Countering the classical renin–angiotensin system

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It is well-established that Ang-(1-7) counteracts the effects of Ang II in the periphery, while stimulating vasopressin release and mimicking the activity of Ang II in the brain, through interactions with various receptors. The rapid metabolic inactivation of Ang-(1-7) has proven to be a limitation to therapeutic administration of the peptide. To circumvent this problem, Alves et al. (Clinical Science (2021) 135(18), https://doi.org/10.1042/CS20210599) developed a new transgenic rat model that overexpresses an Ang-(1-7)-producing fusion protein. In this commentary, we discuss potential concerns with this model while also highlighting advances that can ensue from this significant technical feat.

The renin–angiotensin system (RAS) regulates hydromineral balance, cardiovascular, renal, neuronal, and other organ systems, maintaining body homeostasis. These actions involve the ‘classic’ angiotensin-converting enzyme (ACE), angiotensin (Ang) II, and AT1 receptor (AT1R) axis. This ACE/Ang II/AT1R axis increases blood pressure via several mechanisms, including sympathetic nervous system (SNS) activation and arginine vasopressin (AVP) release. Hyperactivation of the ACE/Ang II/AT1R axis also causes pathological changes: inflammation, fibrosis, hypertrophy, and cellular proliferation. Blocking elements of the ACE/Ang II/AT1R axis typically reduces blood pressure, cellular proliferation, and inflammatory/oxidative stress-induced injury to tissues [1].

It is now known that there are counterregulatory components of the RAS, which are generally considered to oppose the ACE/Ang II/AT1R axis. Ang-(1-7) is viewed as a major component of an ACE2/Ang-(1-7)/Mas Receptor (MasR) axis counter-regulatory system. Angiotensin-converting enzyme-2 (ACE2), a homolog of ACE, present in the epithelial and endothelial cells of the heart and kidney, lung epithelial cells, and small intestinal enterocytes, is the most well-known enzyme of this axis; it inactivates Ang II as an AT1R agonist by cleaving phenylalanine from the C-terminus, giving rise to the heptapeptide Ang-(1-7) [2–4]. Enhancement of the activity of ACE2 and other carboxypeptidases that cleave the omega phenylalanine from Ang II to form Ang-(1-7) has been proposed as a mechanism to inhibit and reverse the activity of the ACE/Ang II/AT1R axis. Additional pathways for Ang-(1-7) formation include metabolism of Ang I to Ang-(1-7) by endopeptidases nephrilysin, thimet oligopeptidase, neprilysin, and prolylendopeptidase (Figure 1, see also [5]).

Angiotensin-(1-7) acts mainly through the MasR, a member of the G-protein–coupled receptor family expressed in the brain (mainly hippocampus and cerebral cortex), testis, kidneys, heart, and vessels [6,7]. However, Ang-(1-7) may also interact with the AT1R as an antagonist or a biased agonist, the AT2R, and the MrgD receptor [8–10] (Figure 1). Unfortunately, the concentrations of Ang-(1-7) used to characterize its effects in study models are often used at such high concentrations as to question its physiological and pharmacological relevance. While Ang-(1-7) is generally recognized as opposing the ACE/Ang II/AT1R axis in the periphery, in the brain it stimulates vasopressin release, mimicking Ang II [11]. When microinjected into the rostral ventrolateral medulla (RVLM) of normotensive rats, Ang II and Ang-(1-7) cause a similar pressor response [12]. Additionally, a recent review showed that microinjection of both Ang-(1-7) and Ang II into the nucleus tractus solitarius (NTS), reduced blood pressure in urethane-anesthetized rats [13]. However, as noted in the recent study by Alves et al. [14], there is considerable ambiguity regarding the Ang II-like versus the Ang II oppositional effects in the brain and kidneys, also discussed in a previous review by these authors [13].
multiple pathways of Ang-(1-7) formation and promiscuity of Ang-(1-7) binding partners

The Mas receptor is the primary receptor for Ang-(1-7). However, Ang-(1-7) has also been reported to interact with other angiotensin receptor types. Of note, there is much ambiguity regarding the physiological significance of these interactions, due to questions regarding the concentrations of Ang-(1-7) used to demonstrate the interactions. Ang-(1-7) has been proposed to have pharmacological significance as a biased agonist for the AT1R, acting via the β-arrestin signaling pathway.

A review of strategies to enhance ACE2 activity, leading to an increase in Ang-(1-7) levels, reports beneficial effects in multiple disease conditions [15]. Lentiviral vector-mediated overexpression of ACE2 in the RVLM significantly reduces blood pressure in spontaneously hypertensive rats (SHR), but not to a normotensive level, possibly because the beneficial effect of ACE2 is only to reduce Ang II levels in the RVLM instead of generating Ang-(1-7) [16]. Another approach has been to administer Ang-(1-7) to directly activate the MasR; however, this approach is plagued by the need to administer the peptide non-parenterally, or as a chemically modified molecule (inclusion with cyclodextrins to enable oral administration with intestinal absorption) [17]. However, these approaches are still plagued by rapid metabolic inactivation of Ang-(1-7). Another approach to bypass the limitations of Ang-(1-7) for therapeutic use is to administer the Ang-(1-7) mimetic AVE0991, which is orally active and resistant to enzymatic breakdown [18].

