Examination of Disease Causing Mutations in the V2 Vasopressin Receptor

Ph. D. thesis

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Introduction

G protein–coupled receptors (GPCR)

Members of the G protein–coupled receptor superfamily are the major group of cell surface receptors, which recognize hormones, neurotransmitters, and sensory information; thus, they play essential roles in physiological processes. Function of the GPCRs is based on the activation of heterotrimeric G protein dependent signaling pathways. Activity in the absence of ligands is the basal or constitutive activity of the GPCRs.

The desensitization (decoupling the GPCR from heterotrimeric G proteins) and the internalization (decreasing cell surface receptor presence) processes play fundamental role in the regulation of GPCR function. In the process of homologue desensitization, the GPCRs are phosphorylated by G protein dependent kinases (GRK). Binding of β-arrestin to the receptor inhibits the G protein coupling and activation sterically. Beside of inducing receptor internalization, activated β-arrestin also regulates G protein-independent signaling pathways in the cells. However, there are β-arrestin-independent internalization processes: the internalization is

insensitive to the presence of dominant negative β-arrestins but may be blocked with dominant negative dynamins.

Biased agonism is a widely investigated topic in the GPCR research. Biased agonism describes the phenomenon when the ligand-GPCR complex does not activate the complete spectrum of cell specific pathways and process (i.e. activation of G proteins, desensitization, internalization, activation of β-arrestin dependent pathways). The complex is biased for specific pathways which may be selectively activated. Either the binding of an artificial ligand or a mutation in the sequence of the receptor may alter the conformation formed after the binding of physiological ligand (biased receptor).

In addition to their physiological importance, the pathological significance of GPCRs cannot be emphasized enough because mutations of GPCRs are responsible for numerous human diseases. Based on activity of the receptor mutations may be divided in two groups. Loss-of-function mutations lead to decreased or lacking basal and/or ligand induced activity. Gain-function-mutations increase the basal or constitutive activity compared to wild type receptors.

Physiology of the arginine-vasopressin system

The arginine-vasopressin hormone (AVP) has essential role in the regulation of water balance. The physiological and pathophysiological effects of AVP are generated by its binding to GPCRs. The cellular consequences depend on the activation of signaling pathways by three possible receptor subtypes: type 1a vasopressin receptor (V1aR), type 1b vasopressin receptor (V1bR) and type 2 vasopressin receptor (V2R) may be responsible for the effects.

Activation of the V1aR expressed in vascular smooth muscle cells leads to vasoconstriction. The V2R expressed in the collecting duct cells of the kidney is fundamental in the regulation water homeostasis. The water permeability of these cells – and thus the regulation of concentrating-diluting function of the kidney – can be altered by the V2R dependent change of aquaporin (AQP) distribution in the cells. Binding of AVP to the V2R activates the adenylate cyclase – cAMP (3',5'-cyclic adenosine monophosphate) – protein kinase A pathway which leads to increasing cell surface expression of AQP2.

Pathophysiology of the arginine-vasopressin system

Decreased function of the arginine-vasopressin system results in diabetes insipidus (DI) disease. DI may be described with three symptoms: polyuria, hyposthenuria and consecutive polydipsia. Nephrogenic diabetes insipidus (NDI) is characterized by the inefficiency of AVP due the defect of the target cells. The molecular bases of congenital NDI are the mutations in either the *AQP2* or *AVPR2* genes. Mutations in the *AVPR2* are called X-linked NDI (XNDI).

Loss-of-function mutations of the V2R leading to XNDI are classified according to their cellular mechanisms and functions. Class I mutants do not have effective synthesis of the V2R protein. Impaired mRNA synthesis, instability may lead to decreased receptor amount. Amino acid substitution in the V2R may result in an impaired protein folding. These class II mutants are recognized by the quality control system of the endoplasmic reticulum (ER). ER retention also leads to decreased cell surface expression of the V2R. Typical mutants of the class III have impaired function but they reach the plasma membrane of the cells. One subtype of this class (IIIa) can be characterized with impaired G protein binding and/or G protein activation ability upon physiologic hormone binding to the receptor with altered conformation. Mutants in class IIIb

have decreased function based on impaired ligand affinity. Identification of the R137H mutation in the V2R revealed a new class in the classification system: this receptor is internalized constitutively in the absence of ligands. The receptor accumulates in intracellular vesicles and suffers from decreased plasma membrane expression.

Pathologically increased activity of the arginine-vasopressin system leads to syndrome of inappropriate antidiuretic hormone secretion (SIADH) due to increased AVP secretion. The syndrome results in hyponatremia based on the altered urinary concentration. Nephrogenic syndrome of inappropriate antidiuresis (NSIAD) is a recently discovered disease of the arginine-vasopressin system. NSIAD is based on the gain-of-function mutation of the V2R leading increased basal (in the absence of agonist) cAMP concentration of the cells. This increased basal activity was shown to result constitutive internalization of the first discovered R137C/L mutants of the V2R. Until today, R137C, R137L and F229V mutation are known to lead to NSIAD disease.

