3-15-2016

A 5'-upstream short open reading frame encoded peptide regulates angiotensin type 1a receptor production and signalling via the β-arrestin pathway

Gina L. Yosten
Saint Louis University School of Medicine

Jun Liu
Georgetown University School of Medicine

Hong Ji
Georgetown University School of Medicine

Kathryn Sandberg
Georgetown University School of Medicine

Robert Speth
Nova Southeastern University, rs1251@nova.edu

See next page for additional authors

J Physiol 594.6 (2016) pp 1601–1605

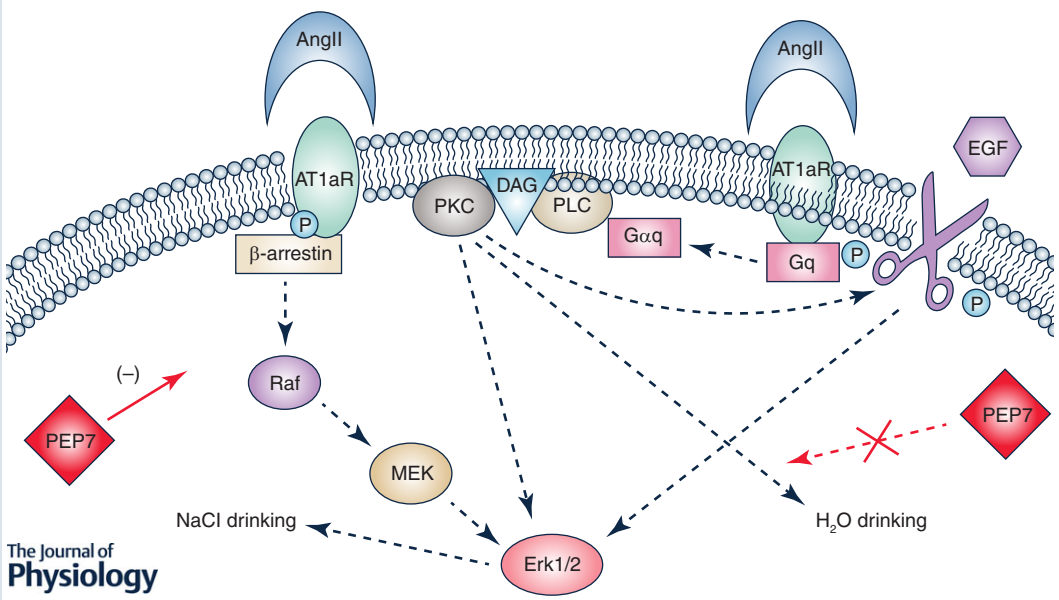
The Journal of Physiology

SYMPOSIUM REVIEW

A 5′-upstream short open reading frame encoded peptide regulates angiotensin type 1a receptor production and signalling via the β-arrestin pathway

Gina L.C. Yosten[1], Jun Liu[2], Hong Ji[2], Kathryn Sandberg[2], Robert Speth[3] and Willis K. Samson[1]

[1]*Department of Pharmacology and Physiology, Saint Louis University School of Medicine, St. Louis, MO, USA*
[2]*Department of Medicine, Georgetown University School of Medicine, Washington, DC, USA*
[3]*Department of Pharmaceutical Sciences, Nova University, Fort Lauderdale, FL, USA*



Abstract AUG sequences and short open reading frames are commonly present in the 5′-leader sequence of G protein-coupled receptor mRNAs. The presence of these upstream AUG sequences has been demonstrated to inhibit downstream receptor translation efficiency and, most recently,

G. L. C. Yosten is an Assistant Professor and **W. K. Samson** a Professor in Pharmacology and Physiology at the Saint Louis University School of Medicine. They collaborate to discover novel peptide sequences using a bioinformatics approach and employ a deductive reasoning strategy to match biologic ligands with orphan G protein-coupled receptors. Recently, they announced the discovery of two novel peptides, neuronostatin and phoenixin, and de-orphanized the receptors for neuronostatin and connecting peptide (C-peptide). With their valued colleagues **K. Sandberg**, **J. Liu** and **H. Ji** at Georgetown University and **R. Speth** at Nova University, they recently characterized the pharmacological actions of a novel peptide sequence encoded in the 5′-upstream leader sequence of the angiotensin type 1a receptor gene.

