CONTRACTILE EFFECTS OF ANGIOTENSINS ARE INDEPENDENT OF RECEPTORS INTERNALIZATION IN RAT AORTA

COSTIN ŞERBAN[1], BOGDAN GURZU[2], LILIANA CHELARU[3], ROXANA IRINA IANCU[1], IRINA LUCIANA DUMITRIU[2], SIMONA MIHAELA SLĂTINEANU[2], GHEORGHE PETRESCU[2], MARCEL COSTULEANU[1]*

Keywords: angiotensin, receptor, β-arrestin, vascular smooth muscle, rat.

Abstract: β-Arrestins-mediated signaling downstream of seven transmembrane receptors is a relatively new paradigm for signaling by these receptors. The inhibitors of AT_1 receptor internalization we tested, that means nigericin, concanavalin A and monensin, did not significantly alter the contractions induced by Ang II in the rat aortic smooth muscle preparations. The same effects we obtained also when we used as agonists Ang I, Ang III and Ang IV. Thus, the contractile effects induced by angiotensin peptides administered in the rat aortic smooth muscle preparations are independent of their receptors internalization. Since β-arrestins are mediating G-protein independent signaling *via* AT_1 receptors their involvement in the contractile effects of angiotensin peptides must be further explored.

INTRODUCTION

Angiotensin II (Ang II) activates a wide spectrum of signaling responses via the AT_1 receptor (AT_1R) that mediate its physiological control of blood pressure, thirst, and sodium balance and its diverse pathological actions in cardiovascular, renal, and other cell types. Ang II-induced AT_1R activation via $G_{q/11}$ stimulates phospholipases A_2, C, and D, and activates inositol trisphosphate/Ca^{2+} signaling, protein kinase C isoforms, and MAPKs, as well as several tyrosine kinases (Pyk2, Src, Tyk2, FAK), scaffold proteins (G protein-coupled receptor kinase-interacting protein 1, $p130^{Cas}$, paxillin, vinculin), receptor tyrosine kinases, and the nuclear factor-kappaB pathway (NF-kB). The AT_1R also signals via $G_{i/o}$ and $G_{11/12}$ and stimulates G protein-independent signaling pathways, such as beta-arrestin-mediated MAPK activation and the Jak/STAT. Alterations in homo- or heterodimerization of the AT_1R may also contribute to its pathophysiological roles. Many of the deleterious actions of AT_1R activation are initiated by locally generated, rather than circulating, Ang II and are concomitant with the harmful effects of aldosterone in the cardiovascular system. AT_1R-mediated overproduction of reactive oxygen species has potent growth-promoting, proinflammatory, and profibrotic actions by exerting positive feedback effects that amplify its signaling in cardiovascular cells, leukocytes, and monocytes. In addition to its roles in cardiovascular and renal disease, agonist-induced activation of the AT_1R also participates in the development of metabolic diseases and promotes tumor progression and metastasis through its growth-promoting and proangiogenic activities (Hunyady and Catt, 2006).

The selective AT_1R belongs to family A of 7 transmembrane (7TM) receptors. The receptor has important roles in the cardiovascular system and is commonly used as a drug target in cardiovascular diseases. Its activation mechanism most likely involves concerted movements of the transmembrane helices, but remains to be completely resolved (Bonde *et al.*, 2010).

Ang II rapidly induces AT_1R internalization, desensitization, nuclear translocation, as well as nuclear *de novo* synthesis of this receptor. Endocytosis modulates cell responses by removing and recycling AT_1R receptors from the cell surface (Becker *et al.*, 2004).

