Deciphering the Roles of Tissue Renin-Angiotensin Systems in Whole Animals

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I am both grateful and humbled to have been invited to give the 2010 Arthur C. Corcoran memorial lecture. I want to thank not only those who nominated and selected me for this prestigious recognition but also all of the trainees, colleagues, and collaborators who have helped to refine the ideas that motivate the research and provide the engine for its realization. Although my group has worked on many aspects of renin-angiotensin system (RAS) function, I have chosen to focus this lecture on the development and application of an engineered protein with the unique ability to target peptide production in whole animals.

RAS Components Scattered Throughout the Body
With the systematic cloning of the various components of the RAS in the mid-1980s, it became possible to use molecular approaches to directly test an idea that had been circulating for some time: that the kidney might not be the only site of renin production and that angiotensin peptides might be formed in tissues where they could have roles beyond blood pressure regulation. In fact, the genes coding for angiotensinogen, renin, angiotensin-converting enzyme (ACE), and various angiotensin receptors appeared to be turned on to different extents in a number of tissues including the brain, kidney, adrenal and pituitary glands, heart and vasculature, and reproductive tissues, leading to the suggestion that tissue RASs might function in a locally restricted manner (reviewed in Ref.1). Thus, although the RAS has an undisputed role in the regulation of blood pressure and volume homeostasis and has become the primary target in the treatment of hypertension, many intriguing questions regarding the physiology and biochemistry of the RAS remain. Chief among these are the nature of the active peptides and whether observed tissue effects are due to the direct action of angiotensin peptides generated within those tissues or whether they are secondary to the hemodynamic effects of angiotensins.

More Complex RAS Than Originally Thought
In addition to the classical circulatory pathway whose main product is Ang II, it has now become clear that enzymes can generate a number of additional biologically important angiotensin peptides (Figure 1). Of particular interest is the ability of the ACE2 enzyme to convert Ang II to Ang-(1-7) and the generation of Ang III and Ang IV by the action of amino-peptidases. Notably, the degree to which any of these metabolites of Ang II are made will depend on the concentration of the starting material (eg, Ang I or Ang II) and the presence of the necessary enzyme for the conversion. As such, these peptides are most likely to act in tissues rich in the conversion enzymes or in conditions of high accumulation of the starting peptide (eg, with ACE inhibition or AT1 receptor antagonism). These conditions pose a particular challenge in determining whether these peptides play a local role in tissues.

Challenges in the Study of the Tissue RAS
Genetically modified mice and rats provide a powerful tool for testing biochemical hypotheses in the context of the whole animal. Indeed, several groups have made use of transgenic mice to overexpress components of the RAS resulting in mice and rats with hypertension dependent on a humanized RAS. Gene knock-out experiments have also led to additional insights into the role of the RAS. Nevertheless, these approaches are limited by 3 main factors. First, many of them lead to whole-body alterations in the RAS. For example, a knock-out of the AT1 receptor inactivates the gene in every organ, thus making it difficult to distinguish between direct effects of angiotensin on the tissue and those effects that are secondary to hemodynamic effects of the peptide. This limitation can be overcome, to some degree, by performing a conditional cell-type-specific inactivation of the gene of interest. Second, the generation of angiotensin peptides requires the concerted action of both renin and ACE on angiotensinogen, making it necessary to ensure that all of the components are in the appropriate tissue compartment and at the appropriate concentrations to work effectively. Third, using standard transgenic approaches, it is impossible to control the relative abundance of the different peptides of the RAS (ie, you cannot make Ang 1-7 or Ang IV directly). Osmotic minipumps can partially overcome the latter problem, but they cannot be used to target release of the peptide to well-defined tissues or cell types. For these reasons, we have developed a novel technique for targeted production of...
Engineered Protein to Directly Generate Peptides of Biological Interest

