Investigation of two clinically important G protein-coupled receptors: V2 vasopressin receptor and AT1 angiotensin receptor

Ph.D. Thesis

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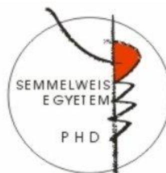

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Introduction

G protein-coupled receptors (GPCRs) are key cell-surface proteins which constitute the largest class of receptor and membrane proteins in the human proteome. They transduce environmental signals to various pathways across the membrane and mediate the majority of cellular responses to external stimuli, including taste, smell and light, and the effects of hormones, paracrine factors and neurotransmitters on cells, thus playing essential roles in physiological processes. Because of that and, not surprisingly, GPCRs are commonly targeted by pharmacological agents.

The arginine-vasopressin system

The diluting and concentrating function of the kidney plays a crucial role in regulating the body's normal water balance. These processes are regulated by the release of the antidiuretic hormone, arginine vasopressin (AVP), from the posterior pituitary gland. The secreted AVP hormone has multiple actions in the body. In addition to its antidiuretic effect, the AVP is a potent neurohormone involved in the regulation of arterial blood pressure, sympathetic activity, baroreflex sensitivity, glucose homeostasis, release of glucocorticoids and catecholamines, stress response, anxiety, memory, and behavior. These multiple actions of AVP are mediated by at least three G protein-coupled receptors: type 1 vasopressin receptor (V1R), type 2 vasopressin receptor (V2R) and type 3 vasopressin receptor (V3R).

AVP exerts its water-conserving effect through the V2R, which is localized on the basolateral side of the principal cells of the kidney's collecting duct. When AVP reaches the kidney, it binds to the V2R receptor and initiates a signaling cascade that mediates the redistribution of aquaporin 2 (AQP2) water channels from intracellular membranes to the apical membrane. This mechanism increases the water permeability, and, thus, water is able to pass the apical membrane passively via AQP2, following the osmotic gradient between the tubular fluid and the hyperosmotic interstitium.

Mutations in GPCRs can lead to pathological conditions such as mutations in the arginine vasopressin type 2 receptor (*AVPR2*) gene, which cause X chromosome-linked nephrogenic diabetes insipidus (X-NDI). The cellular manifestations of the *AVPR2* gene mutation can be classified into five classes. The class I mutations of the *AVPR2* gene result in improperly processed or unstable mRNA, frameshift or nonsense mutations that lead to truncation of V2R receptors. The class II mutations are the most common, and are caused by missense/nonsense mutations that result in misfolding of the receptor protein. They are recognized by the endoplasmic reticulum (ER) quality control system. These mutant receptors are usually trapped in the ER and their degradation occurs often. The class III mutations also cause misfolding of receptor proteins, although here the V2R receptor reaches the plasma membrane and interacts with AVP, but it does not interact fully with G proteins, resulting in impaired cAMP production. The class IV mutations also result in V2R misfolding, but in this case the mutated V2R receptors reach the plasma membrane,

but do not interact properly with AVP, while class V mutations are missorted to an incorrect cellular compartment.

Renin-angiotensin-aldosterone-system (RAAS)

In its classical view, the renin-angiotensin-aldosterone-system (RAAS) is involved in hormonal regulation of blood pressure and the salt/water homeostasis. However, RAAS is now considered as an “ubiquitous” system that is expressed locally in various tissues and it exerts a number of paracrine / autocrine effects that are involved in tissue physiology and homeostasis. It plays a key role in processes such as cell growth, proliferation, differentiation, migration, apoptosis, extracellular matrix (ECM) remodeling and inflammation. The main effector peptide of this system is angiotensin II (AngII) which mainly acts through the angiotensin II type 1 receptor (AT1R). The AngII participates not only in physiological, but also in pathological mechanisms leading to cardiovascular diseases. The excessive action of AngII leads to pathophysiological changes such as hyperplasia and hypertrophy in the blood vessels, vascular transformation, atherosclerosis and hypertension. Given its diverse range of functions and its potency in affecting cardiovascular physiology, the extensive study of RAAS and the understanding of its physiological and pathophysiological function is essential because it is frequently pharmacologically targeted in clinical practice for treatment of heart failure, hypertension, kidney complications of diabetes mellitus, acute myocardial infarction, atherosclerosis and stroke. In particular, it is imperative to understand the characteristics

of AngII receptors, and to investigate the mechanisms of AngII-induced signaling, and the diverse roles of AngII, given the beneficial effects of angiotensin converting enzyme inhibitors (ACE-I) and angiotensin II type-1 receptor blockers (ARBs).

