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ABSTRACT:
Accumulation of a large body of evidence during the past two decades testifies to the complexity of the renin–angiotensin system (RAS). The incorporation of novel enzymatic pathways, resulting peptides, and their corresponding receptors into the biochemical cascade of the RAS provides a better understanding of its role in the regulation of cardiovascular and renal function. Hence, in recent years, it became apparent that the balance between the two opposing effector peptides, angiotensin II and angiotensin-(1-7), may have a pivotal role in determining different cardiovascular pathophysiologies. Furthermore, our recent studies provide evidence for the functional relevance of a newly discovered rat peptide, containing two additional amino acid residues compared to angiotensin I, first defined as proangiotensin-12 [angiotensin-(1-12)]. This review focuses on angiotensin-(1-7) and its important contribution to cardiovascular function and growth, while introducing angiotensin-(1-12) as a potential novel angiotensin precursor.

Introduction:
It seemed for a long time that all components of the renin–angiotensin system (RAS) and their physiological roles were well defined. In this traditional view, the RAS is viewed as a classical endocrine system with the octapeptide angiotensin II (Ang II; Asp1-Arg2-Val3-Tyr4-Ile5-His6-Pro7-Phe8) as an effector hormone expressing its vasoconstrictor, sodium retention, mitogenic, and proliferative effects upon its binding to Ang II type 1 receptors (AT1). Renin and angiotensin converting enzyme (ACE) were thought to be the only enzymes responsible for angiotensin I (Ang I; Asp1-Arg2-Val3-Tyr4-Ile5-His6-Pro7-Phe8-His9-Leu10) and Ang II synthesis, respectively. However, over the last two decades, increasing evidence has accumulated that indicates an exceeding complexity of the system, particularly in tissues such as the heart and kidneys. The evidence for a fully operational RAS in local tissues with tissue-specific enzymatic pathways for the processing of Ang I and Ang II has been detailed in a number of publications from this laboratory [1–6]. Furthermore, the pleiotropic actions of the resulting fragment of Ang I or Ang II, the heptapeptide Ang-(1-7), have been gradually appreciated over the last decade. In general, Ang-(1-7) [Asp1-Arg2-Val3-Tyr4-Ile5-His6-Pro7] counterbalances biological actions of Ang II, and in that way, an inadequate balance between these two peptides may determine different cardiovascular pathophysiological states. Interestingly, the spectrum of novel peptides within RAS continues to expand showing that a peptide containing two amino acids more than Ang I, the dodecapeptide angiotensin-(1-12) [Ang-(1-12); rat sequence: Asp1-Arg2-Val3-Tyr4-Ile5-His6-Pro7-Phe8-His9-Leu10-Leu11-Tyr12], could also be a key player in the regulation of cardiovascular function. This review will therefore focus on Ang-(1-7), its important contribution to cardiovascular function and growth, while introducing Ang-(1-12) as a potential novel angiotensin precursor.

Biochemical pathways for Ang-(1-7) synthesis and degradation ::: Angiotensin-(1-7):
Ang-(1-7) may be derived from either Ang I or Ang II (Fig. 1). Different tissue-specific endopeptidases, including neprilysin (NEP), thimet oligopeptidase (TOP), and prolyl oligopeptidase (POP) catalyze the hydrolysis of the decapeptide Ang-I at the Pro7-Phe8 bond to release the three terminal amino acids and Ang-(1-7) [7–9]. Both POP [10] and TOP [7] have been reported by us to mediate Ang-(1-7) formation in cultures of vascular endothelial and smooth muscle cells. Neprilysin has been shown to be particularly abundant in the kidney [11]. Importantly, as NEP is a membrane-bound enzyme, its localization on the luminal side of the endothelium makes it accountable for most of the Ang-(1-7) production in the circulation [12–14]. Neprilysin degrades vasodilatory atrial natriuretic peptide as well, but its high substrate specificity for Ang-(1-7) formation in hypertensive humans may explain, at least in part, the lack of significant beneficial effects of its inhibitors in the treatment of hypertension [15]. However, neprilysin also degrades Ang-(1-7) into Ang-(1-4) [11], and further studies are necessary to clarify its role in hypertensive disease.