The essential features of the protein designed to release angiotensin peptides are shown in Figure 2A. A signal peptide from human prorenin is linked to a portion of the mouse heavy chain constant region of IgG2b, which we have referred to as the Fc fragment (although it does not exactly correspond to a true Fc fragment of IgG). The signal peptide ensures that the protein will enter the secretory pathway for eventual release from the cell. Although the heavy chains of immunoglobulins normally form disulfide-bridged dimers in the secretory pathway where they bind to a protein called BiP while awaiting the arrival of the light chain, the immunoglobulin fragment chosen does not contain either the intermolecular disulfide bridge or the BiP-binding region. Not only does the immunoglobulin portion of the molecule provide mass for the efficient production of the protein precursor in the secretory pathway, it also greatly facilitates expression of a broad assortment of linked peptides, perhaps because it adopts a stable conformation, thereby avoiding the degradation of poorly folded proteins that occurs in the secretory pathway. Another advantage of using this portion of the mouse IgG molecule is that it contains a protein A-binding region, making it possible to easily purify the fusion protein from culture supernatants. The Fc fragment is linked to the peptide of interest by a portion of the human prorenin prosegment containing a cleavage site at its carboxyl terminus for a protease. In early experiments, we found that this molecular spacer was necessary to expose the protease processing site for the cleavage, which results in the release of the peptide.

The protease processing site between the molecular spacer and the peptide is the key to achieving efficient production of peptide in targeted cells. For several reasons, we chose the protease furin. Furin is a membrane-bound protease that cleaves its substrates in the trans-Golgi network of the secretory pathway of virtually all mammalian cells. Because a fair amount is known about the cleavage preference of furin, we were able to design the peptide cleavage site in the fusion protein to be attractive to furin by modifying the natural prorenin prosegment cleavage site (PMKR to RVRTKR; Figure 2A). After cleavage, the encoded peptide is released in the secretory pathway and secreted by the cell (Figure 2B).

Although the amino acids on the amino-terminal side of the cleavage site are crucial in defining which proteases can cleave a given substrate, the amino acids on the carboxy-terminal side of the cut site are also very important in determining the efficiency of cleavage. The selectivity of furin can be deduced in part by examining its natural substrates. This analysis reveals a broad, but finite, array of sequences that can be used in engineering the fusion protein downstream of the furin cleavage site (reviewed in Ref.5). In practical terms, since the first amino acid after the cleavage site will constitute amino acid 1 of the released peptide, the types of peptides that can be generated using the engineered proteins are limited to those that conform to the cleavage preference of furin.

One of the most interesting extensions of this method is the possibility of making use of a vast literature on the substitution of single natural amino acids in peptides, which give them either antagonist or reverse agonist activity, an altered receptor affinity, or which alter or restrict the signaling of the bound receptor or increase their stability. Some examples of non-natural and xenobiotic peptides we have targeted in vivo using this method are shown in Figure 3A. This technique is, to my knowledge, the only means to investigate

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**Figure 1.** Schematic representation of the RAS pathway and its enzymes, resulting peptides, and putative receptors. Angiotensin (Ang) peptide products are boxed, enzymes are in blue, and receptors are in red. NEP indicates neutral endopeptidase; PEP, prolyl endopeptidase; AP, amino-peptidase; IRAP, insulin-regulated amino peptidase; AT1, angiotensin type 1 receptor; AT2, angiotensin type 2 receptor.

**Figure 2.** Characteristics of the fusion protein engineered to release peptides from cells. A, Schematic diagram of the protein with the function of its various domains. B, The protein is directed to the secretory pathway where the peptide domain is released by the action of the ubiquitous protease furin. The resulting peptide as well as the remnant of the fusion protein are subsequently released from the cell.
the effects of tissue restricted and chronic administration of these peptides in whole animals.

Checking Out the System in Cell Culture
It is quite likely that the conformation of the fusion protein around the furin cleavage site is subtly affected by the structure of the peptide attached. Both this and the sequence of the peptide at the scissile bond have the potential to affect the efficiency of peptide release from the fusion protein. Because it is difficult to predict the efficiency of the cleavage event from the amino acid sequence of the peptide alone, it is useful to have an in vitro test of the efficiency of this processing event before embarking on more costly transgenic experiments.

