) is higher in males. Intrarenal concentrations (femtomole peptide per milligram protein – fmol/mg) of Ang II are higher in males, but Ang-(1–7) content is lower. ACE2 activity (femtomole product per milligram protein per minute – fmol/mg/min) is higher in males, but neprilysin (NEP) activity is lower in males. NEP expression assessed by Western blot was lower in males (M) as compared to females (F). Data are mean ± SEM; $n = 4$–8 per group; $*P < 0.05$ or $**P < 0.01$. Adapted from Pendergrass et al. (81).

rats, renal ACE2 activity and expression were higher in the male mice (143). Ovariectomy increased ACE2 expression in the female kidney, and estradiol replacement reduced the peptidase in both males and females; however, there was no influence of gonadal steroids on either cardiac or pulmonary ACE2 (143). These data suggest that at least under non-pathological conditions, estrogen exhibits an inhibitory influence on kidney ACE2 and raises the issue of whether this response contributes to the deleterious effects of estrogen replacement in older women. Cassis and colleagues reported the sex-dependent expression of circulating Ang-(1–7) in mice fed a high fat (HF) diet (144). Males exhibited a marked decline in plasma levels of Ang-(1–7) that was associated with higher circulating Ang II; however, plasma levels of Ang-(1–7) increased in the HF-fed females. Interestingly, ovariectomy of the HF-fed female mice reduced circulating Ang-(1–7) and ACE2 activity in adipose tissue but did not influence renal ACE2. The fall in circulating Ang-(1–7) was associated with a marked increase in nocturnal blood pressure. Moreover, administration of D-Ala^7-Ang-(1–7) increased blood pressure in female mice maintained on the HF diet suggesting that the ACE2-Ang-(1–7)-Mas axis may buffer obesity-induced hypertension to a greater extent in females.

In regards to the Ang-(1–7) receptor, expression of the Mas receptor was increased in females but not in males following the infusion of Ang II which may explain the attenuated blood pressure to Ang II in the females (142). Pretreatment with the AT_7 receptor antagonist D-Ala^7-Ang-(1–7) enhanced the blood pressure response to Ang II in the female SHR. Similar findings were recently reported in the aldosterone salt-sensitive model where the mRNA levels for both ACE2 and the Mas receptor increased 1.5- and 5-fold, respectively, in the lamina terminalis (LT) of female rats (145). Chronic ICV treatment with the D-Ala^7-Ang-(1–7) antagonist markedly augmented the blood pressure response in intact females treated with aldosterone and sodium chloride. Moreover, ovariectomy exacerbated the blood pressure response to aldosterone/salt; however, the mRNA expression of ACE2 or the Mas receptor in the LT area was not changed suggesting an inability to upregulate the central Ang-(1–7) axis contributes to the increase in blood pressure in this model (145). Denton and colleagues reported that D-Ala^7-Ang-(1–7) alone decreased renal blood flow in female but not male normotensive Wistar rats, although the antagonist did not influence the renal response to acute Ang II infusion in either sex (64). In a separate study, the mRNA levels of the Mas protein were markedly higher in the kidney of adult female rats as compared to the males (146). Interestingly, Mas expression tended to decline in the male kidney while the mRNA levels of the receptor increased in females over the postnatal period (1–110 days). Our studies in the sheep model of fetal programing also provide evidence for sex differences in the responsiveness to Ang-(1–7). Although the exposure to betamethasone results in a similar increase in blood pressure and reduction in nephron number in the male and female sheep, the renal response to Ang-(1–7) or the antagonist differs with sex. An acute infusion of Ang-(1–7) results in a robust natriuretic response in control females but not the adult males (130). Moreover, betamethasone exposure was associated with reduced natriuresis in the males with or without Ang-(1–7) treatment, but significantly blunted the natriuretic actions of Ang-(1–7) in females. At this time, the mechanism underlying the sex-dependent effects of Ang-(1–7) on sodium excretion in control and betamethasone-exposed sheep are not known, but we speculate that it may involve the altered expression or signaling of the Mas receptor on the tubular elements of the kidney.