It has been only recently that direct conversion of Ang II into Ang-(1-7) by a newly discovered homolog of ACE, angiotensin converting enzyme 2 (ACE2), was demonstrated (Fig. 1) [16, 17]. As a carboxypeptidase, ACE2 also mediates the conversion of Ang I into Ang-(1-9), which can be further metabolized into Ang-(1-7) by ACE. However, the higher substrate preference of ACE2 towards Ang II than Ang I underscores the significance of this enzyme in the regulation of tissue Ang II/Ang-(1-7) balance [16, 17]. Consequently, higher cardiac Ang II level was associated with genetic deletion of ACE2 in mice and resulted in the development of severe cardiac dysfunction [18]. On the other hand, local ACE2 overexpression by systemic lentiviral delivery was followed by an attenuation of cardiac remodeling in hypertensive rats [19]. Furthermore, a recent report from our laboratory showed that the hypertensive heart predominantly depends on ACE2 for the production of Ang-(1-7) [20]. Together with evidence for increased ACE2 expression in failing human [21] and rat [22] hearts, our study suggests a preserved compensatory response of injured hearts to maintain Ang-(1-7) levels even in the advanced stage of the disease, although it was obviously not sufficient to counteract the deleterious effects of Ang II.
Besides breaking down bradykinin and Acetyl-Ser-Asp-Lys-Pro, ACE hydrolyzes Ang-(1-7) as well. It acts upon the Ile5-His6 bond to form the inactive metabolite Ang-(1-5) [23–25], and ACE inhibitors increase the short half-life of Ang-(1-7) in the circulation [26]. On the other hand, neprilysin hydrolysis of the Tyr4-Ile5 bond of Ang-(1-7) to form Ang-(1-4) seems to be the predominant pathway for Ang-(1-7) metabolism in the kidney [23, 27–29].

Ang-(1-7) receptor and signaling mechanisms ::: Angiotensin-(1-7):
Prior to the identification of a specific Ang-(1-7) receptor, a modified form of Ang-(1-7), D-Ala7-Ang-(1-7) was designed as a selective antagonist for the putative Ang-(1-7) receptor. Thus, D-Ala7-Ang-(1-7) inhibited Ang-(1-7)-induced systemic and renal vasodilation, did not block pressor or contractile response to Ang-II, and did not compete for binding of 125I-Ang II to rat adrenal AT1 or AT2 receptors [30]. Subsequent studies from our group identified specific non-AT1/AT2 Ang-(1-7) binding sites on bovine aortic endothelial cells [31] and endothelium of coronary artery rings [1] that were selectively competed by D-Ala7-Ang-(1-7). This finding was in agreement with nitric oxide (NO) release from bovine aortic endothelial cells stimulated by Ang-(1-7) that was blocked by D-Ala7-Ang-(1-7) [32]. It was also consistent with previously demonstrated Ang-(1-7)-induced vasodilation of endothelium-intact coronary arteries through release of kinins and NO [33, 34]. However, it was not before the discovery that endothelium-mediated vasodilation by Ang-(1-7) was abolished in mas-knockout mice that the “orphan” mas proto-oncogene receptor was linked to the intracellular signaling of Ang-(1-7) [35–37]. More recent studies revealed that Ang-(1-7), acting on this G protein-coupled receptor, activated endothelial nitric oxide synthase and NO production via Akt-dependent pathways [38]. Furthermore, we showed recently that the presence of an antisense probe directed against mas abolished the Ang-(1-7)-induced inhibition of protein synthesis in cardiomyocytes [39]. This study also revealed that Ang-(1-7) decreased serum-stimulated ERK1/ERK2 mitogen-activated protein kinase activity, a response that was blocked by D-Ala7-Ang-(1-7). These findings agree with the observation that genetic deletion of mas elicits cardiac dysfunction [37, 40]. Thus, it is clear that a reduction in the counterbalancing arm of the renin–angiotensin system via the ACE2/Ang-(1-7)/mas axis may have a major influence in determining cardiac structural and functional development [18, 37, 40]. In addition, a recent report suggests another Ang-(1-7) receptor subtype sensitive to the Ang-(1-7) antagonist [D-Pro7]-Ang-(1-7) but not D-Ala7-Ang-(1-7) [41]. This finding, as well as an intriguing interaction between AT1 and mas [42, 43], clearly warrants further investigation.