This review was presented at the symposium "*Bristol-São Paulo Research Summit*" - *Autonomic and neuroendocrine dysfunction in chronic diseases*, which took place at the University of São Paulo, Brasil, between 7–10 August 2014.

DOI: 10.1113/JP270567

receptor signal transduction. A seven amino acid peptide encoded by a short open reading frame in exon 2 of the angiotensin type 1a receptor has been shown to inhibit non-G protein-coupled signalling of angiotensin II, without altering the classical G protein-coupled pathway activated by the ligand. This finding may lead to the development of a new class of angiotensin receptor antagonists with activities biased for one, but not all, of the signalling cascades activated by angiotensin II, which could have therapeutic implications for the myriad hormones and neurotransmitters that signal through G protein-coupled receptors.

(Resubmitted 17 March 2015; accepted after revision 20 August 2015; first published online 1 September 2015)
Corresponding author W. K. Samson: Saint Louis University, 1402 South Grand, St. Louis, MO 63104, USA.
Email: samsonwk@slu.edu

Abstract figure legend PEP7 interrupts angiotensin II-induced saline but water drinking: proposed mechanism of action. PEP7 interferes with the action of angiotensin II (Ang II) to stimulate sodium chloride drinking via an action on the non-G protein-coupled signal cascade leading to a reduction in peptide stimulated phosphorylation of extracellular regulated kinases 1/2 (Erk1/2). PEP7 does not interrupt the action of angiotensin II to stimulate water drinking, nor does it interfere with angiotensin II activation of Erk1/2 via the G protein-dependent pathway leading to activation of phospholipase C (PLC), protein kinase C (PKC), or the epidermal growth factor (EGF) receptor.

Abbreviations AT_1R, angiotensin II receptor type 1; 5′-LS, 5′-leader sequence; PKC, protein kinase C; sORF, short open reading frame.

As a result of the Human Genome Project and earlier gene sequencing efforts it became apparent that multiple AUGs and short open reading frames (sORFs) exist in the 5′-leader sequence (5′-LS) of many genes including most G protein-coupled receptors. This has led to several important questions: do the multiple start codons in the 5′-LS regulate translation (Kobilka *et al.* 1987)? Where does the ribosome start translation when AUGs in excellent Kozak consensus sequence (Kozak, 1999) exist upstream of the major open reading frame? To what extent do ribosomes use internal ribosome entry, leaky scanning or other methods to reach the downstream major open reading frame if translation occurs at an upstream sORF? How often are peptides encoded within upstream sORFs produced (Hunt, 1985)? What is the function of biologically active peptides encoded within upstream sORFs?

Several studies suggest upstream sORFs inhibit expression of the downstream major open reading frame (Mori *et al.* 1996; Nomura *et al.* 2001; Ji *et al.* 2004; Rabadan-Diehl *et al.* 2007). In addition to inhibiting translation, these sORFs may serve other functions, for instance regulation of distinct signalling cascades activated by a receptor encoded within the downstream major open reading frame. Upon binding some ligands induce distinct G protein-coupled receptor conformations that only activate a specific signal transduction pathway (Wei *et al.* 2003). In contrast, angiotensin II is known to activate multiple, intracellular signalling cascades, via both G protein-coupled and non-G protein-coupled angiotensin II receptor type 1 (AT_1R)-mediated pathways (Wei *et al.* 2003; Hunyady & Catt, 2006; Godin & Ferguson, 2012). The mechanism whereby angiotensin II selectively activates only one signalling cascade has been a longstanding mystery. Here we describe a mechanism for biasing the action of angiotensin II signalling through the $AT_{1a}R$ by a peptide encoded within a sORF in the 5′-LS of the receptor mRNA.