Objectives

During this Ph.D. study, the functions and signaling mechanisms of two clinically important GPCRs, the V2R and the AT1-R were investigated.

The aims in the characterization of the disease-causing S127F mutant V2 receptor studies were:

- Investigation of the cellular consequences of the missense mutation of the V2R (S127F-V2R), such as examination of the localization, cAMP signal generation capability of the mutant receptor and classification of the type of the disease-causing mutation based on the obtained data.
- Investigation of the possible functional rescue of the mutant receptor by pharmacochaperones.

The aims in the investigation of long-term effect of AngII in VSMCs were:

- Investigation of the AngII-induced upregulation of *DUSP* genes in the VSMCs.
- Examination of the signaling pathways affecting the expression of the different *DUSPs*, including the identification of the involved receptor and G protein type.
- Investigation of the role of β-arrestin and calcium signal in the AngII-induced upregulation of *DUSP* genes.

Results

Identification of the S127F mutation in the *AVPR2* gene

Our collaborating researchers have identified a nephrogenic diabetes insipidus patient in a Hungarian family. The male patient has manifested the classic symptoms of NDI since birth. The clinical diagnosis is based on results of the water deprivation test and on the ineffective response to arginine vasopressin analogue, 1-deamino-8-D-arginine-vasopressin (desmopressin) therapy. DNA sequencing revealed a point mutation in the *AVPR2* gene in the proband, and showed that the mother also carries the same mutation, which confirmed that this is not a *de novo* mutation and confirmed that the mother is a heterozygous carrier. This missense mutation caused a cytosine to thymine single-base substitution (g.741C→T) in the exon 2, resulting in an amino acid substitution from serine-127 to phenylalanine (S127F).

Characterization of the mutant receptor

Firstly, we determined the intracellular location of the fluorescence-labeled S127F mutant and wild type (WT) V2Rs in non-permeabilized and permeabilized HEK-293 cells. The results of the study showed that the WT V2Rs were located on the plasma membrane, whereas the S127F mutant V2Rs were not detectable on the cell surface in non-permeabilized cells. In permeabilized cells, WT-V2R was mainly present on the cell surface and showed only mild intracellular staining. While the S127F-V2R mutant receptor showed

marked intracellular staining confirming receptor synthesis in cells, the synthesized receptors were apparently not able to reach the cell surface.

Next, a functional assay of the S127F-V2R mutant receptor was performed. The bioluminescence resonance energy transfer (BRET) technique was used for comparing cytoplasmic cAMP production of the WT-V2R and S127F-V2R in response to AVP stimulus. The AVP stimulation of S127F-V2R expressing cells caused only minimal cAMP production compared to the cAMP signal generation of the WT receptor expressing cells, indicating partial functionality of the mutant receptor.

Functional rescue of the S127F-V2R

Firstly, the pharmacochaperone effect of the V2R inverse agonist tolvaptan in functional rescue of S127F-V2R was investigated. The increase in intracellular cAMP levels upon 1 μM AVP stimulation in WT-V2R expressing cells showed a rapid and robust change in both vehicle or 100 nM tolvaptan pretreated cells. However, when the mutant receptor expressing cells were pretreated with the vehicle, only a minimal cAMP response was generated upon 1 μM AVP stimulus, but pretreatment with 100 nM tolvaptan significantly increased the cAMP response.

Thereafter, the role of the pharmacochaperone biased agonist of V2R in functional rescue of S127F-V2R was examined. Pretreatment of wild-type receptor-expressing cells with 10 μM MCF14 caused an increase in basal cAMP levels, and this cAMP level could not be

further increased by 1 μM AVP stimulation. S127F-V2R expressing cells did not show basal activity upon MCF14 pretreatment, but they showed a rapid and robust increase in intracellular cAMP level upon 1 μM AVP stimulation.

In the next experiment, we examined whether an acute stimulus with MCF14 could directly activate misfolded receptors in S127F-V2R expressing cells. Based on our measurements, MCF14 stimulation was able to induce cAMP signal similar to ones induced in AVP stimulation in WT-V2R cells, but the MCF14 stimulation did not result in significant cAMP signal generation in S127F-V2R cells.