Internalization of a G-protein-coupled receptor (GPCR) is essential to the desensitization, endocytosis, and signal transduction of the receptor. It has been the general view that conventional homologous internalization of a GPCR requires activation of the G-protein(s) coupled to the receptor. However, whether and how GPCR-mediated G-protein-independent signals trigger receptor internalization remains unknown, although G-protein-independent internalization has been reported. It was particularly shown (Feng *et al.*, 2005) that an AT_1R mutant incapable of activating any G-protein still undergoes normal internalization. Substitution of Asp^{125} with Ala and Arg^{126} with Leu at the highly conserved DRY motif of the AT_1 receptor disabled the ability of the receptor to activate G-proteins, as shown by various Ang II binding studies, GDP-GTP exchange, and inositol phosphate production assays. Surprisingly, the mutant internalized normally in the presence of Ang II and transactivated the epidermal growth factor receptor (EGFR). Similar to the wild-type receptor, overexpression of a dominant-negative K220R mutant GRK2 diminished the internalization of D125A-R126L but not the transactivation of EGFR. These data indicate that G-protein-independent specific signals may also trigger homologous internalizations of the AT_1 receptor through beta-arrestin-dependent and -independent pathways, suggesting a possible mechanism for G-protein-independent activation of G-protein-coupled receptor kinases (GRKs). This may represent a general mechanism for triggering GPCR internalization.

The goal of our study was represented by the effects of some very well known blockers of GPCRs internalization on angiotensin peptides contractions in isolated rat aortic smooth muscle preparations.

MATERIALS AND METHODS

For the experiments we used Wistar adult male rats (Băneasa source), weighing 150-200 g, kept under normal and the same laboratory conditions. They were beheaded and exsanguinated after anesthesia. Thoracic aorta was quickly removed and cut into rings 2 mm long. Endothelium was removed and the rings mounted in organ baths of 2 ml. Mechanical activity was recorded using isometric force transducers and recorders (type Radelkis, Budapest, or Carl Zeiss) or a data acquisition card PC-LPM-16 Multifunction I/O with associated software, NiDaqWin v.4.8. (National Instruments Inc.). The 2 ml organ baths contained Krebs-Henseleit solution (pH 7.4) with the following composition (in mM): NaCl, 118; KCl, 4.8; $CaCl_2$, 2.5; $MgSO_4$, 1.6; KH_2PO_4, 1.2; $NaHCO_3$, 25; glucose 5.5. Solution containing 40 mM K^+ had the same composition, but Na^+ was replaced by equimolar K^+. Krebs-Henseleit solution was maintained at 37ºC and aerated continuously with 95% O_2 and 5% CO_2 (Carbogen, Linde Romania). The initial tension was 2 g, preparations being left to equilibrate in the Krebs-Henseleit solution for 2 hours before the experiments were performed. Lack of a functional endothelium was verified by the inability of aorta rings to relax in response to administration of carbachol 10^{-5} M, when they were pre-contracted with phenylephrine 10^{-6} M.

All experiments were performed on rings capable of contractions reproduction to 40 mM K^+ without changes greater than 10% after stabilization. Thus, after the stable contractions induced by 40 mM K^+, in the bath were administered for 15 minutes before Ang II, Ang I, Ang III and Ang IV: nigericin (10^{-5} M), which induces K^+ depletion; concanavalin A (250 μg/ml), which primarily blocks EGF receptor transactivation; and monensin (10^{-5} M), inhibitor of endosomes acidification. All of the above pretreatments are known to block the receptor internalization which is G protein-coupled. In addition, their actions on Ang II (80 nM) were compared with those on Ang I (600 nM), ANG III (1 μM) and Ang IV (10 μM) (Petrescu *et al.*, 2001).

The statistical significance of test results was highlighted using the Variance One-Way ANOVA (possibly complemented by Bonferroni test) and Student t-test and the results were expressed as mean ± S.E.M (n = 6). Value of $p<0.05$ was always considered as being statistically significant.

Present studies were carried out in accordance with the "Guide for Care and Use of Animal Experiments" of U.S. National Institutes of Health (NIH), published by the U.S. National Academy in 1996, and approved by the Ethics Committee of the University of Medicine and Pharmacy "Gr T. Popa" Iaşi.

