Tissue Targeting of Angiotensin Peptides*

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Angiotensin II (Ang II) is an octapeptide generated by the sequential proteolytic action of renin and angiotensin converting enzyme on the glycoprotein angiotensinogen. While numerous mammalian tissues have been shown to express some or all of the components of the renin-angiotensin system (RAS), the function of most of these tissue RAS remains a matter of conjecture. To test for tissue-specific functions of Ang II and as an alternative to co-expressing all the components of the RAS, we have engineered a fusion protein that leads to direct Ang II release within specific tissues. The angiotensin peptide is cleaved from the fusion protein within the secretory pathway by the ubiquitous endoprotease furin and is released from the cell by constitutive secretion. Direct injection of an expression vector encoding such a fusion protein into rat cardiac ventricles results in a highly localized expression of atrial natriuretic peptide mRNA (an angiotensin responsive marker of cardiac hypertrophy), demonstrating the utility of this approach for local targeting of mature peptides to tissues in animal models.

Angiotensin II (Ang II) is the peptide product of the renin-angiotensin system (RAS) and is generated via two sequential proteolytic steps. First, renin, a circulating aspartyl protease, cleaves the decapeptide angiotensin I (Ang I) from the amino terminus of the hepatic glycoprotein angiotensinogen. Angiotensin-converting enzyme then removes two amino acids from the carboxyl terminus of Ang I to release the vasoactive peptide Ang II. This enzymatic cascade that occurs in the circulation has also been suggested to take place within certain tissues. Brain, kidney, adrenal and pituitary glands, heart, vasculature and reproductive tissues have been shown to express protein and/or mRNA for many of the components of the RAS including angiotensin receptors (1–6). Activity of local or tissue RAS has been implicated in a variety of physiologic pathways and pathophysiologic conditions including sympathetic nerve transmission, pituitary hormone secretion, migration of eggs in the oviduct, renal development, hypertension, end-stage renal disease, cardiac hypertrophy, and restenosis following vascular injury (reviewed in Refs. 7–10). However, two findings make it difficult to differentiate between the possibilities that the Ang II mediating these activities is either generated and acts locally, comes from the circulation, or is synthesized locally and acts elsewhere to generate the observed effects. First, in tissues, components of the RAS are often expressed in different cell types or are present in only extremely low levels, making it difficult to be certain that all of the necessary components would encounter each other in biologically relevant concentrations. Second, renin is synthesized as a zymogen that is activated before secretion from the juxtaglomerular cells of the kidney. Removal of the kidneys results in virtual disappearance of renin from the circulation, whereas its precursor, prorenin, remains (11–14). This finding has raised the question about whether non-renal tissues have the capacity to activate prorenin and thereby carry out the first reaction in the RAS. For these reasons and despite much circumstantial evidence, the functions of tissue RAS remain a matter of conjecture.

By using transgenic animals, it is possible to test for the biological effect of tissue expression of the RAS by inducing either loss of function or gain of function mutations. Mice lacking all RAS activity have been generated via homologous recombination by insertional mutagenesis of the angiotensinogen gene (the only known substrate for generation of the angiotensin peptides) (15, 16). These mice are hypotensive and exhibit some defects in the development of the kidney, but these experiments shed little light on the normal physiologic functions of tissue RAS. On the other hand, generalized over-expression of components of the RAS in transgenic mice and rats is clearly linked to an increase in blood pressure (17–20). To discriminate between circulatory and local effects of transgene expression, it is necessary to target expression of the RAS transgenes to specific tissues. However, this approach is complicated by the fact that all of the RAS components (i.e. angiotensinogen, prorenin, angiotensin-converting enzyme, Ang II receptors, and a prorenin convertase) need to be expressed in the target tissue. In addition, the Ang II generated can elicit biological responses at other sites.

