# THE FATE OF TYPE I ANGIOTENSIN II RECEPTOR AFTER BIASED ACTIVATION

**PhD Thesis Booklet** 

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### Introduction

The renin-angiotensin-aldosteron system is an important pharmacological target due to it's essential role in the regulation of cardiovascular system as well as salt and water balance. This has been supported by the morbidity and mortality reduction effects of the type 1 angiotensin II receptor ( $AT_1R$ ) blockers and the inhibitors of the angiotensin convertase ensyme.

One of the main executing molecules of this irregular endocrine system is the angiotensin II (AngII), a hormone which regulates blood pressure and plasma volume mainly by expanding vascular tone and boosting aldosterone secretion, heart muscle contractility and sympathetic nervous system activity. The AngII produces these effects through the AT<sub>1</sub>R, which belongs to the family of the G protein-coupled receptors (GPCRs). The active conformation of the AT<sub>1</sub>Rtriggers a widespread signal transduction network, which encompasses mechanisms both related to and independent of G-protein functions. G-protein-independent signal transduction mechanisms are mainly regulated by the central regulation roles of the  $\beta$ -arrestins, which are known to play a role in disconnecting the receptor from the G-protein (desensitization) and triggering receptor internalization. Studies confirm that different ligands in active conformation can stabilize the receptor, enabling the activation of different signal transduction pathways to varying degrees. This phenomenon is called selective activation of signal transduction. Selective activation of signal transduction has been one of the main areas of GPCR research for years, and several selective signalling analogues of AngII AT<sub>1</sub>R agonists are known. Amongst them is a newer formula, TRV120027, which has been clinically researched for treating heart failure. The AT<sub>1</sub>R activated by this ligand cannot bind G-proteins, however, its  $\beta$ -arrestin bind remains, along with its G-protein independent signaling, which results in an effect combination with major clinical benefits.

GPCRs are also known to phosphorylate, desensitize, then internalize into the cell, after activation. The internalized vesicle is transformed into an early endosome, from which the receptor can proceed towards lysosomal disassembly, or to the plasma membrane, with the help of the recycling endosomes. The sum of these internalization and externalisation processes fundamentally determines the sensitivity and response of the receptors, and consequently, the sensitivity and response of the cells.

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#### **Objectives**

We have much information on the mechanisms and signal transduction consequences of the functionally selective activation of GPCRs, including the AT<sub>1</sub>R. However, we have less knowledge on the later fate of the selectively activated receptor inside the cell, which has a defining role in the sensitivity of the cell. Therefore, we aim to investigate the intracellular fate of the AT<sub>1</sub>R, induced by signal-selective ligands, as a significant functionally selective pharmacological target.

We sought answers for the following questions:

- How does the internalization of the AT<sub>1</sub>R change after signal transduction selective activation?
- Which mechanism has a defining role in the appearance of the biased ligand activated AT<sub>1</sub>Rin early endosomes?
- Is there a difference in the later intracellular fate of the signal transduction activated AT<sub>1</sub>Rs, compared to AngII stimulated receptors?

#### Methods

#### **DNA Constructs**

The cDNA of yellow fluorescent protein-tagged (eYFP)  $\beta$ arresztin2 and *Renilla* luciferase (Rluc)-tagged AT<sub>1</sub>Rs (AT<sub>1</sub>R-Rluc, DRY/AAY AT<sub>1</sub>R-Rluc) were constructed as described previously. The DRY/AAY mutant AT<sub>1</sub>R (Asp125 and Arg126 was substituted with alanine residues) is unable to activate G proteins.

To investigate the vesicular transport of the AT<sub>1</sub>R eYFPlabeled Rab4, Rab5, Rab7 and Rab11 were constructed by replacing the green fluorescent protein (eGFP)-coding region with eYFP in the GFP-tagged constructs as described previously. The eYFP-tagged The eYFP-tagged version of PLC $\delta$ 1 pleckstrin homology (PH) domain, which can bind to the phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)*P*<sub>2</sub>), was constructed as described previously (PLC $\delta$ 1-PH-YFP). The PLC $\delta$ 1-PH–super *Renilla* luciferase (Sluc) was constructed by replacing the eYFP coding region of PLC $\delta$ 1-PH-YFP with sequence of super *Renilla* luciferase.

#### **Cell Culture and Transfection**

The experiments were performed on human embrionic kidney cell lines (HEK293 and HEK293T). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 100 IU/ml penicillin, 100  $\mu$ g/ml streptomycin and 10% heatinactivated fetal

bovine serum (FBS) in 5% CO2 at 37°C. For the experiments, the cells were cultured in 10 cm plastic dishes, trypsinized prior to transfection, transiently transfected by using Lipofectamine 2000 and Opti-MEM<sup>®</sup> medium.

## Bioluminescence resonance energy transfer (BRET) Measurement

The principle underlying this method is the energy transfer between a bioluminescent donor and a fluorescent acceptor molecule that occurs when the two molecules are in appropriate proximity (less than 10 nm distance) to each other. Therefore light emission can be detected on the spectum of the excited fluorescent acceptor. In our experiments we fused Renilla luciferase as donor molecule and yellow fluorescent protein-tagged (YFP) as acceptor molecule to the interaction partners.