Drugs used. Angiotensin I, angiotensin II, angiotensin III and angiotensin IV were all obtained from *Sigma-Aldrich Co*. All other compounds used were of analytical grade. Angiotensin peptides were stored frozen as aliquots, which were defrosted as required on the day of experiment. Dilutions of all drugs were made in Krebs-Henseleit solution.

RESULTS AND DISCUSSIONS

As previously shown (Petrescu *et al.*, 2001a), the contractions induced by the administration of 80 nM Ang II, 600 nM Ang I, 1 μM Ang III and 10 μM Ang IV were, respectively, of 21±2.06%, 21.32±1.25%, 21.83±2.83% and 21.58±1.92% of the control contractions induced by K^+ 40 mM ($n=6$, $p<0.05$). We administered concentrations of angiotensin peptides that induced very similar contractions in isolated rat aorta.

All the used blockers of the G protein-coupled receptor internalization, that means nigericin (Nig, 10^{-5} M), which induces K^+ depletion, concanavalin A (Con, 250 μg/ml), which primarily blocks EGF receptor transactivation, and monensin (Mon, 10^{-5} M), also inhibitor of endosomes acidification, did not have significant inhibitory effects on contractions induced by Ang II in the rat aortic smooth muscle (figure no. 1).

On the other hand, nigericin (10^{-5} M), concanavalin A (250 μg/ml) and monensin (10^{-5} M) had no significant inhibitory effects on contractions induced by Ang III, Ang I and Ang IV in the rat aortic smooth muscle.

Ang II initiates cellular responses by activation of type I (AT_1) and type 2 (AT_2) angiotensin receptors. Both AT_1 and AT_2 receptors have seven transmembrane structures characteristic of G protein-coupled receptors, but only the AT_1 receptor undergoes rapid internalization upon agonist binding. In addition to the agonist hormone, the peptide antagonist

[Sar[1],Ile[8]]Ang II can also induce internalization of the AT_{1a} receptor expressed in mammalian cell lines. AT_1 receptor internalization occurs via clathrin-coated pits. Mutagenesis studies demonstrated that AT_1 receptor internalization requires two regions in the cytoplasmic tail of the receptor, but it is independent of G protein activation. The dependence of AT_1 receptor internalization on the presence of a serine-threonine-rich region suggests that phosphorylation of the receptor tail may regulate the internalization process (Hunyady, 1999).

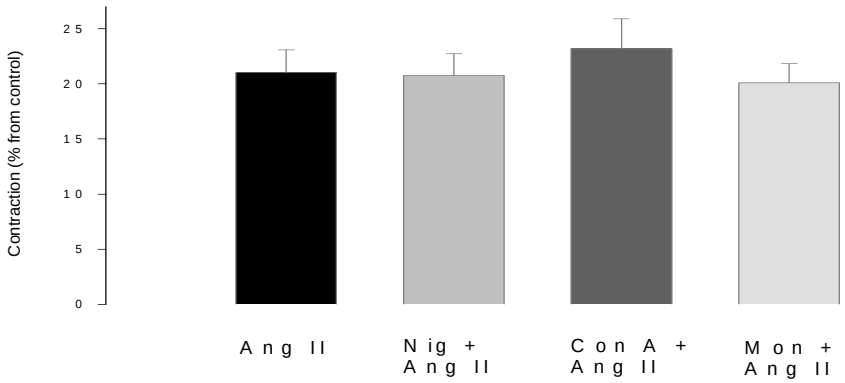

Figure no. 1: Nigericin (Nig, 10^{-5} M), concanavalin A (Con, 250 μg/ml) and monensin (Mon, 10^{-5} M) do not have significant inhibitory effects on contractions induced by Ang II (80 nM) in the rat aortic smooth muscle. Results were expressed as a percentage (mean ± S.E.M., n = 6) versus 100% contraction induced by 40 mM K^+.

Internalized ligand-receptor complex is considered to be extremely important for angiotensin AT_1 receptor desensitization and recycling. This may also help to generate a sustained cellular signal supported by AT_1 stimulation and/or intracellular accumulation of angiotensin peptides (Modrall *et al.*, 2001).