SUMMARY

The current review has examined several aspects from the recent literature on the non-classical or alternative ACE2-Ang-(1–7)-Mas receptor axis of the RAS. The mounting biochemical and functional evidence clearly supports the tenet that this pathway may antagonize the ACE-Ang II-AT_1-receptor arm of the RAS either directly through metabolism of Ang II to Ang-(1–7) by ACE2 or via distinct pathways that limit the activation of Ang II-AT_1-receptor signaling. Indeed, the demonstration of an intracellular ACE2-Ang-(1–7)-Mas axis that attenuates the Ang II-dependent stimulation of ROS on renal nuclei is in keeping with the concept of a balanced RAS even within the cell and emphasizes the importance of targeting the intracellular system as a therapeutic approach to enhance the functional ratio of Ang-(1–7) to Ang II. The evidence that an altered Ang-(1–7) system within the brain and the kidney following antenatal glucocorticoid exposure

implicates an interaction between the Ang II and Ang-(1–7) pathways that contribute or promote the cardiovascular dysfunction associated with fetal programing events. Finally, sex differences apparent in blood pressure regulation and cardiovascular pathologies may reflect alterations in the ACE2-Ang-(1–7)-Mas receptor axis of the RAS in addition to those effects typically associated with the ACE-Ang II-AT_1-receptor pathway.

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Clinical relevance of local renin angiotensin systems

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The concept of a "local" renin angiotensin system (RAS) can mean different things to different people. Its main purpose is to differentiate the "local" RAS operating in tissues from the classical "circulating" RAS, but it is difficult to differentiate between the two systems because of their extensive overlap. The circulating RAS comprises kidney-derived renin acting on liver-derived angiotensinogen to generate angiotensin (Ang) I that is converted to Ang II by angiotensin converting enzyme (ACE). However, tissues are the main site of production of angiotensin peptides by the circulating RAS, whereby plasma-derived renin acts on plasma-derived angiotensinogen to generate Ang I, which is converted to Ang II by endothelial ACE (1–4).

Local RAS refers to tissue-based mechanisms of Ang peptide formation that operate separately from the circulating RAS. Although many different concepts of local RAS have been described, a key feature is the local synthesis of RAS components including angiotensinogen and enzymes such as renin that cleave angiotensinogen to produce Ang peptides independently of the circulating RAS. ACE and Ang II type 1 (AT1) and type 2 (AT2) receptors are invariably locally synthesized, but these are also components of the circulating RAS. Many other potential components of local RAS have been described that may contribute to tissue-specific mechanisms of Ang peptide formation, and that may either participate in disease processes or contribute to mechanisms that protect from tissue injury. These include the (pro)renin receptor (5), renin-independent mechanisms of Ang peptide generation from Ang- (1-12) (6), intracellular (or intracrine) RAS that may contribute to cardiovascular disease (7, 8), and AT2 receptors (7) and the ACE2/Ang-(1-7)/Mas receptor pathway (6–8) that may mediate therapeutic benefit in cardiovascular disease. In addition, novel Ang peptides with novel pharmacology, including Ang IV, Ang A, alamandine, and angioprotectin (6, 8), have the potential to contribute to disease or to protective mechanisms. Moreover, the brain RAS, including the ACE2/Ang-(1-7)/Mas receptor and the Ang IV/insulin regulated aminopeptidase pathways may play a role in Alzheimer's and Parkinson's diseases (9). Local production of aldosterone may have a pathogenic role (7, 10), ACE, AT2 receptors, Ang-(1-7) and acetyl-Ser-Asp-Lys-Pro may have a role in hematopoiesis (11), and the ACE2/Ang-(1-7)/Mas receptor pathway may contribute to fetal programing, reproduction, and cancer (6, 12).

This short opinion piece discusses the potential clinical relevance of local RAS. The challenge in demonstrating the independence of local from circulating RAS, and the potential interaction of ACE inhibitor and AT1 receptor blocker (ARB) therapies with local RAS are discussed. Attempts to define local RAS that are independent of the circulating RAS have been primarily based on animal models and the clinical relevance of local RAS is uncertain. However, this area of research continues to evolve, and today's opinions may change as we gain better understanding of how these novel components and mechanisms impact on clinical medicine.

HOW CAN LOCAL RAS BE SHOWN TO BE INDEPENDENT OF THE CIRCULATING RAS?