Because of the important role played by angiotensin II in fluid and electrolyte homeostasis and cardiovascular and renal function, angiotensin receptor antagonists and angiotensin converting enzyme inhibitors are widely used clinically for the treatment of hypertension and associated cardio-renal diseases. These agents, however, are not without adverse side-effects and they do not control hypertension in all individuals. Therefore, the search for alternative strategies for the regulation of the renin–angiotensin–aldosterone system continues to be an important aspect of cardiovascular and kidney research and of drug development. Is it possible that biased antagonists might be developed that block activation of the non-G protein signalling , but not the G protein-coupled receptor signalling pathway, or vice versa, leading to enhanced efficacy and fewer side-effects? We summarize here our findings demonstrating the feasibility of this approach.

In the rat, two distinct RNA transcripts are synthesized from the $AT_{1a}R$ gene by alternative splicing. The longer, relatively less abundant transcript contains exons 1, 2 and 3 (E1,2,3-$AT_{1a}R$), while the shorter, more abundant transcript contains only exons 1 and 3 (E1,3-$AT_{1a}R$). The coding region for the receptor resides in exon 3. Therefore both transcripts encode the same receptor protein (Ji *et al.* 2004). These variants differ by the presence and absence of a sORF in exon 2, which encodes a novel seven amino acid peptide that we have named PEP7 (Fig. 1). The start codon for this sORF is in excellent Kozak consensus sequence, which is predictive of endogenous production of

PEP7 from the E1,2,3-$AT_{1a}R$ splice variant of the receptor (Kozak, 1999). Because the sequence is highly conserved across mammalian species (Fig. 1), we hypothesized that PEP7 is biologically active.

Human embryonic kidney cells expressing the rat $AT_{1a}R$ respond to angiotensin II exposure with robust increases in phosphorylation of extracellular signal-regulated kinases 1/2 (Erk1/2), which can be attenuated, in a concentration-dependent manner, by pretreatment with synthetic rat PEP7. This effect was selective for mitogen-activated protein (MAP)-kinase activation via the non-G protein-coupled signalling pathway because PEP7 pretreatment did not alter angiotensin II stimulated inositol trisphosphate production (Liu *et al.* 2014). Further evidence of that selectivity (Abstract Figure) was provided by our observation that PEP7 did not inhibit Erk1/2 phosphorylation induced by epidermal growth factor or phorbol 12-myristate 13-acetate activation of protein kinase C (PKC).

PEP7 did not inhibit binding of [Sar^1, Ile^8]-angiotensin II to rat liver membranes suggesting that the abrogation of Erk1/2 phosphorylation by PEP7 was not due to modulation of angiotensin II binding to the $AT_{1a}R$ by PEP7. Instead, these data suggest that PEP7 interferes with the non-G protein-coupled signalling pathway activated by angiotensin II.

Physiologically relevant consequences of selective inhibition of the Erk1/2 dependent pathway of angiotensin II signalling have been demonstrated. Angiotensin II stimulates water and saline drinking through two separate pathways (Daniels *et al.* 2005, 2007, 2009; Felgendreger *et al.* 2013). Angiotensin II-stimulated water, but not saline drinking, in rats can be blocked by inhibitors of PKC activity, indicating signalling via the G protein-dependent pathway. Saline drinking, on the other hand, is abrogated by pretreatment with MAP-kinase inhibitors, but not PKC inhibitors, suggesting this behaviour is activated by angiotensin II signalling via the non-G protein-coupled, β-arrestin pathway (Daniels *et al.* 2009). Thus the β-arrestin dependent pathway appears to be important for the ability of angiotensin II to stimulate salt appetite. These behavioural models provided the opportunity to determine if a peptide encoded in a short open reading frame in the upstream 5′-LS of a receptor-encoding gene might determine selectivity of ligand activated signalling.