The immunoglobulin domain of the engineered protein is particularly useful in this regard for two reasons. First, it allows a rapid batch purification of the fusion proteins using protein A or G. Second, it is common among all of the engineered proteins, thus making it unnecessary to use antibodies directed against each new fusion protein. In our own group, we transfected a cell line expressing good levels of furin (eg, GH4C1 cells) with an expression vector encoding one of the engineered proteins under the control of a strong viral promoter (eg, the Rous sarcoma virus or RSV long terminal repeat). The secreted fusion proteins can then be rapidly purified from the culture supernatants using protein A-Sepharose and analyzed by SDS gel electrophoresis (see Figure 3B for an example). The full length fusion protein and the remnant after removal of the peptide by furin can be distinguished by their size. Such results provide two important pieces of information. First, the simple presence of the engineered protein in the culture supernatant is evidence that the particular construction is secreted from the cell (and by implication, correctly folded). In the example shown in Figure 3B, it can be seen that all of the engineered proteins are expressed at roughly equivalent levels in the transfected cells regardless of the encoded peptide. This is remarkable for engineered proteins in the mammalian secretory pathway because of a very efficient structural proofreading machinery in the endoplasmic reticulum and may be due to the use of the immunoglobulin domain as the engineered protein core. Second, the ratio of the full-length to the cleaved form is an indication of the efficiency of the furin cleavage that results in the release of the peptide.

Leap to In Vivo Systems
To test for the role of various angiotensin peptides in vivo, we made use of the ability of gene control elements (promoters) to target the expression of genes to very specific tissues in transgenic mice and rats. In the example shown in Figure 4, linking the a-myosin heavy chain promoter to the fusion protein results in the expression of the transgene in cardiomyocytes and the release of Ang II into the interstitium of the heart. The accumulated Ang II can then stimulate signaling either in an autocrine or paracrine fashion in the surrounding tissue. By simply changing either the promoter or the portion of the transgene coding for the peptide, it is possible to target the chronic release of various peptides in tissues of choice. Following is a brief overview of experiments that illustrate the flexibility and unique features of this tool in the analysis of tissue RAS function.

Brain Ang II and Kidney Development/Function
Inactivation of the RAS by gene mutation in humans has lethal consequences, caused primarily by a failure of renal tubules to develop. Likewise, inactivation of key components of the RAS in mice (renin, angiotensinogen, ACE) results in a number of defects including prenatal lethality, increased thirst and urine production, hypotension, development of postnatal hydrenephrosis, and an inability to concentrate urine when challenged with dehydration. Because the same severity of symptoms required the simultaneous inactivation of the AT1a and AT1b receptors, we reasoned that the responsible tissue must be rich in both receptors and, for this and other reasons, suspected a role in the brain. To test this hypothesis, we made use of the direct peptide targeting system to release Ang II in the brain. By expressing an Ang II-releasing fusion protein under the control of an astrocyte-specific promoter (glial fibrillary acidic protein), we were able to target Ang II release specifically to the brain of transgenic animals. By crossing these mice with a strain...
deficient for angiotensinogen (AGT−/−), we were able to generate mice that only made Ang II in the brain. Restoration of Ang II exclusively in the brain of these mice reduced water intake and urine production, restored normal blood pressure, and restored the ability of the mice to concentrate urine.12 Notably, restoration of brain Ang II eliminated the hydronephrosis seen in AGT−/− mice, although it did not correct the obvious disorganization of the renal cortical tubules. These results demonstrated for the first time that many of the defects associated with the inactivation of the RAS can be attributed to loss of brain angiotensin peptides (possibly Ang II).

**Ang IV in the Brain**

As with any enzymatic reaction, generation of the various peptides of the RAS (Figure 1) requires not only a reasonable abundance of the appropriate starting material or substrate but also the presence of sufficient enzyme with a reasonable affinity for that substrate (reviewed in Ref.13). For example, Ang II is probably only converted to Ang III and Ang IV to a significant extent in tissues with abundant aminopeptidases, such as the brain and kidney. Because Ang IV binding sites were reported to be abundant in the brain and show only a partial overlap with Ang II binding sites, we decided to test the function of chronic elevations of brain Ang IV by using a modification of the peptide targeting strategy we had previously used for Ang II. Because of the cleavage preference of the furin protease required to release the peptide from our fusion protein, we had to add an alanine to the amino terminus of the peptide (Figure 3A, ala-AngIV), which is rapidly removed from the majority of the peptide after release from the expressing cells in vivo.14 Second, we substituted the proline that would normally be found at the penultimate position of the peptide with a glycine that had been reported to increase the affinity of Ang IV by 10-fold for its receptor.9 With Gaëtan Thibault (Clinical Research Institute of Montreal, Montreal, Quebec, Canada), we found that the glycine-substituted peptide only binds its receptor with an affinity similar to that of natural Ang IV, but it provided an additional benefit: it allowed us to develop an antibody that distinguished the transgenic Ang IV from endogenous angiotensin peptides (our Ang II antibody did not distinguish between Ang II, Ang III, and Ang IV).