As an alternative to co-expressing RAS components, we have engineered a fusion protein that leads to the direct release of an Ang II peptide within specific tissues. This peptide is a natural variant of Ang II found in the skin of the Australian frog *Crinia georgiana* (fsAngII (21)) which has been reported to have pressor activity similar to that of mammalian Ang II (22). Its amino-terminal alanine residue presents a favorable P1 cleavage site for proteolytic release of fsAngII from the fusion protein by the ubiquitous protease furin (23). In this study, the receptor binding and biological activity of the fsAngII peptide...
were characterized, and experiments were performed to test for the effect of local over-expression of fsAngII in vivo. Direct injection of the expression vector into the apex of rat hearts leads to local expression of fsAngII and induction of atrial natriuretic peptide (ANP) expression, a biochemical molecular marker of cardiac hypertrophy in adult cardiac ventricles (24, 25). These results demonstrate for the first time the feasibility of using an engineered fusion protein to deliver a peptide with local biological activity in whole animals.

MATERIALS AND METHODS

Construction of an Expression Vector for Direct Release of Ang II—Expression vector plg contains the signal peptide of human prorenin followed by a fragment of the heavy chain constant region of mouse IgG2b. The Ig fragment encodes 221 amino acids from the CH2 and CH3 domains while maintaining the protein A binding motif of cardiac hypertrophy in adult cardiac ventricles (24, 25). The expression vector pIg contains the signal peptide of human prorenin following the Ig fragment was amplified from pIg by polymerase chain reaction. The sequences of the forward and reverse primers were 5′-CGCCATTTGACCATTCAC-3′ and 5′-AAGGCCTCTCCCGACAGAC-3′. Construction of this plasmid is described elsewhere (27). This construct is used as a negative control (Fig. 1A).

The expression vector plgPfsAngII (Fig. 1B) contains the human prorenin signal peptide and Ig fragment from plg linked to a portion of the human prorenin prosegment followed by a furin consensus cleavage site and the coding sequence of the fsAngII peptide. plgPfsAngII was constructed as follows. A cDNA fragment encoding the signal peptide of human prorenin following the Ig fragment was amplified from plg by polymerase chain reaction. The sequences of the forward and reverse primers were 5′-GGCCATTGTGACACATC-3′ and 5′-AAGGCCTCTCCCGACAGAC-3′. A StuI restriction site in the reverse primer is underlined. A portion of the human prorenin cDNA encoding the first 35 amino acids of the prosegment was generated by polymerase chain reaction from the expression vector pHrR1100 (26) using the following primers: forward, 5′-AAGGCCTCTCCCGACAGAC-3′; reverse, 5′-GAA- GATCTGGGACCAAGGCTG-3′ (StuI and BglII restriction sites in the amplifying oligonucleotides are underlined). The StuI-BglII restriction fragment containing the human prorenin prosegment was ligated to oligonucleotides containing a 5′ BglII site, a sequence encoding the furin consensus cleavage site (RVKTRK (29, 30)), the coding sequence for fsAngII (APGDRIYVHPF), a stop codon, and a 3′ EcoRI site and inserted at the 3′ end of the Ig coding sequence. The forward oligonucleotide sequence was 5′-GGCCATTTGACCATTCAC-3′, the reverse sequence was 5′-GGCCATTTGACCATTCAC-3′. The entire coding sequence was placed in the expression vector Rous sarcoma virus-globin where it is under the transcriptional control of the Rous sarcoma virus-long terminal repeat promoter/enhancer. Transcribed RNA is provided with an intron and polyadenylation signal from a portion of the rabbit β-globin gene placed 3′ of the fusion protein coding sequence (28).