The BRET measurements were performed after 24 hours of the transfection on white 96-well plates. The DMEM of the cells which contains 10% FBS was changed prior to measurements to a modified Krebs-Ringer buffer.

The BRET measurements were started after addition of the cell-permeable substrate coelenterazine h (5  $\mu$ M), and the counts were recorded at 37 °C by using either Berthold Mithras LB 940 or Varioskan Flash readers at 37 °C. The BRET signal was determined by calculating the ratio of the light emitted by the energy acceptor YFP (485 nm) to the light emitted by the energy donor Rluc (530

nm). The BRET signal is increased when the donor and acceptor molecules are closer to each other, and decrease when the distance is greater between them. BRET ratios were baseline corrected to the vehicle curve.

#### Western blot expreiments

Twentyfour hours after transfection with Rluc, AT<sub>1</sub>R-Rluc or DRY/AAY AT<sub>1</sub>R-Rluc constructs, the HEK293 cells were stimulated for 5 minutes with 100 nM AngII or 10 mM SII-AngII at 37°C. Cells were scraped into an SDS sample buffer and separated on 12% SDS-polyacrilamide gels. The proteins were transferred to polyvinylidene fluoride membranes and incubated with the primary antibodies in 1:1000 dilution ( $\alpha$ -p-ERK1/2 and  $\alpha$ -total-ERK1/2) and appropriate secondary antibodies. The antibodies were visualized by enhanced chemiluminescence.

## Cytoplasmic Ca<sup>2+</sup> Measurements in Cell Suspensions

HEK293 cells were transfected with the various  $AT_1R$  constructs by using Lipofectamine 2000. After 24 hours, the cells were removed by mild trypsinization, and aliquots of cells were loaded in medium containing Fura-2/ AM for 45 minutes at room temperature in dark. The cells were centrifuged rapidly before the measurements and dispersed in medium without Fura-2/AM. Calcium measurements were performed at room temperature in a PTI

Deltascan spectrofluorometer with excitation at 340 nm and 380 nm, and detection of emission at505 nm. 340/380 nm excitation ratio for fura-2 allows accurate measurements of the intracellular Ca2+ concentration.

#### **Confocal microscopy**

The localisation of the  $AT_1R$  was analysed with Zeiss LSM 710 confocal laser-scanning microscope during the stimulation of  $AT_1R$ -GFP stably expressing HEK293 cells with AngII or TRV120027. The GFP was excited with argon laser at 488 nm. Postacquisition data analysis of the confocal images was performed with either ZEN or MetaMorph software

#### **Statistical analysis**

Data analysis and figures were processed with GraphPad Prism 4.03 and Sigmaplot 10.0. Differences between ligands were tested by one-way or two-way ANOVA and Bonferroni or Tukey post-hoc tests. A p value less than 0.05 was considered significant.

#### Results

# Tracking the induced endocytosis of the $AT_1R$ with BRET method.

In our first experiments, we have used cytoplasmatic Ca<sup>2+</sup>measurement and ERK1/2 phosphorylation tracking to verify the operability of the AT<sub>1</sub>R marked with Renilla luciferase (Rluc). After this, we have investigated the appearance of  $AT_1R$  in early endosomes with the BRET method. In our experiments, we have tracked the energy transfer between the Rluc marked  $AT_1R$  and the YFP marked Rab5 protein. We have observed that AngII stimulation results in the faster appearance of the G protein-uncoupled DRY/AAY signaling selective mutant  $AT_1R$  in the early endosomes containing Rab5, compared to the wild-type AT<sub>1</sub>R. Following this, we have examined some other  $\beta$ -arrestin signaling selective AngII analogues: [Sar<sup>1</sup>,Ile<sup>8</sup>]-AngII (SI-AngII), [Sar<sup>1</sup>,Ile<sup>4</sup>,Ile<sup>8</sup>]-AngII (SII-AngII), TRV120023(TRV3), TRV120027 (TRV7). We have observed similiar phenonema in these ligands: the faster appearance of AT<sub>1</sub>R in early endosomes compared to AngII stimulation. The angiontensin IV (AngIV), which has similarly low affinity as the signalling selective ligands and is able to activate both G-proteindependent and G-protein-independent pathways, resulted in similiar internalization kinetics.

# Description of the differences in the internalization of the signaling selective activated $AT_1R$

We have proved with confocal microscopy that the early internalization of the signaling selectively activated  $AT_1R$  has accelerated, for which HEK 293 cells stably expressing GFP-tagged  $AT_1R$  were used, developed earlier by our workgroup. In order to investigate the different early endocytic pathways, we have blocked clathrin-mediated endocytosis with 300mM saccharose and the caveolae-mediated pathway with filipin. Saccharose pretreatment blocked AngII and SII-AngII induced internalization, while filipin pretreatment did not influence ligand-induced internalization difference. BAPTA pretreatment blocking of the Ca<sup>2+</sup>signal has not influenced early internalization either.