To our great surprise, the mice with increased Ang IV in the brain exhibited chronic hypertension that responded to an Ang II AT1 receptor antagonist. In collaboration with Rhian Touyz (University of Ottawa, Ottawa, Ontario, Canada), we subsequently found that Ang IV potentiates a long-lasting increase in intracellular calcium concentration only after the cell has been stimulated with Ang II, which likely explains the efficacy of the AT1 receptor antagonist in our mice. These results were the first to propose a mechanistic interaction between Ang IV and Ang II, and they made use of two of the characteristics of the peptide-targeting system that set it apart from other transgenic strategies. First, we were able to obtain mice with a chronic (lifetime) increase in a specific Ang II metabolite in a target tissue, something that is not possible with transgenic animals that overexpress renin and angiotensinogen. Second, it allowed us to study the chronic, tissue-specific effects of a non-natural peptide with potentially interesting properties. To my knowledge, this is not possible with any other approach.

**Ang-(1-7), the Heart, and the Kidney**

Although Ang-(1-7) had long been considered a breakdown product of the RAS, there existed numerous reports of its beneficial properties including vasodilation, regulation of cardiac function and remodeling, growth of cardiomyocytes, smooth muscle and mesangial cells, regulation of salt balance in the kidney, wound healing, stem cell activation, and cancer (reviewed in Ref.15). Research concerning its biological properties took on new importance with the demonstration that its concentration greatly increased with ACE inhibition and angiotensin receptor blockade16 and the identification of an enzyme (ACE2; Figure 1) that mediates its direct synthesis from Ang II.17 Because most of the properties of Ang-(1-7) were demonstrated either in vitro or in acute infusions, we used the peptide-targeting strategy to examine the chronic in vivo effects of this interesting peptide. In collaboration with Michael Bader (Max Delbrück Center for Molecular Medicine, Berlin-Buch, Germany) and Robson Santos (Federal University of Minas Gerais, Belo Horizonte, Brazil), we generated a transgenic rat that expressed the Ang-(1-7) fusion protein under the control of a viral promoter with the intention of driving whole-body increases in Ang-(1-7).18
Surprisingly, the transgene was only expressed in the testicle of male rats. Nevertheless, the resulting 2- to 3-fold chronic increase in circulating Ang-(1-7) levels in these rats resulted in a decrease in hypertensive cardiac remodeling; decreased vascular resistance in selected beds including the lung, spleen, kidney, adrenals, brain, testis, and brown fat tissue; and a decrease in urine output with no apparent effect on salt balance or renal histology. Notably, the animals were not hypotensive despite the reported vasodilatory properties of Ang-(1-7) underscoring the importance of testing the in vivo chronic responses to the peptide.

Using the fusion protein to release Ang-(1-7) exclusively in the heart revealed that the cardioprotective effects of the peptide largely are due to a direct action on the heart rather than being secondary to peripheral (eg, renal) effects of the peptide. In collaboration with Rhian Touyz, we obtained evidence that the cardio-protective effects correlated with an induction of mitogen-activated protein kinase phosphatase activity, which pointed to a unique mechanism of action of the Ang-(1-7) peptide.

More recently, Mike Katovich and Mohan Raizada (University of Florida, Gainesville, FL) packaged the Ang-(1-7)-releasing fusion protein into a lentivirus expression vector. When administered by such viral therapy to the lungs, Ang-(1-7) significantly reduced the pulmonary fibrosis and hypertension resulting from monocrotaline injury. In all of these experiments, the use of the peptide-releasing fusion protein has solidified the position of the Ang-(1-7) peptide as a potentially important therapeutic complement to extend the clinical benefits of RAS blockade.