Cardiovascular and renal effects of Ang-(1-7) ::: Pleiotropy of Ang-(1-7) biological actions:
A series of studies after our initial characterization of Ang-(1-7) actions in brain [44] established the basis for exploring the systemic and regional vasodilatory and hypotensive effects of this peptide [33, 34, 45]. In these studies, it was demonstrated that the vasodilator effect of Ang-(1-7) was mediated through different vasoactive autocoid release [2, 14, 46–52]. Moreover, it was also shown that Ang-(1-7) potentiated bradykinin vasodilatory action [49, 53] and that this interaction was exaggerated after ACE inhibition. Although the precise mechanisms of this potentiation remains controversial [54, 55], data suggest that the release of prostaglandins, NO, endothelium-derived hyperpolarizing factor [56–58] as well as the ability of Ang-(1-7) to inhibit ACE activity [24, 25, 59] may be involved.
Early studies from our laboratory strongly suggested that Ang-(1-7) may represent an intrinsic counterbalancing factor to the pressor and trophic actions of Ang II [1]. This unique concept was confirmed in the experiments in which hyperreninemia was stimulated through induction of

renovascular hypertension [51] or a low-salt diet [47]. Despite increased levels of Ang II during salt depletion, blood pressure remained unchanged, at least in part, due to the opposing actions of Ang-(1-7). Indeed, Ang-(1-7) blockade by either the selective Ang-(1-7) receptor antagonist D-Ala7-Ang-(1-7) or specific Ang-(1-7) antibodies caused a dose-dependent increase in arterial pressure in salt-restricted rats [47], underscoring the importance of Ang-(1-7) in counterbalancing the effects of Ang II.
The significance of the alternative arm of the RAS comprising ACE2, Ang-(1-7), and mas in blood pressure regulation was further underscored by the demonstration of a considerable contribution of Ang-(1-7) to the hypotensive effects of RAS blockade [14, 46, 48, 60–62]. Importantly, chronic antihypertensive effects of captopril or omapatrilat in hypertensive patients were also associated with increased urinary levels of Ang-(1-7) [28, 63]. The importance of this observation was magnified by the concurrent observation that plasma and urinary excretion levels of Ang-(1-7) are reduced in untreated essential hypertensive subjects [64]. More recently, we showed that chronic administration of irbesartan to normotensive subjects was associated with large increases in plasma Ang-(1-7) [65, 66]. These results suggest an important contribution of Ang-(1-7) in mediating the antihypertensive effects of both ACE inhibitors and AT1 receptor antagonists.
It was then in our laboratory that the effects of RAS blockade on the Ang-(1-7)-forming enzyme, ACE2, were evaluated for the first time [67]. From the preceding study, we knew that heart failure due to coronary artery ligation was associated with compensatory increase in cardiac Ang-(1-7) levels [68]. It was in this experimental model that we subsequently showed that AT1 receptor antagonism further augmented plasma Ang-(1-7)/Ang II ratio suggesting increased formation of Ang-(1-7) from Ang II [67]. Indeed, AT1 receptor antagonism attenuated cardiac remodeling and dysfunction, and these changes were associated with a threefold increase in ACE2 mRNA expression in the left ventricle. The changes in the cardiac ACE2 gene activity and the profile of plasma angiotensin peptides after RAS inhibition were confirmed in following experiments including different strains of normotensive and hypertensive animals [60–62]. The pathophysiological relevance of Ang-(1-7) in the heart was further highlighted by studies demonstrating that chronic infusion of either Ang-(1-7) [69] or its stable non-peptide analog AVE-0991 [70] was cardioprotective in experimental heart failure. Finally, several studies demonstrated that Ang-(1-7) was protective against cardiac ischemia-induced injury and arrhythmias [71–73]. The beneficial antiarrhythmic effects of Ang (1-7) on the failing heart result from the combined effect of the peptide on the sodium pump, hyperpolarization of cardiac cell membranes, and increased conduction velocity [74]. However, in isolated hearts, supra-pharmacological concentrations of Ang-(1-7) enhanced reperfusion arrhythmias [75]. We also showed that Ang-(1-7) at higher concentrations (10−7 M), induces early-after depolarization [74]; therefore, an optimal tissue concentration of Ang (1-7) must be achieved to permit a protective role of the heptapeptide on cardiac arrhythmias.
Numerous studies indicate that the Ang-(1-7) effects on the kidney are opposite to those of Ang II. Thus, Ang-(1-7) infusion induced vasodilation of pre-constricted afferent arterioles [45], increased glomerular filtration rate, and induced natriuresis and diuresis [76–78] by inhibiting the Na+–K+–ATPase [79]. These vascular and tubular effects were attenuated by the selective Ang-(1-7) antagonist D-Ala7-Ang-(1-7). Interestingly, these counterbalancing effects of Ang-(1-7) were noticeable under conditions of RAS activation, such as during salt depletion or renal hypertension, but not in the salt-replete state [80].