As reviewed elsewhere (5–12), many lines of evidence suggest the possibility of local RAS that may operate independently of the circulating RAS and play a pathogenic or protective role. This evidence includes the widespread tissue expression of angiotensinogen, the only known precursor of the Ang peptides and an essential requirement for an independent local RAS (13–16). However, local production of RAS components does not prove their functional significance, and proving their clinical relevance presents many challenges. One approach to study of the role of locally synthesized RAS components is their targeted deletion from specific tissues. This approach has been applied to the kidney.

Both clinical experiences with ACE inhibitor and ARB therapies during pregnancy, and ACE, renin, angiotensinogen, and AT1 receptor gene mutation and knockout models demonstrate a critical role for the RAS in renal development and function in animals and humans (17–23). Moreover, ACE inhibition demonstrates a differential regulation of Ang II levels in kidney and blood (24). However, these data do not prove a specific role for the local RAS in the kidney. Matsusaka et al. investigated the role of the local RAS in renal development and function by producing mice with genetic deletion of angiotensinogen synthesis in the kidney. In contrast to the morphological and functional consequences of whole body or liver specific deletion of angiotensinogen gene expression, deletion of angiotensinogen production in the

kidney had no effect on renal morphology or function (25). Moreover, contrary to the expectation that locally produced angiotensinogen was the main contributor to renal Ang II levels, Matsusaka et al. showed deletion of renal angiotensinogen production had no effect on renal Ang II levels, and that liver angiotensinogen is the primary source of Ang II in the kidney (25). With the caveat that the studies of Matsusaka et al. were not in pathophysiological models (25), these data show that evidence for local synthesis of a RAS component is not sufficient to establish a role for the locally synthesized component in physiology or pathology, whether by an intracellular (intracrine) or extracellular mechanism. Proof that a locally synthesized RAS component contributes to physiology or pathology requires demonstration that deletion of the locally synthesized component impacts on physiology and/or pathology.

Similar to the case for angiotensinogen, mice with reduced renal expression of ACE had normal histology and urine concentrating ability (26), suggesting that locally synthesized ACE does not play an essential role in normal renal development and function. Moreover, the marked reduction in Ang II levels in kidney, heart, and other organs caused by global ACE gene deletion, despite near-normal Ang I levels (27, 28), indicates that an intracellular (intracrine) ACE-independent mechanism of Ang II formation is unlikely to exist in these tissues.

Evidence for a pathogenic role of renal ACE is the demonstration that genetic deletion of renal ACE expression prevented hypertension produced by subcutaneous administration of Ang II (26), suggesting a specific renal ACE-dependent mechanism of hypertension in this model. However, the significance of this finding is uncertain because ACE inhibition does not modify hypertension produced by intravenous Ang II administration in either animal of human studies (29–33), and it is questionable whether the subcutaneous Ang II model of hypertension has any physiological or pathological relevance (34).

An alternative approach to defining a local tissue RAS was to use recombinant technology to express ACE as a reporter gene on the cardiomyocyte membrane (35). In this model, ACE expression on the cardiomyocyte membrane (where it is not normally expressed) would be expected to increase cardiac Ang II levels if Ang I were also present in this tissue compartment. Expression of ACE on the cardiomyocyte membrane increased cardiac Ang II levels in mice without endothelial expression of ACE, but not in rats or mice with endothelial ACE expression (35, 36). These studies do not therefore provide evidence in support of Ang I formation in the extravascular compartment of the heart of animals with endothelial ACE expression. By contrast, deletion of testicular ACE reduced male fertility (37), indicating a specific role for testicular ACE. However, ACE has many substrates (38) and the reduction in male fertility may reflect an action of testicular ACE that is independent of Ang peptides.

Part of the challenge in identifying a local RAS that is independent of the circulating RAS is the difficulty in measuring *in vivo* levels of Ang peptides in tissues. For example, initial reports of substantial amounts of Ang II and Ang-(1-7) in the brain (39, 40) were not confirmed when more rigorous methodology was applied (41, 42).

DO THE THERAPEUTIC BENEFITS OF ACE INHIBITOR AND ARB THERAPIES ESTABLISH THE CLINICAL RELEVANCE OF LOCAL RAS?