The Ang II cellular internalization, totally dependent on the internalized complex with its own type AT_1 receptor, protects it from degradation by endothelial peptidases, which explains the longer half-life for internalized Ang II as compared with circulating hormone. The question is whether the internalization of angiotensin-receptor AT_1 complex has every important role in Ang II-mediated processes. AT_1 receptor binding of Ang II requires more points of contact and induces the conformation of the receptor agonist affinity, so that ligand dissociation is a relatively slow process (van Kats *et al.*, 1997). Acidification of endocytosis vesicles appears to accelerate the dissociation of the ligand from AT_1 receptor (Hein *et al.*, 1997) and the receptor is recycled to the cell membrane in a low affinity conformation.

For the beginning, there was evidence that, in contrast to the internalization of other G protein-coupled receptors, the internalization of the AT_1 receptor might be independent of dynamin and beta-arrestin (Hunyady, 1999).

More recently, ligand-induced interaction between AT_{1a} receptors and beta-arrestins was measured by Bioluminescence Resonance Energy Transfer 2. AT_{1a}-β-arrestin1 and AT_{1a}-β-

arrestin2 fusion proteins were cloned and tested for differences using immunocytochemistry, inositol phosphate hydrolysis and competition radioligand binding. Bioluminescence Resonance Energy Transfer 2 analysis showed that beta-arrestin1 and 2 were recruited to AT_{1a} receptors with similar ligand potencies and efficacies. The AT_{1a}-β-arrestin fusion proteins showed attenuated G protein signaling and increased agonist binding affinity, while antagonist affinity was unchanged. Importantly, larger agonist affinity shifts were observed for AT_{1a}-β-arrestin2 than for AT_{1a}-β-arrestin1. It was concluded that β-Arrestin1 and 2 are recruited to AT_{1a} receptors with similar ligand pharmacology and stabilize AT_{1a} receptors in distinct high-affinity conformations. However, β-arrestin2 induces a receptor conformation with a higher agonist-binding affinity than β-arrestin1. Furthermore, it was demonstrated that β-arrestins interact with AT_{1a} receptors in different ways and suggested that AT_{1a} receptor biased agonists, with the ability to recruit either of the β-arrestins selectively, would be possible to design (Sanni *et al.*, 2010).

β-Arrestins, originally discovered to desensitize activated GPCRs, also mediate 7TMR internalization and G-protein independent signaling *via* these receptors. More recently, several regulatory roles of β-arrestins for atypical 7TMRs and non-7TM receptors have emerged. Thus, there was uncovered an entirely novel regulatory role of β-arrestins in cross talk between the AT_{1a} receptor and a member of the transient receptor potential (TRP) ion channel family, $TRPV_4$. $AT_{1a}R$ and $TRPV_4$ form a constitutive complex in the plasma membrane and angiotensin stimulation leads to recruitment of β-arrestin1 to this complex. Surprisingly, angiotensin stimulation results in ubiquitination of $TRPV_4$, a process that requires β-arrestin1, and subsequently to internalization and functional downregulation of $TRPV_4$. β-Arrestin 1 interacts with, and acts as an adaptor for AIP4, an E3 ubiquitin ligase responsible for $TRPV_4$ ubiquitination. These data provides the first evidence of a functional link between β-arrestins and $TRPV_4$ and uncovers an entirely novel mechanism to maintain appropriate intracellular Ca^{2+} concentration in order to avoid excessive Ca^{2+} signaling (Shukla *et al.*, 2010).

An emerging concept in GPCR signaling is β-arrestin-biased agonism, in which specific ligand-activated GPCR conformational states selectively signal through β-arrestins, rather than through G proteins. It was shown that mechanical stretch induced β-arrestin-biased signaling downstream of AT_1Rs in the absence of ligand or G protein activation. Mechanical stretch triggered an AT_1R-mediated conformational change in β-arrestin similar to that induced by a β-arrestin-biased ligand to selectively stimulate receptor signaling in the absence of detectable G protein activation. Hearts from mice lacking β-arrestin or AT_1Rs failed to induce responses to mechanical stretch, as shown by blunted extracellular signal-regulated kinase and Akt activation, impaired transactivation of the epidermal growth factor receptor, and enhanced myocyte apoptosis. These data show that the heart responds to acute increases in mechanical stress by activating β-arrestin-mediated cell survival signals (Rakesh *et al.*, 2010).