The expression vector plgfsAngII (Fig. 1C) is constructed as plgPfsAngII except that the portion of the human prorenin cDNA encoding the first 35 amino acids of the prosegment is missing, and the 3′ splice and polyadenylation signal comes from the simian virus 40 T-antigen splice and polyadenylation signal.
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**Cleavage and Secretion of the Fusion Protein in Transfected Rat Pituitary GH,C1 Cells—**Rat pituitary GH,C1 cells were grown in a mixture of Dulbecco's modified Eagle's medium supplemented with 5% (v/v) fetal calf serum, 0.1% streptomycin (Irvine Scientific, CA), and geneticin at 10 µg/ml (Life Technologies, Inc.) in a humidified incubator (5% CO₂, 95% air) at 37 °C. GH,C1 cells, plated at 8 x 10⁵ cells per 35-mm dish, were transfected 20 h after plating with Lipofectin (Life Technologies, Inc.) in serum-free medium using 20 µg of plasmid DNA per dish. The cells were transfected with an expression vector for fsAngII (plgPfsAngII) or its control counterpart plg (Fig. 1). After 48 h, cells were transferred to 12-well plates at a concentration of 4 x 10⁴ cells/well. Twenty four hours later parallel wells of transfected cells were depleted of methionine for 1 h in methionine-Dulbecco's modified Eagle's medium containing 10% dialyzed fetal calf serum. The cells were then labeled for 30 min with 300 Ci of [35S]methionine/well, washed with complete medium, and incubated for 2.5 h. Culture supernatants were immunoprecipitated with protein G-agarose (Life Technologies, Inc.). Immunoprecipitated proteins were fractionated by SDS-polyacrylamide gel electrophoresis, and gels were subjected to fluorography. SDS-polyacrylamide gel electrophoresis, and gels were subjected to fluorography. 

**Expression of fsAngII in Transfected Myocytes—**Ventricular myocyte cultures from 4-day-old Sprague-Dawley rats (Charles River, St-Constant, Quebec, Canada) were prepared as described elsewhere for atrial myocyte cultures (33). Twenty four hours before transfection, cardiac myocytes were plated at 1.5 x 10⁶ per 35-mm Primaria-coated dish (Falcon). The cells were grown in complete serum-free medium (CSFM-I, Life Technologies, Inc.) supplemented with 10% (v/v) fetal calf serum and ampicillin-streptomycin-fungizone (Life Technologies, Inc.) at 10 µg/ml in a humidified incubator (5% CO₂, 95% air) at 37 °C. The cells were transfected with plgPfsAngII or plg as a control. For transfection, a DNA-calcium phosphate precipitate (250 µl) formed with 10 µg of plasmid DNA was added, and the cells were incubated at 37 °C. After 24 h the medium was changed, and the cells were incubated an additional 48 h. Supernatants were collected and cells were rinsed twice with phosphate-buffered saline and sonicated in 2 ml of ice-cold extraction medium (5% formic acid, 15% trifluoroacetic acid, 10% hydrochloric acid, 1% sodium chloride). Lysates were cleared by centrifugation, and pellets were retained for protein quantitation by the method of Bradford (38). Ang II content of cell lysates and supernatants from transfected cardiac myocytes was extracted on disposable octadecyl cartridges (38). Ang II content of cell lysates and supernatants from transfected cardiac myocytes was extracted on disposable octadecyl cartridges (38). An antiserum against Ang II (designated CD4) was used. This antiserum displayed 50–60% cross-reactivity to fsAngII (Ala-Pro-Gly-[Ile³,Val⁵]Ang II), purchased from Bachem, and less than 1% cross-reactivity to Ang I (data not shown).

**In Vivo Injection of DNA Into Rat Hearts—**This protocol was approved by the Care of Experimental Animals Committee of Henry Ford Hospital and is in accordance with federal guidelines. 250–300-g rats (Harlan Sprague Dawley, Charles River, Kalamazoo, MI) were anesthetized, intubated, and ventilated. Following thoracotomy, the heart was injected with 95 µg of DNA in 100 µl of phosphate-buffered saline as described previously (40). Rats were randomly divided into two groups, one group receiving injection of plgPfsAngII and the other receiving plg as a control. These animals were used to generate data on plasma Ang II levels, heart weight to body weight ratios, and blood pressure. The two DNA constructions were also injected into a separate group of rats for Northern blot analysis of injected hearts. Systolic blood pressure of conscious rats was measured by the tail-cuff method. Four to seven determinations were made for each rat. Measurement were made twice prior to DNA injection and on day 6 post-injection. All other parameters were determined at the time of sacrifice (7 days post-injection).