We observed that intracerebroventricular PEP7 pretreatment failed to alter angiotensin II-induced water drinking in male rats, but significantly blocked the action of intracerebroventricular angiotensin II to stimulate saline drinking (Liu *et al.* 2014). Female rats, which consumed more saline under *ad libitum* conditions than males, also failed to drink saline in response to angiotensin II when pretreated with PEP7. As in male rats, the water drinking response to angiotensin II of female rats was not altered by PEP7 pretreatment. We are investigating now the ability of PEP7 to interrupt sodium appetite in models of hyponatraemia associated with hypovolaemia and hypernatraemia associated with hypervolaemia. We need also to determine if the action of locally produced PEP7 in the structures of the rostral lamina terminalis reflects an interruption of brain- or peripherally derived angiotensin II.

Are other consequences of $AT_{1a}R$ activation interrupted by PEP7? A hallmark effect of angiotensin II is its ability to elevate arterial pressure, which is why angiotensin converting enzyme inhibitors and angiotensin receptor antagonists are used widely in clinical medicine. We have preliminary evidence that intravenous infusion of PEP7 in male rats significantly reduces the pressor effect of subsequently infused intravenous angiotensin II, suggesting that the pressor response to angiotensin II is mediated in part by the β-arrestin pathway that is inhibited by PEP7 acting as a biased antagonist in the peripheral vasculature (Liu *et al.* 2014).

These *in vivo* results agree with those from our *in vitro* studies demonstrating selective inhibition of the β-arrestin-mediated MAP-kinase signalling pathway of angiotensin II. Thus depending upon which splice variant is expressed, the actions of angiotensin II could be directed toward one, but not both, of the signalling cascades activated by angiotensin II. Is alternative splicing regulated in a physiological fashion, for instance dependent upon sex hormones or ageing? Cycling female rats express lower densities of $AT_{1a}Rs$ than male rats, perhaps explaining in part the results of experiments showing that ovarian hormone replete animals are more resistant to the

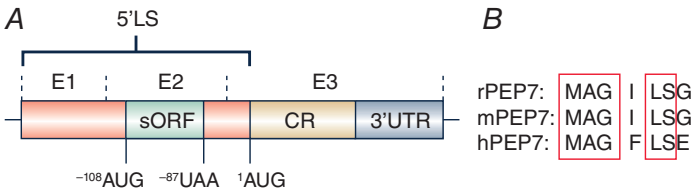


Figure 1. The rat angiotensin type 1a receptor mRNA transcript containing exons 1, 2 & 3 (E1,2,3-$AT_{1a}R$)
A, Exon 2 (E2) contains a short open reading frame (sORF) that encodes PEP7. *B*, The rat PEP7 (rPEP7) is identical to mouse PEP7 (mPEP7) and homologous to human PEP7 (hPEP7).

The Journal of Physiology

development of hypertension than ovarian hormone deficient animals (Sandberg & Ji, 2012). Could this mean that PEP7 therapy in postmenopausal women might be a novel therapeutic option for the treatment of hypertension? Might PEP7 be a therapeutic agent to help reduce salt appetite and intake in individuals with salt-sensitive hypertension?

Many questions remain to be answered in this newly emerging field, not the least of which is where and how PEP7 interrupts the non-G-protein-coupled signalling cascade activated by angiotensin II. Because of its small size and lack of a conjugation motif, we have endeavoured to generate antibodies selective to the peptide by using a keyhole limpet haemocyanin conjugate linked with a cysteine at the carboxy terminal as antigen. While we have made some progress, we are still in the process of establishing the specificity of those antibodies. They will become critically important tools with which we hope to demonstrate PEP7 cellular localization and tissue distribution. We do not at this point know whether the proposed interruption of the $AT_{1a}R$ signalling via the non-G protein-coupled pathway is due to an interaction of PEP7 with the receptor, with β-arrestin itself or through some unidentified mechanism. We plan both imaging and immunoprecipitation studies to address those questions.