Ang II and the Heart

Of all of the studies we have undertaken with the peptide-targeting system, those involving the role of Ang II in the heart yielded results that were the most surprising and had the most direct implications for clinical care. By targeting the expression of an Ang II-releasing fusion protein to the heart of transgenic mice (Figure 4), we obtained mice with 30- to 50-fold increases in cardiac Ang II with no apparent spillage of the peptide into the circulation. Despite the abundant data showing that Ang II is hypertrophic to cardiomyocytes placed in tissue culture, these mice showed no evidence of cardiac hypertrophy. The transgenic mice had not lost the ability to respond to Ang II or develop hypertensive cardiac remodeling; however: infusion of Ang II over a 2-week period resulted in an appropriate hypertension and cardiac hypertrophy in these mice. Furthermore, engineering the fusion protein to release a degradation-resistant variant of Ang II (fsAng II; Figure 3) led to spillage of Ang II from the heart into the circulation, leading to hypertension and cardiac hypertrophy. Although the large accumulation of Ang II in the heart of these mice has no apparent effect on contractility or heart rate, there were more subtle changes detectable in these hearts including a doubling of interstitial fibrosis and a reduction in ventricular capillary density.

These subtle changes may have big effects when the heart is challenged, however. Oscar Carretero and Xiao-Ping Yang (Henry Ford Hospital Research, Detroit, MI) experimentally induced a myocardial infarction (MI) in the hearts of mice by coronary artery ligation and found that those engineered to have high cardiac Ang II had significantly decreased cardiac function 8 weeks after the MI compared to control mice. More recently, these same investigators found that deoxycorticosterone acetate/salt-induced hypertension led to more severe cardiac remodeling (inflammation, oxidative stress, and cell death contributing to cardiac hypertrophy and fibrosis) in mice with high cardiac Ang II than was seen in control animals. These results are consistent with Ang II setting up the heart to perform poorly when challenged. Indeed, several clinical trials have demonstrated decreased morbidity and mortality post-MI with RAS inhibition, but it has been extremely difficult to determine whether these benefits were due to reductions in blood pressure or other mechanisms.

Overall, the results obtained using the Ang II-releasing protein in the heart clearly identify targets beyond blood pressure control in improving outcomes in hypertension and after MI.

Other Applications

Although we have primarily used the peptide-releasing system to study the importance of the tissue RAS, the approach has the potential for much broader applications. As an example, the peptide being released does not have to be a member of the angiotensin family but could include any number of other peptide hormones or growth factors. In addition, since this approach is not limited to naturally occurring peptides (see above), there is tremendous potential for expanding its application to non-natural peptides with particular biological properties.

Another modification of the approach is to engineer the peptide-releasing fusion protein into viral vectors as was reported recently by Katovich, Raizada, and their colleagues. This approach makes it possible to express peptides in sites of injection or lavage in animal models without the long delay normally required to generate transgenic animals.

Finally, the peptide-releasing protein has tremendous, but as yet untested, potential in the transient expression of peptides or growth factors in stem or progenitor cells where paracrine or autocrine action might help in targeting, engraftment, or maintenance of differentiation of these cells for regenerative therapy. Although we designed the protein with a mixture of domains from human and mouse proteins (Figure 2), the fusion protein could be easily humanized for therapeutic applications.

Perspectives

In our efforts to develop a better method to study the role of the tissue RAS, we designed a peptide-releasing protein that allows the precise targeting of peptide production in tissues of whole animals. This approach has led us to discover roles of the angiotensin peptides that were not predicted based on the results of experiments treating isolated cells or organs in vitro or by infusing peptides over short periods of time in vivo. It is my conviction that this approach has a unique potential to enlighten our understanding of problems of clinical importance related to RAS therapy and to potentially identify new treatment avenues to extend the benefits of RAS blockade in the treatment of cardiovascular disease.
Acknowledgments

The work described in this brief review would not have been possible without the expert technical support of Chantal Mercure, Marie-Josée Lacombe, and Manon Laprise.

Sources of Funding

This research was supported by grants from the Canadian Institutes for Health Research.

Disclosures

None.

References


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*Hypertension*. 2011;57:532-537; originally published online December 28, 2010;
doi: 10.1161/HYPERTENSIONAHA.110.167114

*Hypertension* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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