Effect of V2R pharmacochaperone ligands on the cell surface expression of the S127F-V2R

In the following stage, the cell surface expression of the rescued mutant receptor by flow cytometry and confocal microscopy was examined. Therefore, to perform the assay, HEK293 cells were transiently transfected with HA-WT-V2R or HA-S127F-V2R, and, after 24 h of transfection, the cells were pretreated with vehicle or 100 nM tolvaptan or 10 μM MCF14 for 18 hours. Based on our measurements, both tolvaptan and MCF14 slightly increased the cell surface expression of the WT-V2R, while no notable change in the cell surface expression level of S127-V2R could be detected. That may reflect the fact that only a very small proportion of intracellular retained receptors were rescued by treatment with tolvaptan or MCF14.

In the second part of the thesis, the other medically important GPCR, the AT1-R, was examined. This receptor is implicated in the development of many cardiovascular diseases, and in order to get new data about the effect of the long-term of its stimulation, AngII-induced gene expression in vascular smooth muscle cells was analyzed.

Affymetrix GeneChip analysis of the AngII stimulation upregulated genes in VSMCs

In this experiment, we compared the gene expression profile of vehicle and 100 nM AngII-treated VSMC cells by using the Affymetrix GeneChip Rat Gene 1.0 ST array.

For our experiments, primary rat vascular smooth muscle cells were isolated. In order to confirm that these isolated cells have VSMCs-specific properties, smooth muscle α-actin immunostaining and agonist stimulation were performed on these early passages. The results confirmed that these cells have their characteristic properties of VSMCs, inasmuch as they showed smooth muscle α-actin expression and the typical ERK1/2 pattern upon 50 ng/ml EGF or 100 nM AngII stimulation.

In our Affymetrix GeneChip experimental setup, more than 100 gene expressions were altered by AngII stimulation in smooth muscle cells. Among the upregulated genes, several *DUSP* genes were significantly upregulated. However, one *DUSP* was selected from each *DUSP* subfamily, namely *DUSP5, DUSP6*, and *DUSP10*, for further study in this dissertation.

qRT-PCR measurements validate the upregulation of *DUSP5*, *DUSP6* and *DUSP10* gene expressions. Time kinetics of gene expression changes in response to AngII stimulation of VSMCs.

The primary aim was to confirm the Affymetrix GeneChip results and to determine the time course of the AngII-induced upregulation of examined *DUSPs* expression levels by qRT-PCR. Based on this determination, it was clearly observed that *DUSPs* mRNA levels were highest 2 hours after AngII stimulation. Thus, the 2-hour AngII stimulation was chosen for further experiments to analyze gene expression changes.

Investigation of signaling pathways involved in the AngII-mediated responses

Thereafter, we wanted to determine which angiotensin receptor type and associated G protein play a role in the expression regulation of the studied *DUSP* genes. 10 μM candesartan (a selective AT1-R antagonist) pretreatment was used, which completely inhibited the AngII-induced upregulation of *DUSP* mRNA levels. This result indicated that the AT1-R mediates the observed AngII-induced gene expression changes. EGF stimulation was also used to demonstrate the amplitude of the direct EGFR activation-induced expression changes.

Furthermore, the YM-254890, a selective $G_{q/11}$ inhibitor, was used to investigate the role of the $G_{q/11}$ pathways. The application of YM-254890 at a concentration of 1 μM completely abolished the AngII-mediated gene expression upregulation in all examined *DUSPs*.

Next, we wanted to investigate the role of the β-arrestin mediated signaling pathway in the regulation of AngII-induced gene expression changes. For this study, a biased agonist of the AT1-R receptor, the TRV3 peptide, was used, which induces β-arrestin-mediated signaling through β-arrestin binding. In contrast to the AngII stimulus, the use of 3 μM TRV3 did not induce significant changes in *DUSP* gene expression, suggesting that *DUSP* upregulation is regulated exclusively in a G protein-dependent manner.

The role of calcium signaling and calcium dependent kinases in the upregulation of DUSP levels

Prior to the agonist stimulation, the VSMCs were pretreated with 50 μM BAPTA-AM, a calcium chelator, to investigate the role of intracellular calcium in the regulation of *DUSP* mRNA levels. The results obtained with BAPTA-AM pretreatment show that the induced intracellular calcium increase is essential for the upregulation of the *DUSP* genes in response to both AngII and EGF stimulations in VSMCs.