A key argument in support of the clinical relevance of the RAS, whether local or circulating, is the therapeutic benefit from inhibition of this system. De Mello and Frohlich proposed that the local RAS mediates in part the therapeutic benefits of ACE inhibitor and ARB therapies (7), but there are difficulties in establishing such a role for local RAS. For example, the claim that the beneficial effects of these therapies occurred independently of blood pressure (7) suggests, but does not prove, a role for local RAS. The complexity of blood pressure regulation means that alternative explanations are possible and ambulatory blood pressure monitoring may be necessary to demonstrate an effect of therapy on blood pressure not detected by office blood pressure measurement (43). Furthermore, the different benefits of ACE inhibitor and ARB therapies in comparison with antihypertensive agents that act independently of the RAS (7) do not prove that these benefits were due to inhibition of local rather than the circulating RAS.

Ang II administration is a well-recognized model of cardiovascular and renal disease (44–46), and the therapeutic benefits of RAS inhibition are almost certainly in large part a consequence of reduced Ang II stimulation of the AT1 receptor in high renin, high Ang II conditions such as renal artery stenosis and heart failure. Reduced AT1 receptor stimulation may also play an important role in the renal effects of RAS inhibition, including the side effects of these therapies (47, 48). Many studies investigating the combination of ACE inhibitor, ARB, and renin inhibitor therapies were based on the assumption that the therapeutic benefits of these agents are the consequence of reduced AT1 receptor stimulation, and that combination of these therapies would produce greater therapeutic benefit by producing greater reduction in AT1 receptor stimulation (47–53). What may not have been appreciated was the large body of preclinical and clinical data indicating that these drugs also produce benefits by mechanisms separate from reduced AT1 receptor stimulation. Moreover, many of these mechanisms separate from reduced AT1 receptor stimulation involve novel RAS components implicated in local tissue RAS (**Figure 1**). For example, ARB therapies, by blocking the negative feedback control of renin secretion, also increase Ang II levels that stimulate the AT2 receptor, leading to cardioprotection (54, 55). Moreover, both ACE inhibitor and ARB therapies increased Ang-(1-7) levels (56) that may produce therapeutic effects mediated by the Ang-(1-7)/Mas receptor pathway (6). In addition, ACE inhibitor, ARB, and renin inhibitor therapies increase bradykinin levels that may contribute to their antihypertensive and cardioprotective actions (54, 55, 57–63). Consequently, therapeutic benefit from ACE inhibitor, ARB, and renin inhibitor therapies does not prove a pathogenic role for the RAS, either local or circulating.

An important aspect of these additional mechanisms of therapeutic benefit from RAS inhibition is that combination of ACE inhibitor, ARB, and/or renin inhibitor therapies may block some of these mechanisms of benefit, thereby explaining the many clinical studies, apart from heart failure (49), that showed no additional benefit

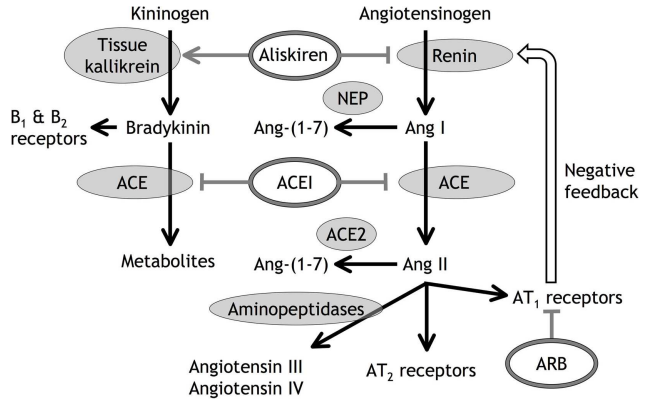

FIGURE 1 | Diagrammatic representation of angiotensin (Ang) and bradykinin peptide formation and metabolism, with the sites of action of angiotensin converting enzyme (ACE) inhibitors (ACEI), angiotensin type 1 (AT_1) receptor blockers (ARB), and the renin inhibitor aliskiren. In addition to inhibiting renin, aliskiren increases tissue kallikrein activity and bradykinin levels that may act on bradykinin type 1 (B_1) and type 2 (B_2) receptors (55). Neutral endopeptidase (NEP) converts Ang I to Ang-(1-7), ACE2 converts Ang II to Ang-(1-7), and aminopeptidases convert Ang II to Ang III and Ang IV.