In the recent study published in Clinical Science, Alves et al. [14] have engineered a new hypotensive transgenic rat model (TG7371) whose tissues overexpress the mRNA of an Ang-(1-7)-producing fusion protein using human glial fibrillary acidic protein (hGFAP) promoter, signal peptide from human renin, immunoglobulin fragment from mouse IgG2b, a furin cleavage site preceding the mRNA for Ang-(1-7) followed by a stop codon, to counter-regulate the ACE/Ang II/AT1R axis, circumventing current limitations to the therapeutic administration of Ang-(1-7), similar

Figure 1. Multiple pathways of Ang-(1-7) formation and promiscuity of Ang-(1-7) binding partners

In the brain:
• Vasopressin release
• Increased sympathetic activity
• Pressor effect (RVLM)
• Depressor effect (NTS)
• Thirst and salt appetite

In the periphery:
• Opposes AT1R
• Decreased sympathetic activity
• Vasodilation
• Decreased fibrosis
• Natriuresis
to previous methodology [19,20]. The authors compared the TG7371 rats to wild-type Sprague-Dawley (SD) rats, reporting that TG7371 rats have decreased total peripheral resistance (TPR), increased plasma atrial natriuretic peptide (ANP), and lower blood pressure, compared with SD rats. Additionally, TG7371 rats showed increased plasma AVP and peripheral sympathetic drive. By observing the mRNA expression of the Ang-1(7)-producing fusion protein in multiple organs and tissues, they concluded that the changes observed in the TG7371 rats arose from changes in the levels of Ang-(1-7) in the brain and aortic vascular endothelial cells. They also demonstrated increased transgene protein expression in the aorta and the brain by Western blotting, with the hypothalamus expressing significantly higher levels of Ang-(1-7) than SD rats. Of note, Ang II levels in the same region were not significantly different between the two strains. In the plasma, levels of both Ang-(1-7) and Ang II were similar between TG7371 and SD rats, which is somewhat surprising considering that an increase in Ang II levels would have been expected following the observed increase in sympathetic drive in the transgenic model [21]. The rapid metabolism of Ang-(1-7) by ACE in the vasculature may contribute to the inability to demonstrate an increase in plasma Ang-(1-7). Future studies should address the importance of ACE on plasma levels of Ang-(1-7) in the TG7371 rat strain. It will also be of interest to see to what extent Ang-(1-7) is overexpressed in other brain areas and peripheral tissues, so that its expression can be related back to the mRNA expression of the fusion protein. Minimal, or lack of, spillover of Ang-(1-7) into the bloodstream suggests local action of the released peptide, which, when overexpressed, elevates blood flow in multiple organs and tissues by decreasing TPR, thereby lowering baseline blood pressure in TG7371 rats. Additionally, the study reported increased plasma ANP and sympathetic drive in the kidneys, resulting in increased excretion of sodium in the urine, increased urinary osmolality along with increased plasma AVP, making the urine more concentrated, reducing free water clearance. The presumed reduction in TPR in the kidney, along with the observed increase in renal sympathetic drive, fuels the thought that an increase in Ang II levels should have been observed, as increased renal sympathetic activity, increased plasma vasopressin and reduced blood pressure are known to increase renin release from juxtaglomerular cells via facilitated production of prostaglandin E2 and nitric oxide, ultimately leading to increased Ang II production [22,23].

This novel transgenic rat strain creates a potential model to study the pharmacological effects of Ang-(1-7) in multiple disease states, e.g., hypertension, diabetes mellitus, neurodegenerative diseases such as Alzheimer’s/vascular dementia, cardiac and renal fibrosis, thrombosis, etc., for which Ang-(1-7) or an Ang-(1-7)-mimetic, such as AVE0991, could be therapeutic. However, such a model raises some concerns, begging the question: was a possible pathological transgenic animal created? While this model overexpresses Ang-(1-7), it also introduces potentially adverse side effects, e.g., sustained increased sympathetic drive on the heart and other tissues, with increased stimulation of the kidneys by AVP. When overexpressed, AVP increases blood pressure, glomerular filtration rate, and renin release, which, in the long term, can result in chronic kidney disease [24], as is occurring in Central American farm workers subjected to increasing temperatures due to global warming [25]. While the authors view this model as examining Ang-(1-7) expression, an alternative outlook could be an animal model expressing chronic sympathetic, AVP and ANP overactivation.