In the next experiment, a specific calcium/calmodulin-dependent protein kinase II (CaMKII) inhibitor, 50 μM CK59, was used to explore additional mechanisms involved in calcium signaling. The pretreatment with 50 μM CK59 significantly inhibited the increase in AngII-induced *DUSP5* mRNA levels, but not the EGF caused response. The AngII-induced regulation of *DUSP6* and *DUSP10* gene expression was also significantly reduced by CaMKII inhibition, but the effect was much lower in EGF-stimulated cells.

In the next step, the role of calcium-dependent PKC was determined in the presence of a highly selective inhibitor of PKC, RO31-8425. The pretreatment decreased the AngII-induced *DUSP5* mRNA level increase, but not the EGF-induced response. In the case of *DUSP6* and *DUSP10*, the pretreatment significantly reduced AngII-induced upregulation, but had only a moderate effect on EGF-stimulated cells.

Furthermore, the role of Pyk2 (a regulator of EGFR transactivation in VSMCs) in the AngII-induced upregulation of *DUSPs* was investigated, using 1 µM PF-562271, a potent ATP-competitive FAK and Pyk2 kinase inhibitor. Pretreatment with 1 µM PF-562271 reduced the AngII-induced upregulation of *DUSP5* and *DUSP6* slightly, but not significantly. Based on our data on the Pyk inhibitor, AngII-induced *DUSP5* upregulation is partially, but not completely Pyk2 dependent. It also reduced not just the AngII-induced *DUSP6* upregulation, but also the *DUSP6* mRNA baseline. However, based on the results of multiple linear regression, the effect of Pyk2 inhibition remained significant. As for *DUSP10*, the 1 µM PF-562271 pretreatment did not affect the increase in AngII-mediated gene expression.

Conclusions

In our experiments, we examined the cell physiological consequences of the S127F-V2R mutation and the possibility of restoring the mutant receptor function by pharmacochaperones. In the second part of the dissertation, the AngII-induced upregulation of *DUSPs* in the VSMCs and the signaling pathways affecting the expression of the different *DUSPs* were examined. Based on our results, the following conclusions can be drawn:

The S127F mutation belongs to class II mutation category of V2R, which leads to the production of misfolded and intracellularly, ER retained receptors. They can initiate cAMP signaling after their pharmacochaperone induced translocation to the plasma membrane. In our experiments, S127F-V2R was able to generate a very low cAMP signal by the AVP stimulus, indicating partial functionality of the mutant receptor. This partial functionality of the S127F-V2R mutant receptor was successfully enhanced by pretreatment of V2R ligands with either tolvaptan and MCF14, which demonstrated their ability to reach and bind to ER-trapped receptor molecules, and, thus, to act as a pharmacochaperone. Our data shows that the pharmacochaperone treatments, with administration of either tolvaptan or MCF14, caused the translocation of only a negligible proportion of the intracellular trapped receptors to the plasma membrane and that this small number of translocated receptors did not yield a significant detectable increase in the cell surface receptor level.

The results obtained in the study of the pathways responsible for the upregulation of *DUSP* genes showed that AngII effects are due to AT1-R activation caused by $G_{q/11}$-protein binding. Subsequently, our results confirmed that the Ca^{2+} signal plays an important role in all the investigated *DUSP* expression changes. Our data demonstrated that the regulation of expression changes of *DUSP* genes is probably determined by the interaction of the signaling cascades involved.

Bibliography of the candidate's publications

Publications related to the thesis:

1. Gem JB, Kovacs KB, **Szalai L**, Szakadati G, Porkoláb E, Szalai B, Turu G, Toth AD, Szekeres M, Hunyady L, Balla A. (2021) Characterization of Type 1 Angiotensin II Receptor Activation Induced Dual-Specificity MAPK Phosphatase Gene Expression Changes in Rat Vascular Smooth Muscle Cells. Cells, 10(12):3538. doi: 10.3390/cells10123538. IF: 7,666

2. **Szalai L**, Sziraki A, Erdelyi LS, Kovacs KB, Toth M, Toh AD, Turu G, Bonnet D, Mouillac B, Hunyady L and Balla A. (2022) Functional rescue of a nephrogenic diabetes insipidus causing mutation in the V2 vasopressin receptor by specific antagonist and agonist pharmacochaperones. Front Pharmacol, 13: 811836. doi: 10.3389/fphar.2022.811836. IF: 5,811