from combination of ACE inhibitor, ARB, and renin inhibitor therapies (47, 48, 50–53). For example, the benefits of ARB therapy produced by increased Ang II levels and AT2 receptor stimulation will be blocked if combined with renin inhibitor or ACE inhibitor therapies, because renin inhibitor and ACE inhibitor therapies attenuate the increase in Ang II levels produced by ARB therapy (55, 56, 64, 65). Moreover, the benefits of ACE inhibitor and ARB therapies produced by increased Ang-(1-7) levels and Mas receptor stimulation will be blocked if combined with renin inhibitor or neutral endopeptidase inhibitor therapies because renin inhibitor and neutral endopeptidase inhibitor therapies attenuate the increase in Ang-(1-7) levels produced by ACE inhibitor and ARB therapies (66). In addition, neutral endopeptidase inhibitor therapy may increase Ang II levels by reducing Ang II metabolism (66, 67).

CONCLUSION

Current concepts of the local RAS have expanded to include the (pro)renin receptor, renin-independent mechanisms of Ang peptide generation from Ang-(1-12), AT2 receptors, the ACE2/Ang-(1-7)/Mas receptor and Ang IV/insulin regulated aminopeptidase pathways, an intracellular (intracrine) RAS, and novel Ang peptides (5–9, 11, 12). Much of the evidence for these new RAS components is based on animal studies and further research is required to establish that local RAS contribute to physiology and disease. Consequently, the clinical relevance of local RAS remains speculative. Nevertheless, the expanding repertoire of local RAS components offers new therapeutic targets and the prospect of new therapies.

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Beyond the circulating renin–angiotensin aldosterone system

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The activation of the classical renin–angiotensin aldosterone system (RAAS) is known to be involved in the regulation of blood volume and blood pressure and plays an important role in cardiovascular pathology including hypertension and heart failure. Evidence is now available that independently of the classical RAAS, several RAAS components are expressed in cells from different organs including the heart and kidney and are able to change important physiological properties like cell communication, heart excitability, and activation of ionic channels and cell volume when applied locally to the cells (1) or systemically, independently of blood pressure. In cardiac cells, swelling induced by angiotensin II (Ang II), is counteracted by angiotensin (1–7) [Ang (1–7)] with consequent decrease of swelling-dependent chloride current helping the re-establishment of cell volume (2). Recently, it was found that Ang (1–7) re-establishes cell communication impaired by cell swelling in cardiac muscle raising the possibility of a beneficial effect of the hexapeptide during myocardial ischemia (3). These findings have important clinical implications (1, 4) and represent a novel and fruitful pathway to be followed to better understand the role of the RAAS in different pathological conditions. Furthermore, they offer the opportunity for the development of new therapeutic agents.

Although studies performed on transgenic animals generated controversial results, evidence is available that the overexpression of some components of RAAS like Ang II on cardiac muscle, elicit ventricular hypertrophy independently of changes in arterial blood pressure (5). Furthermore, the identification of some of the RAAS components inside the cell including the nucleus and mitochondria (6–8) and the results achieved dialyzing Ang II or renin intracellularly (1, 7), supports the notion that there is an intracellular component with functional properties (the intracrine effect) (1, 7). In arterial myocytes from vascular resistance vessels, for instance, intracellular Ang II has an effect opposite to that of extracellular Ang II on vascular tone (9) suggesting an important intracrine effect of the peptide on peripheral resistance. Furthermore, the (pro) renin receptor (PRR), mainly located intracellularly (10, 11), is a new member of the RAS, originally considered to be involved in the regulation of blood pressure. Recent observations using transgenic animals over-expressing PRR demonstrated that PRR is an accessory protein of V-ATPase that plays an important role in the regulation of several cellular homeostatic processes including autophagy (11).

The harmful effects of Ang II on cardiovascular and renal systems inducing remodeling, seems, in part, related to increase in oxidative stress. The discovery of angiotensin converting enzyme 2 (ACE2) (12) and the evidence that it promotes the formation of Ang (1–7) from Ang II in animal models, represented an important chapter in the studies of RAAS because Ang (1–7) counteracts many effects of Ang II (13) including the enhancement of oxidative stress induced by Ang II. Further studies are, however, necessary to confirm if these beneficial effects of Ang (1–7) are present in humans.

In this Research Topic, the pathophysiological role of local RAAS in different tissues and organs are reviewed by different authors, each one expert in their respective fields (14–18). We hope these articles will help the development of future investigation of this important topic.

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