In terms of the pharmacological effect of exogenous PEP7 *in vivo*, we speculate that PEP7 enters the cells to interrupt the β-arrestin dependent signalling cascade. PEP7 may bind to the $AT_{1a}R$ at an allosteric site (different from the angiotensin II binding site) and be internalized via receptor-mediated endocytosis. The availability of a selective PEP7 antibody would help us address that question. An alternative hypothesis is that the peptide, due to its small size and amino acid content, is lipophilic and enters the cells by diffusion.

It will be equally important to determine which actions of angiotensin II can be modified by PEP7 or PEP7 mimetics. Are the effects of PEP7 on Ang II signalling present not only in brain and the vasculature, but also in other tissues, for instance the pituitary gland, kidney, heart and pancreas?

Additional questions remain. For instance, is the effect of PEP7 selective for the $AT_{1a}R$? In other words, does PEP7 have effects on the signal transduction mechanisms activated by ligand binding to other G protein-coupled receptors? Are there tissue specific differences in PEP7 expression? Does endogenously produced PEP7 exert inhibitory effects on Erk1/2 activation under basal conditions or only after angiotensin II stimulation? Is PEP7 translation regulated under physiological and pathophysiological conditions? Does PEP7 serve as a brake on the powerful actions of angiotensin II in some but not all tissues?

Our alternative model for biasing the action of endogenous ligands may apply to other G protein-coupled receptors. The decision to activate G protein-dependent versus G protein-independent signalling may, under physiological conditions, not be due to ligand biasing, which may be the case for pharmacological analogues, but instead be due to expression of endogenous peptide sequences encoded in the 5′-LS receptor mRNA itself. Thus unlike the hypotheses of biased ligand binding or altered receptor confirmation biasing (Shenoy & Lefkowitz, 2011, Shukla *et al.* 2011), this model allows for physiological regulation at the cellular level of biased signalling dependent upon selective expression of unique splice variants of the receptor itself. This model predicts a novel targeting strategy for the control of selective cellular responses to endogenous ligands under distinct physiological conditions and the opportunity to therapeutically determine (i.e. bias) the action of endogenous ligands in pathological states of receptor over activation.

In a broader sense, our work elucidating the consequences of alternative splicing of the $AT_{1a}R$ transcripts suggests that peptides like PEP7 encoded in open reading frames of numerous G protein-coupled receptors may control not only translational activity but also ligand-receptor signalling. This may have a direct impact on the development of novel therapeutic agents in the future. Finally since upstream open reading frames are present in numerous G protein-coupled receptors, proto-oncogenes, signalling molecules and immune modulators (Zimmer *et al.* 1994, McGraw *et al.* 1998), PEP7 may serve as a prototype for the elucidation of novel modulators of cellular communication in multiple tissue systems.

References

Daniels D, Mietlicki EG, Nowak EL & Fluharty SJ (2009). Angiotensin II stimulates water and NaCl intake through separate cell signalling pathways in rats. *Exp Physiol* **94**, 130–137.

Daniels D, Yee DK, Faulconbridge LF & Fluharty SJ (2005). Divergent behavioral roles of angiotensin receptor intracellular signaling cascades. *Endocrinology* **146**, 5552–55560.

Daniels D, Yee DK & Fluharty SJ (2007). Angiotensin II receptor signalling. *Exp Physiol* **92**, 523–527.

Felgendreger LA, Fluharty SJ, Yee DK & Flanagan-Cato LM (2013). Endogenous angiotensin II-induced p44/42 mitogen-activated protein kinase activation mediates sodium appetite but not thirst or neurohypophysial secretion in male rats. *J Neuroendocrinol* **25**, 97–106.

Godin CM & Ferguson SS (2012). Biased agonism of the angiotensin II type 1 receptor. *Mini Rev Med Chem* **12**, 812–816.

Hunt T (1985). False starts in translational control of gene expression. *Nature* **316**, 580–581.

Hunyady L & Catt KJ (2006). Pleiotropic AT1 receptor signaling pathways mediating physiological and pathogenic actions of angiotensin II. *Mol Endocrinol* **20**, 953–970.