Ang-(1-7) functions in the brain ::: Pleiotropy of Ang-(1-7) biological actions:
Ang-(1-7) is present in brain tissue, and its distribution throughout the hypothalamus, medulla oblongata, and amygdala underlines its importance in the regulation of blood pressure, fluid balance, and osmoregulation [81]. Although the action of Ang-(1-7) in the brain sometimes mimics the action of Ang II, such as stimulation of vasopressin release [44], their overall effects are in general opposite. The involvement of different receptors, neurotransmitter pathways, and complex integrative regulatory brain mechanisms implicated in the action of the two angiotensins have been already reviewed elsewhere [4]. In brief, intracerebroventricular administration of an Ang-(1-7) antibody elevated arterial pressure, while endogenous neutralization of Ang II had an opposite effect [50]. Ang-(1-7) at the nucleus of the solitary tract evoked bradycardic and depressor response [82], augmented baroreceptor reflex control of heart rate [83–85], and these effects were enhanced in hypertensive animals when compared to the controls [86, 87]. In the rostral ventrolateral medulla, Ang-(1-7) elicited pressor responses [88]; however, in the caudal ventrolateral medulla, Ang-(1-7) lowered arterial pressure by inhibiting the pressor action of the

rostral ventrolateral medulla [89, 90]. More unexpected actions of Ang-(1-7) include its ability to enhance long-term potentiation, a process thought to be involved in learning and memory [91].

Ang-(1-7) relevance in cardiovascular and cancerous growth ::: Pleiotropy of Ang-(1-7) biological actions:
Similar to their actions in the circulation and the control of blood pressure, Ang II and Ang-(1-7) elicit opposing effects on tissue growth as well. Ang-(1-7) inhibited proliferation of aortic vascular smooth muscle cells in culture [92], and this antiproliferative effect was later confirmed in in vivo studies. Indeed, Ang-(1-7) infusion reduced neointimal proliferation after vascular injury in rat carotid arteries [93]. Recent reports demonstrated that Ang-(1-7) also inhibited Ang II-induced protein synthesis in neonatal cardiomyocytes by activating mas [39]. Consistently, Ang-(1-7) infusion reduced myocyte surface area in rats subjected to coronary artery ligation [94]. These results are in keeping with the beneficial effects of RAS blockade on cardiac remodeling and dysfunction after myocardial infarction where activation of ACE2/Ang-(1-7) system has been verified [67]. In addition, Ang-(1-7) inhibited collagen synthesis in adult rat cardiac fibroblasts acting on receptors that are distinct from the AT1 and AT2 receptors [95]. Subsequent studies confirmed that Ang-(1-7) prevented an excessive accumulation of cardiac collagen fibrils in different models of experimental hypertension [69, 96]. Excitingly, the antiproliferative and antiangiogenic ability [97] of Ang-(1-7) found an important application in inhibiting cancerous growth as well. Thus, experimental evidence that Ang-(1-7) inhibited lung [98] and breast cancer growth in vitro [99] as well as in vivo [100] now provides a solid foundation for the initiation of clinical trials in which the chemotherapeutic potential of Ang-(1-7) is being tested.

Ang-(1-7) in pregnancy ::: Pleiotropy of Ang-(1-7) biological actions:
All components of the RAS are expressed in placenta including Ang-(1-7) and ACE2 [101], and activation of the RAS during normal pregnancy has been described in plasma and urine [102, 103]. Adequate balance between the two opposing arms of RAS might be of extreme importance in normal pregnancy, as the predominance or deficit of either one might lead toward adverse outcomes. For example, unopposed antiangiogenic properties of Ang-(1-7) may have a harmful effect, particularly in early pregnancy during which vascularization of tissue beds is critical. On the other hand, decreased plasma levels of Ang-(1-7) were associated with preeclamptic pregnancies characterized by elevated arterial pressure and proteinuria [102]. To further confirm this relationship, the most recent study from our group related an experimental model of preeclampsia with failure to increase Ang-(1-7) in kidney as well [104].