A major limitation of this study was the use of only male rats. Without using both male and female test subjects, findings cannot be generalized to females. It is crucial to study both sexes, as this is a significant factor that must be taken into account. A second limitation is the lack of measurements of angiotensin peptides, renin, ACE, and ACE2 in the brain, plasma, and aorta, and of AT1R and MasR in the brain, aorta, and other tissues. Without these data, the mechanisms of the phenotypic expression of the transgenic modification cannot be elucidated. Since the ACE2/Ang-(1-7)/MasR axis counterbalances the ACE/Ang II/AT1R axis, obtaining quantifications for the associated renin, ACE, and ACE2 levels would give insight as to how this counterbalancing occurs. While the RNAscope staining corresponds to the anatomical location of vascular endothelial cells; in the brain, the lack of containing of the transgene mRNA-positive cells for either GFAP, CD34 or NeuN precludes identification of the labeled cells as astrocytes, vascular endothelial cells, or neurons, an ambiguity revealed by the inconsistent characterization of the labeled cells in the brain. Yet another limitation is the lack of comparison of body weight differences between TG7371 and SD rats. Both drinking and cardiac index are dependent on body weight, and the apparent tendency for the TG7371 rats to have a lower body weight may skew these comparisons. A final limitation would be that the authors only measured Ang-(1-7) in the hypothalamus and plasma. Thus, the extent of expression of Ang-(1-7) by the transgene in other brain areas and tissues is unknown, as noted above. They did however provide Western blot data for the expression of the 32 kDa Ang-(1-7)-producing fusion protein in various tissues and organs. While this measurement is less than ideal, it provides good insight into expression of the transgene throughout the body, setting the stage for future research.

This novel transgenic rat strain must be acknowledged as a significant technical feat, as it is still fairly uncommon to create transgenic rat models. Transgenic models are typically created using mice, which are much easier to work
with while less costly to obtain and maintain, but rat models of diseases are superior to mouse models [26]. The larger size of rats makes them better models for cardiovascular studies, since the bigger the animal, the less relative tissue damage is caused by surgical techniques [26,27]. Additionally, rats are more easily trained, thereby easier to handle and monitor behavioral changes. They are also less aggressive and less territorial [27], obviating the need for single housing.

Multiple questions arise from the findings and the TG7371 transgenic rat model created by Alves et al. [14], the first one being: is overexpression of Ang-(1-7) in the model indicating physiological function of the peptide, or is this an animal model of the effects of overexpression? Different explanations can be given to the observed hyperactivity of AVP and the SNS. One is that it can be a compensatory baroreflex response to reduced blood pressure arising from Ang-(1-7)'s vasodilatory effect. Another explanation is that the sympathetic hyperactivity and increased plasma AVP can be due to direct effects of Ang-(1-7) mimicking Ang II effects in the brain. The means by which this could occur are uncertain, but could include differences between AT1R in the brain and the periphery, or similar biased agonist effects of brain Ang II and Ang-(1-7) on the AT1R. The biased agonist effect occurs at higher concentrations than the G-protein–coupled response, so the exogenous administration of Ang II and Ang-(1-7) may preferentially generate such effects [28]. Another possible explanation is that there is little or no MasR in the RVLM, NTS, and paraventricular nucleus of the hypothalamus, so the action of Ang-(1-7) in these brain regions involves a different mechanism of activation of these neuronal pathways.

It is important to note that the observed increase in SNS and AVP activity is not a good thing. In the future, this model could be used to determine if there is also an increase in renal sympathetic nerve activity, which would be expected to increase renin release from the kidneys, consequently increasing plasma Ang II, leading to renal failure later in life. Additionally, hypersympathetic drive overstimulates cardiac β1 adrenergic receptors, resulting in cardiomyocyte hypertrophy, proliferation, mitogenesis of cardiac fibroblasts, and apoptosis, all of which can trigger cardiovascular diseases, including heart failure [29]. In all, the TG7371 rat promises to be a welcome, but complicated, addition to our arsenal of animal models that can be used to enhance our knowledge of physiological regulatory mechanisms.

Competing Interests
The authors declare that there are no competing interests associated with the manuscript.

Funding
Yazmin M. Restrepo and Robert C. Speth are supported by NIH-NINDS [grant number R01-NS124204].

Acknowledgements
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Abbreviations
ACE, angiotensin-converting enzyme; ACE2, angiotensin-converting enzyme 2; Ang II, angiotensin II; Ang-(1-7), angiotensin-(1-7); ANP, atrial natriuretic peptide; AT1R, angiotensin II type 1 receptor; AT2R, angiotensin II type 2 receptor; AVP, arginine vasopressin; hGFAP, human glial fibrillary acidic protein; MasR, Mas receptor; MrgD, Mas-related G-protein–coupled receptor D; NTS, nucleus tractus solitarius; RAS, renin–angiotensin system; RVLM, rostral ventrolateral medulla; SD, Sprague-Dawley; SHR, spontaneously hypertensive rats; SNS, sympathetic nervous system; TPR, total peripheral resistance.

References