**Northern Blot Analysis—**Total RNA was extracted by homogenization of hearts in RNA STAT-60 (Tel-Test, Friendswood, TX). Northern blot analysis for atrial natriuretic peptide (ANP) and glyceraldehyde-3-phosphate dehydrogenase has been described previously (41). ANP mRNA was quantitated using scanning densitometry of autoradiograms.

**RESULTS**

**Receptor Binding of fsAngII—**Binding of frog skin Ang II (fsAngII) to the AT₂ receptor was tested using membrane preparations from transfected COS-1 cells. As shown in Fig. 2A, binding of ¹²⁵I-labeled [Sar¹,Ile⁸]Ang II was completely displaced with cold Ang II with an apparent Kᵢ of 0.66 ± 0.25 nM. fsAngII also completely displaced the radiolabel, but the apparent affinity was approximately 43-fold lower than that of Ang II. Losartan, an AT₁-specific antagonist, completely displaced binding, confirming that all of the receptors in this membrane preparation are of the AT₁ subtype. Using membrane prepared from PC12W cells, we have determined that the fsAngII peptide also bound the AT₂ receptor with an affinity only slightly lower than that of native Ang II (apparent Kᵢ of fsAngII = 6.18 ± 1.01 nM; apparent Kᵢ of Ang II = 1.94 ± 0.30 nM) (Fig. 2B). PD123319, an AT₂-specific antagonist, completely displaced binding in PC12W membranes, confirming that all of the receptors in this membrane preparation are of the AT₂ subtype. Thus fsAngII binds to both the AT₁ and AT₂ Ang II receptor subtypes; however, binding to the AT₁ receptor occurs with roughly 40-fold less affinity than native Ang II.

**Biological Activity of fsAngII—**Intracellular free calcium concentration ([Ca²⁺],) measurements were conducted to determine the AT₁ receptor-mediated biological response of cells to fsAngII (Fig. 9). Basal [Ca²⁺] in AT₁-transfected COS-1 cells was 76.5 ± 2 nmol/liter (n = 36 experiments comprising 180 cells). Ang II and fsAngII induced a rapid and acute [Ca²⁺]i response that reached peak levels within 20 s after addition of the agonist. Both peptides increased [Ca²⁺]i, with [Ca²⁺]i sensitivity to Ang II and fsAngII being similar (pD₂ for Ang II = 8.7 ± 0.3; pD₂ for fsAngII = 8.6 ± 0.7; pD₂ = −log(EC₅₀ (mol/liter))). Ang II at concentrations greater than 10⁻⁹ mol/liter induced significantly larger...
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Fig. 3. Biologic activity of fsAngII. Intracellular calcium mobilization in response to Ang II or fsAngII was measured on AT1 receptor-transfected COS-1 cells. A, line graphs demonstrate dose-response curves for Ang II and fsAngII. Each data point represents the mean ± S.E. of 4–6 experiments with each experimental field comprising 5–10 cells. *, p < 0.05; **, p < 0.01 versus corresponding fsAngII concentration using Student’s t test. Calculated pD2 values are as follows: Ang II, 8.7 ± 0.3; fsAngII, 8.6 ± 0.7. B, line graphs demonstrate the time course of [Ca2+]i, recovery following 10–8 mol/liter agonist stimulation. The peak response was set at time 0 s, and [Ca2+]i levels remained persistently elevated above basal levels 300 s after the peak response (Fig. 3B).