However, examination of the  $\beta$ -arrestin2 binding revealed that after sginalling selective activation the strength of the  $\beta$ -arrestin2 binding is weaker compared to the AngII induced one. Similarly weakened  $\beta$ -arrestin2 binding was observed when using AngIV. Dose-response curves confirmed that the observed differences were not due to submaximal ligand concentrations.

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# Role of the plasma membrane $PtdIns(4,5)P_2$ synthesis and depletion in the full agonist and function selective agonist induced internalization of the $AT_1R$

We have also examined the effects of the PtdIns(4,5) $P_2$  found in the plasma membrane to the early internalization of the AT<sub>1</sub>R. First, we have confirmed that our ligands selective for  $\beta$ -arrestin signal transduction (SI-AngI, SII-AngII, TRV3 és TRV7) do not lead to the depletion of PtdIns(4,5) $P_2$  by G-protein activation.

Blocking phosphatidylinositol 3-kinases with small dose wortmannin did not result in changes to early internalization. However, blocking the phosphatidylinositol 4-kinases(PI4K) resulted in complete blocking of AngII and AngIV induced internalization, while TRV3 induced internalization remained unaffected. After this, we have blocked PI4KA with A1-blocking agent and PIKB isoforms with PIK93. Inhibiting the PI4KA isoform resulted in the disappearance of AT<sub>1</sub>R in early endosomes when stimulated with AngII or AngIV, but remained unaffected in case of TRV3 stimulation.

After examining the synthesis of PtdIns(4,5) $P_2$  we have also observed the role of depletion of PtdIns(4,5) $P_2$ . We inhibited the activation of Gq proteins thus the depletion of PtdIns(4,5) $P_2$ molecules using an overexpression system of a dominant-negative GRK2 (DN-GRK2) construct. DN-GRK2 accelerated AngII-evoked AT<sub>1</sub>R internalization, but had no effect ont he TRV3-induced response. Subsequently, we have analyzed the role of GFKR PtdIns(4,5) $P_2$  induced separation. Previous stimulation of the  $\alpha$ 1-adrenerg receptor slowed the early internalization of the AT<sub>1</sub>R, when induced by AngII or TRV3.

# Following the intracellular fate of $AT_1R$ with Rab small G-proteins

Our final objective was to examine the appearance of  $AT_1R$ in early recycling endosomes containing Rab4, late recycling endosomes and lysosomes containing Rab7, and late recycling endosomes containing Rab11. After signal selective activation  $AT_1R$ appeared earlier in all examined compartments compared to AngII stimulation. The receptor appeared faster and to a greater extent after both signal selective activation and AngIV stimulation, compared to AngII stimulation in lysosomes containing Rab11. However, it appeared less in lysosomes containing Rab7.

Our final experiments confirmed that  $Ca^{2+}$  chelation pretreated with BAPTA does not effect the long term intracellular fate of  $AT_1R$ .

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#### Conclusions

Based on our results, we conclude the following:

After  $\beta$ -arrestin signaling selective activation, AT<sub>1</sub>R appears earlier in early endosomes containing Rab5, compared to AngII stimulation. The  $\beta$ -arrestin binding strength of signal selective ligands is weakercompared to AngII induced ones.

The altered early internalization is not created through different endocytic pathways, nor does the different  $\beta$ -arrestin strength or the absence of the Ca<sup>2+</sup> signal play a role in the process. However, the depletion of PtdIns(4,5)*P*<sub>2</sub> induced by the G-protein activation and the subsequent resynthesis is key to the slower internalization induced by the AngII. Based on this, we can theorize that G-protein activation and the absence of PtdIns(4,5)*P*<sub>2</sub> depletion are responsible for the faster early internalization, in the case of  $\beta$ -arrestin signal selective biased ligands.

Furthermore, the appearance of  $AT_1R$  in endosomes containing Rab4, Rab7, and Rab11 also differs following functionally selective activation. The receptor appears earlier in compartments marked with these proteins, when activated by biased ligands, compared to the application of non selective AngII or AngIV. The later intracellular fate of the receptor is not influenced by the absence of Ca<sup>2+</sup> signal, however, β-arrestin binding strength correlates with the process.

#### List of publications

#### Publications related directly to the thesis

- I. <u>Szakadati G</u>, Toth AD, Olah I, Erdelyi LS, Balla T, Varnai P, Hunyady L, Balla A. (2015) Investigation of the Fate of Type I Angiotensin Receptor after Biased Activation. Mol Pharmacol, 87: 972. IF:3,931
- II. Balla A, Toth DJ, Soltesz-Katona E, <u>Szakadati G</u>, Erdelyi LS, Varnai P, Hunyady L. (2012) Mapping of the localization of type 1 angiotensin receptor in membrane microdomains using bioluminescence resonance energy transfer-based sensors. J Biol Chem, 287: 9090-9099. IF:4,651