Ji H, Zhang Y, Zheng W, Wu Z, Lee S & Sandberg K (2004). Translational regulation of angiotensin type 1a receptor expression and signaling by upstream AUGs in the 5′ leader sequence. *J Biol Chem* **279**, 45322–45328.

Kobilka BK, MacGregor C, Daniel K, Kobilka TS, Caron MG & Lefkowitz RJ (1987). Functional activity and regulation of human β2-adrenergic receptors expressed in *Xenopus* oocytes. *J Biol Chem* **262**, 15796–15802.

Kozak M (1999). Initiation of translation in prokaryotes and eukaryotes. *Gene* **234**, 187–208.

Liu J, Yosten GLC, Ji H, Zhang D, Zheng W, Speth RC, Samson WK & Sandberg K (2014). Selective inhibition of angiotensin receptor signaling through Erk1/2 pathway by a novel peptide. *Am J Physiol Regul Integ Comp Physiol* **306**, R619–R626.

McGraw DW, Forbes SL, Kramer LA & Liggett SB (1998). Polymorphisms of the 5′ leader cistron of the human beta2-adrenergic receptor regulate receptor expression. *J Clin Invest* **102**, 1927–1932.

Mori Y, Matsubara H, Murasawa S, Kijima K, Tsukaguchi H, Okubo N, Hamabuko T, Inagami T, Iwasaki T & Inada M (1996). Translational regulation of angiotensin II type 1A receptor: role of upstream AUG triplets. *Hypertension* **28**, 810–817.

Nomura A, Iwasaki Y, Saito M, Aoki Y, Yamamori E, Ozaki N, Tachikawa K, Mutsuga N, Morishita M, Yoshida M, Asai M, Oiso Y & Saito H (2001). Involvement of upstream open reading frames in regulation of rat V1b vasopressin receptor expression. *Am J Physiol Endocrinol Metab* **280**, E780–E787.

Rabadan-Diehl C, Martinez A, Volpi S, Subburaju S & Aquilera G (2007). Inhibition of vasopressin V1b receptor translation by upstream open reading frames in the 5′-untranslated region. *J Neuroendocrinol* **19**, 309–319.

Wei H, Ahn S, Shenoy SK, Karnik SS, Hunyady L, Luttrell LM & Lefkowitz RJ (2003). Independent β-arrestin 2 and G protein-mediated pathways for angiotensin II activation of extracellular signal-regulated kinases 1 and 2. *Proc Natl Acad Sci USA* **100**, 10782–10787.

Sandberg K & Ji H (2012). Sex differences in primary hypertension. *Biol Sex Differ* **3**, 7.

Shenoy SK & Lefkowitz RJ (2011). β-Arrestin-mediated receptor trafficking and signal transduction. *Trens Pharmacol Sci* **32**, 521–533.

Shukla AK, Xiao K & Lefkowitz RJ (2011). Emerging paradigms of β-arrestin-dependent seven transmembrane receptor signaling. *Trends Biochem Sci* **36**, 457–469.

Zimmer A, Zimmer AM & Reynolds K (1994). Tissue specific expression of the retinoic acid receptor-β2: regulation by short open reading frames in the 5′-noncoding region. *J Cell Biol* **127**, 1111–1119.

Additional information

Competing interests

No conflicts of interest, financial or otherwise, are declared by the authors.

Author contributions

All authors contributed to the conception, design, conduct and analysis of the experiments reviewed in this manuscript. Additionally, all authors assisted with the creation and editing of the manuscript and have approved of the final version. Experiments reviewed in this manuscript were performed at the Georgetown University and Saint Louis University Schools of Medicine.

Funding

Work described here by the authors was supported by grants to J.L. from the American Heart Association, to K.S. from the National Institutes of Health (NIH Grants HL121456 & AG/HL-19291), to R.S. from Nova Southeastern University, NIH (HL-113905) and a Pilot Award from the Translational Technologies Component of the Georgetown/Howard Universities Center for Clinical and Translational Science (UL1TR000101), and to W.K.S. from NIH (HL-066023) and the American Heart Association (Midwest Affiliate 10GRNT4470043).