β-Arrestin-mediated signaling downstream of seven transmembrane receptors is a relatively new paradigm for signaling by these receptors. There were examined the changes in protein phosphorylation occurring when HEK293 cells expressing the AT_{1a} receptor were stimulated with the β-arrestin-biased ligand [Sar^1,Ile^4,Ile^8]Ang II (SII), a ligand previously found to signal through β-arrestin-dependent, G protein-independent mechanisms. There were identified 1,555 phosphoproteins (4,552 unique phosphopeptides), of which 171 proteins (222 phosphopeptides) showed increased phosphorylation, and 53 (66 phosphopeptides) showed decreased phosphorylation upon SII stimulation of the AT_{1a} receptor. This study identified 38

protein kinases and three phosphatases whose phosphorylation status changed upon SII treatment. Using computational approaches, there were performed system-based analyses examining the β-arrestin-mediated phosphoproteome including construction of a kinase-substrate network for β-arrestin-mediated AT_{1a} receptor signaling. The analysis demonstrates that β-arrestin-dependent signaling processes are more diverse than previously appreciated. Notably, the analysis identified an AT_{1a} receptor-mediated cytoskeletal reorganization network whereby β-arrestin regulates phosphorylation of several key proteins, including cofilin and slingshot. This study provides a system-based view of β-arrestin-mediated phosphorylation events downstream of an AT_{1a} receptor and opens avenues for research in a rapidly evolving area of 7TMR signaling (Xiao et al., 2010).

Our actually data are strengthened by the previous ones, showing that the intracellular effects of Ang II (administered by the means of liposomes) are associated with AT_1 receptor activation/internalization and might thus be part of the mechanism of angiotensin peptides direct contractile effects in the vascular smooth muscle (Petrescu *et al.*, 2001b).

Moreover, Ang II has no contractile activity in the rat vascular smooth muscle preparations when a specific antibody against AT_1 receptor was administered as pretreatment (preliminary personal, unpublished data). Firstly, this lack of Ang II effect might be due to the competition for AT_1 receptor between Ang II and specific antibody. Secondly, through the interaction between antibody and AT_1 receptor, the later might suffer a conformational change not allowing the interaction between receptor and β-arrestins.

CONCLUSIONS

None of the known and tested inhibitors of AT_1 receptor internalization, that means nigericin, concanavalin A and monensin, did not significantly alter the contractions induced by Ang II in the rat aortic smooth muscle preparations. The same effects we obtained also when we used as agonists Ang I, Ang III and Ang IV. Thus, the contractile effects induced by angiotensin peptides administered in the rat aortic smooth muscle preparations are independent of their receptors internalization. Since β-arrestins are mediating G-protein independent signaling *via* AT_1 receptors, their involvement in the contractile effects of angiotensin peptides must be further explored.

ACKNOWLEDGEMENTS

This study was partially supported by C.N.C.S.I.S. grants IDEI-PCE-1273/2007 (Project director Lect. Dr. Bogdan Gurzu) and IDEI-PCE-2670/2008 (Project director Prof. Dr. Gheorghe Petrescu).

REFERENCES

Becker, B.N., Cheng, H.F., Hammond, T.G., Harris, R.C. (2004): *The type 1 angiotensin II receptor tail affects receptor targeting, internalization, and membrane fusion properties*. Mol. Pharmacol., 65(2), 362-369.

Bonde, M.M., Yao, R., Ma, J.N., Madabushi, S., et al. (2010): *An angiotensin II type 1 receptor activation switch patch revealed through evolutionary trace analysis*. Biochem. Pharmacol., 80(1), 86-94.