Further expansion of the complexity of the renin–angiotensin system: angiotensin-(1-12):
In line with expanding data on the newer angiotensin peptide, Ang-(1-7), Nagata and colleagues [105] recently identified another new angiotensin peptide, the dodecapeptide Ang-(1-12). The authors were probing for analogs of Ang II when they discovered an unidentified immunoreactive peak by high-performance liquid chromatography (HPLC), which the authors found to be a 12-amino acid derivative of angiotensinogen, two amino acids larger than the traditional intermediate peptide Ang I. The dodecapeptide produced pressor responses both in isolated rat aorta and acutely in intact Wistar rats—a finding that was abrogated by coadministration of both an ACE inhibitor or an angiotensin receptor blocker (ARB). These data suggested that "proangiotensin-12," as the authors named it, was exerting its actions through rapid metabolism into Ang II.
Recent data from our laboratory provided further evidence for a biological role of Ang-(1-12) as a new endogenous peptide of the RAS. Because Ang-(1-12) was identified endogenously by RIA in different organs and tissues [105] (Fig. 2), we first undertook studies that investigated the immunolocalization of the dodecapeptide in the hearts and kidneys of normal Wistar–Kyoto (WKY) and spontaneously hypertensive rats (SHR). Jessup et al. [106] found that Ang-(1-12) was localized by immunohistochemistry predominantly in cardiac myocytes, while staining in the medial and endothelial layers of the coronary arteries appeared more faint (Fig. 3) and failed to be detected in all vessels examined. The distribution of Ang-(1-12) within the hearts of SHR was more robust than that found in WKY. This observation was confirmed by tissue content analysis, which revealed significantly higher levels of cardiac Ang-(1-12) in SHR compared to WKY. Renal Ang-(1-12) was localized to the proximal and distal tubules and the collecting duct, but it was scantily observed in glomeruli or intra-renal vessels. These data, in accordance with those from Nagata and colleagues [105] show that Ang-(1-12) is indeed localized endogenously within

tissues, and the distribution of the new angiotensin peptide may reflect the state of the health of that tissue, as shown by differences in distribution between WKY and SHR.

Further enhancements towards the understanding for a biological role for Ang-(1-12) were made by studies from our laboratory which illustrated the metabolic capacity for Ang-(1-12) to yield known downstream bioactive angiotensin peptides. Intriguingly, Chappell et al. [107] found that serum exclusively formed Ang II from Ang-(1-12) by ACE, and renal neprilysin activity converted Ang-(1-12) to Ang-(1-7). Both of these pathways were independent of renin activity. Moreover, we [108] showed that Ang-(1-12) could be metabolized into Ang I, Ang II, and Ang-(1-7) in isolated hearts from five different normotensive and hypertensive rat strains. Collectively, these data provide strong evidence that Ang-(1-12) may be an alternate precursor substrate for the formation of bioactive angiotensin peptides in the heart, kidney, and circulation that may depend on the localization of one of its processing enzymes, ACE, but not renin.

Conclusions:
In conclusion, a large body of evidence emerging from experimental and human studies clearly reveals pathophysiological importance of novel peptides and related enzymes incorporated recently into the biochemical RAS cascade. The consequences of an altered ACE2/Ang-(1-7)/mas axis in hypertensive disease and heart failure is now well recognized. In addition to the potential therapeutic application in the treatment of cardiovascular disease, the ACE2/Ang-(1-7)/mas axis emerges further as a prospective therapeutic target in cancer and preeclamptic patients. Thus, there is a great potential for genetic and pharmacological modulation of the ACE2/Ang-(1-7)/mas axis in the treatment of various diseases that, however, warrants further meticulous investigation. Future studies will certainly provide us with better understanding of the relevance of ACE2 function as a receptor for severe acute respiratory syndrome (SARS) virus. They will also expand our comprehension of the signaling mechanisms and a potential angiotensin receptor interaction. Building upon the complexity unveiled thus far on the RAS as documented by data on the ACE2/Ang-(1-7)/mas axis, the demonstration of endogenous Ang-(1-12) is indeed novel and important. Although the data are still limited to the rat, forthcoming research may provide insight onto anomalies within RAS physiology that we cannot yet explain. Conceptually, Ang-(1-12) may serve as an alternate substrate for the production of bioactive angiotensin peptides as shown in our preliminary studies. Moreover, in lieu of the specific sequence requirements for the generation of Ang I by renin, Ang-(1-12) may be formed directly from angiotensinogen in a renin-independent manner. In support of this notion, Oparil and colleagues [109] found that coadministration of aliskiren and the ARB valsartan produced additive reduction in blood pressure in patients when compared to each drug administered alone—a finding not expected if renin is the limiting step in the formation of angiotensin peptides from angiotensinogen. Identification of a biological role for Ang-(1-12) requires further work; however, expression of the peptide throughout the body argues strongly that this angiotensin intermediate may add further unrecognized complexity to the renin–angiotensin system.