Expression of fsAngII in Transfected Cells—To achieve direct expression of angiotensin peptides, an fsAngII-encoding peptide was linked to the 3’ end of an engineered secretory protein through a consensus cleavage site for furin, a ubiquitous protease (Fig. 1). To test for cell-mediated release and cleavage of the fsAngII fusion protein, GH4C1 cells were transfected with plgfsAngII and its control counterpart, plg, which lacks the fsAngII coding sequences. Immunoprecipitation of the Ig fragment from cells transfected with either plg or plgfsAngII shows that the fusion protein detected in both cases migrated as a single band with plgfsAngII having a slightly higher molecular weight than the plg counterpart (data not shown). This result is consistent with a lack of cleavage of the fsAngII peptide from the Ig fragment in this fusion protein. In the plgfsAngII construct, the 43-amino acid human prorenin prosegment was inserted between the Ig fragment and the furin cleavage site to act as a molecular “spacer” which would favor accessibility of the furin cleavage site. Immunoprecipitation of the Ig fragment from GH4C1 cells transfected with plgfsAngII shows two bands of similar intensity (Fig. 4, arrows). Deduced molecular weights suggest that the higher band represents the intact fusion protein and the lower band the Ig fragment linked to the prorenin prosegment following removal of the fsAngII peptide. These results demonstrate that fsAngII can be cleaved from an appropriate fusion protein in GH4C1 cells with an efficacy of roughly 50% and be secreted into the extracellular space.

To test for cardiocyte-mediated release of fsAngII, primary rat ventricular myocytes were transfected with plgfsAngII and its control counterpart, plg. Ang II content from cell lysates and supernatants was measured by radioimmunoassay (Table I). Introduction of plgfsAngII into neonatal rat primary cardiocytes leads to expression of fsAngII. fsAngII was detected both within the cells and in cell lysates, demonstrating that fsAngII can be cleaved from an appropriate fusion protein and be secreted into the extracellular space.

Local Expression of fsAngII in Vivo Induces ANP mRNA, a Marker of Cardiac Hypertrophy—To test for in vivo synthesis and biological effects of fsAngII, either plg or plgfsAngII was directly injected into the apex of the rat cardiac ventricle. To test whether expressed fsAngII had local or systemic biological effects in vivo, we measured circulating plasma Ang II levels, blood pressure, heart weight, and ANP mRNA, a molecular marker of cardiac hypertrophy. Data in Table II show that there were no differences in the heart to body weight ratios and blood pressures of plgfsAngII- and plg (control)-injected rats. The plgfsAngII-injected group also had no significant elevation in plasma Ang II levels versus controls (data not shown). To examine the effects of overexpression of fsAngII on ANP mRNA, total RNA was isolated from the lower one-third of injected hearts (injection site and surrounding apex and left ventricular free wall) and from the upper one-third of the left ventricle (control tissue, distal to injection area). ANP mRNA was induced 4-fold in the plgfsAngII-injected hearts as com-
pared with the plg-injected hearts (Fig. 5, A and B; p < 0.01, n = 8). Enhanced ANP gene expression was not detected from the upper one-third of the left ventricle of plgPfsAngII- and plg-injected hearts (data not shown). Thus, our data suggest that local overexpression of fsAngII in cardiac myocytes leads to a highly localized induction of ANP mRNA, a molecular marker of cardiac hypertrophy.

**DISCUSSION**

We have engineered a fusion protein that leads to the direct release of an Ang II analog within transfected tissues. The angiotensin peptide used in this study is released by a single cleavage, effected by the processing protease furin, leading to the constitutive secretion of the released peptide. Furin, a mammalian homolog of the yeast precursor-processing Kex2 endoprotease, is a Golgi-anchored convertase expressed in all mammalian cells (29, 30). fsAngII, Ala-Pro-Gly-[Ile3,Val5]Ang II, is an uncharged decapeptide isolated from the skin of the Australian frog C. georgiana (21), which was chosen because its amino-terminal alanine residue is a good substrate for efficient processing by furin (23). Our results show that efficient cleavage and secretion of fsAngII from the fusion protein requires the presence of a molecular spacer between the Ig fragment and the angiotensin peptide presumably due to steric constraints near the cleavage site. The choice of the prorenin spacer as a spacer was dictated by our previous success with the engineered furin cleavage of various peptide-containing fusion proteins in cell cultures (Fig. 4 and data not shown) by the simple use of protein-A- or G-coupled matrices. Evidence for the cleavage-mediated release of fsAngII peptide is also provided by the finding that introduction of plgPfsAngII into primary cultures of rat neonatal ventricular myocytes leads to intracellular accumulation and secretion of immunoreactive fsAngII (Table I).