Feng Y.H., Ding, Y., Ren, S., Zhou, L., et al. (2005): *Unconventional homologous internalization of the angiotensin II type-1 receptor induced by G-protein-independent signals*. Hypertension, 46(2), 419-425.

Hein, L., Meinel, L., Pratt, R.E., Dzau, V.J., Kobilka, B.K. (1997): *Intracellular trafficking of angiotensin II and its AT_1 and AT_2 receptors: evidence for selective sorting of receptor and ligand*. Mol. Endocrinol., 11(9), 1266-1277.

Hunyady, L. (1999): *Molecular mechanisms of angiotensin II receptor internalization*. J. Am. Soc. Nephrol., 10(Suppl 11), S47-S56.

Hunyady, L., Catt, K.J. (2006): *Pleiotropic AT_1 receptor signaling pathways mediating physiological and pathogenic actions of angiotensin II*. Mol. Endocrinol., 20(5), 953-970.

Modrall, J.G., Nanamori, M., Sadoshima, J., Barnhart, D.C., et al. (2001): *ANG II type 1 receptor downregulation does not require receptor endocytosis or G protein coupling*. Am. J. Physiol. Cell. Physiol., 281(3), C801-809.

Petrescu, G., Costuleanu, M., Slătineanu, S.M., Costuleanu, N., et al. (2001a): *Contractile effects of angiotensin peptides in rat aorta are differentially dependent on tyrosine kinase activity*. J. Renin Angiotensin Aldosterone Syst., 2(3), 180-187.

Petrescu, G., Costuleanu, M., Slătineanu, S.M., Foia, L., et al. (2001b): *Contractile effects of intracellularly administered angiotensin II are partially dependent on membrane receptors internalization in isolated rat aorta*. Rev. Med. Chir. Soc. Med. Nat. Iaşi, 105(4), 700-704.

Rakesh, K., Yoo, B., Kim, I.M., Salazar, N., et al. (2010): *beta-Arrestin-biased agonism of the angiotensin receptor induced by mechanical stress*. Sci. Signal., 3(125), ra46.

Sanni, S.J., Hansen, J.T., Bonde, M.M., Speerschneider, T., et al. (2010): *beta-Arrestin 1 and 2 stabilize the angiotensin II type I receptor in distinct high-affinity conformations*. Br. J. Pharmacol., 161(1), 150-161.

Shukla, A.K., Kim, J., Ahn, S., Xiao, K., et al. (2010): *Arresting a TRP channel [beta]-arrestin 1 mediates ubiquitination and functional downregulation of TRPV4*. J. Biol. Chem., Jul 22, [Epub ahead of print].

van Kats, J.P., de Lannoy, L.M., Jan Danser, A.H., van Meegen, J.R., et al. (1997): *Angiotensin II type 1 (AT_1) receptor-mediated accumulation of angiotensin II in tissues and its intracellular half-life in vivo*. Hypertension, 30(1 Pt 1), 42-49.

Xiao K, Sun J, Kim J, Rajagopal S, et al. (2010): *Global phosphorylation analysis of {beta}-arrestin-mediated signaling downstream of a seven transmembrane receptor (7TMR)*. Proc. Natl. Acad. Sci. U. S. A., Aug 4, [Epub ahead of print].

1. Department of General and Oro-Maxillo-Facial Pathology, Faculty of Dental Medicine, 2.Department of Physiology, 3.Department of Histology, Faculty of Medicine, University of Medicine and Pharmacy "Gr. T. Popa" Iaşi, 16, University Str., 700115, Iaşi, Romania.

*Author for correspondence: Prof. Dr. Marcel Costuleanu, Department of General and Oro-Maxillo-Facial Pathology, Faculty of Dental Medicine, University of Medicine and Pharmacy "Gr. T. Popa" Iaşi; 16, University Str., 700115, Iaşi, Romania; tel. +40-745-589050; fax: +40-232-211820; e-mail: mcostuleanu@yahoo.com.