Our results demonstrate that fsAngII binds to both the AT1 and AT2 Ang II receptor subtypes. While fsAngII binds the AT2 receptor with a similar affinity to that of mammalian Ang II (native Ang II), binding to the AT1 receptor, which is thought to mediate most of the cardiovascular effects of Ang II (34, 45), occurs with 40-fold lower affinity than native Ang II (Fig. 2A). However, even though fsAngII binding elicits only approximately 65% of the AT1 receptor-mediated release of intracellular calcium, its duration of action on target cells is significantly longer than that of native Ang II (Fig. 3). The combination of these properties may explain the apparent similarities in effective biological concentrations of Ang II and fsAngII on transfected cells. Indeed, Khosla and co-workers (22) have previously reported that the pressor activity of the synthetic fsAngII in rats was 90.6 ± 5.0% that of human Ang II.

**Fig. 5. Ventricular ANP mRNA expression 7 days post-injection of rat hearts with plg or plgPfsAngII.** A, representative autoradiograph of ANP mRNA from Northern blot hybridizations. mRNA was isolated from the apex and the lower one-third of the left ventricle free wall (injection area) of hearts injected with the designated plasmid. B, densitometry of ANP mRNA. Densitometry was used to quantify the hybridization signals depicted in A. ANP mRNA was corrected for differences in total amount of RNA loaded per lane by comparison with the signal for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. DU, densitometry units. Each bar represents the mean ± S.E. of eight hearts. ***, p < 0.01 (Mann-Whitney nonparametric test) ANP mRNA in plgPfsAngII-injected group compared with ANP mRNA of plg (control) injected group.

**Table I**

| Transfected plasmid | Supernatant Ang II | Total supernatant Ang II | Cell Ang II | Total cell Ang II |
|--------------------|-------------------|--------------------------|-------------|------------------|
| plg                | 8.13              | 195.19                   | 31.27       | 27.22            |
| plgPfsAngII        | 0.87              | 20.99                    | 2.70        | 4.11             |

**Table II**

| Rats injected with | Systolic BP (mm Hg) | Heart wtody wt (mg/g) | Left vent. wt/ear wt (mg/g) | Left vent. wtody wt (mg/g) |
|--------------------|---------------------|-----------------------|-----------------------------|-----------------------------|
| plg                | 125.8 ± 8.0**       | 2.8 ± 0.1**           | 0.73 ± 0.02**               | 2.1 ± 0.1**                 |
| plgPfsAngII        | 129.5 ± 7.0**       | 3.0 ± 0.1**           | 0.70 ± 0.01**               | 2.2 ± 0.1**                 |

**Summary of physiological changes following expression vector injection in rat hearts**

Systolic BP was measured 6 days post-injection. All other parameters were measured seven days post-injection. BP, blood pressure; wt, weight; vent., ventricle. Values are means ± S.E.
and pIgPfsAngII-expressing groups. In contrast to the absence of systemic fsAngII effects, fsAngII induced a significant increase in ANP mRNA levels in the injected portion of the heart (Fig. 5). Enhanced ANP gene expression was not detected distal to the injection site. Coupled with the failure to detect an increase in heart to body weight ratios, these data suggest that the expression of fsAngII is highly localized to cells along the injection site. These data support the in vitro results of Sadoshima et al. (46) who demonstrated the autocrine effects of Ang II in cardiac myocyte hypertrophy in vitro and demonstrate that in vivo local overexpression of fsAngII in the rat heart leads to a highly localized cardiac hypertrophic phenotype.

In conclusion, we have described a novel expression vector that can serve as an alternative to coexpressing all the components of the RAS to generate Ang II. This type of approach could also be extended with the use of tissue-specific genes in transgenic animals to test the importance of other bioactive peptides on organ physiology.

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