Objectives

Despite of the intensive research of the V2R mutations, there may be unknown and uncharacterized mutations. The molecular basis of the pathological conditions may be investigated with microscopic and functional studies. Based on the findings, therapeutic strategy may be proposed.

We wanted to answer the following questions:

1. Which genetic alteration may be responsible for the symptoms of the NDI patient treated in the 2nd Department of Internal Medicine of Semmelweis University? Which cellular consequences does the mutation lead to? What would be optimal therapeutic strategy in the case of the patient?

2. Does the newly identified V2R mutation leading to NSIAD have effect on the receptor function? Which properties can this new receptor conformation characterized by? Which therapy may be chosen for the family carrying this mutation?

Methods

Mutation analysis

Written informed consent was obtained from a male patient with NDI (2nd Department of Internal Medicine). Genomic DNA was extracted from peripheral blood leukocytes using a DNA isolation kit. The *AVPR2* gene was amplified with PCR in two fractions. The sizes of the PCR products were determined in agarose gel. The PCR products were purified for DNA sequencing and sequenced in both directions.

Molecular biology

The untagged and the HA-tagged (hemagglutinin) V2Rs were subcloned into pcDNA3.1. HA tag was in 5' direction from the receptor in the plasmid. For the construction of the super Renilla luciferase (Sluc)–tagged V2R, the receptor sequence was amplified from the cDNA clone and subcloned into a pEYFP-N1 vector containing the sequence of Sluc. To create the mVenus-tagged V2R, the amplified receptor was subcloned into a pEYFP-N1 vector containing the sequence of mVenus. Mutagenesis was performed using standard site-directed mutagenesis techniques to generate N321K and I130N receptor constructs. After verifying the mutations with dideoxy

sequencing, the mutated fragment was exchanged between the wild-type (WT) and mutated portion with suitable restriction sites to avoid the generation of unwanted mutations outside the sequenced regions. For the construction of the Epac-BRET sensor, the mTurquoise part of the $^{T}EPAC^{VV}$ was replaced with Sluc. The Sluc sequence was amplified with PCR and was subcloned into $^{T}EPAC^{VV}$. All constructs were controlled with automatic sequencing.

Cell culture and transfection

HEK-293 (human embryonic kidney) cells used in the experiments were incubated in 37^{o}C and 5% CO_2. For transfections, cells were attached to poly-L-lysine pretreated plates and coverslips. Transfection solutes contained plasmids and Lipofectamine 2000™ transfection reagent. Measurements were performed 24 h after transfection.

Bioluminescence resonance energy transfer (BRET) measurements

We used the Epac-BRET probe to monitor the cAMP generation of the cells. The increase in the intracellular cAMP level results in a decreased BRET ratio in our measurements. BRET measurements were performed with Berthold Mithras

LB 940. Coelenterazine-h was added to the cells at the beginning of the measurement.

Receptors were tagged with mVenus and β-arrestin was tagged with Rluc in the β-arrestin binding studies. Increased BRET ratio reported the approximation of β-arrestin to the receptor.

The internalization was also investigated with BRET technique. MP-YFP construct tagged the plasma membrane with yellow fluorescent protein. Receptors were tagged with Sluc. BRET ratio reflected the distance between the receptors and the plasma membrane. BRET ratio decreased during internalization of the receptors.

Confocal microscopy

Two distinct methods were used for the investigation of the N321K and I130N mutants.

N321K: The cells plated on polylysine-pretreated glass coverslips and were transiently transfected with the HA-tagged receptor constructs or with pcDNA3.1. The cells were fixed with 4% paraformaldehyde solution. After washing cells were stained with anti-HA-Alexa 488 in the presence or absence of saponin. Cells were analyzed using a Zeiss LSM 510 confocal laser scanning microscope.

I130N: The cells were plated on polylysine-pretreated glass coverslips and were transiently transfected with the HA-tagged receptor (or pcDNA3.1) constructs and NLS-mRFP. Cells were stained with anti-HA-Alexa 488 on 4^{o}C without fixation. For the co-localization studies, cells were transiently transfected with the mVenus-tagged receptor constructs NLS-mRFP and MP-Cerulean. The localization and distribution of the targeted probes were analyzed using a Zeiss LSM710 confocal laser-scanning microscope.

Flow cytometry

The cells plated on glass coverslips were transiently transfected with the HA-tagged receptor constructs or pcDNA3.1. The cells were suspended in ice-cold PBS and incubated with diluted anti-HA-Alexa 488 antibodies. Flow cytometry measurements were performed with Beckman-Coulter SC. The fluorescent intensities were analyzed with WinMDI v2.9.

Wire myography

Thoracic aortas from mice were removed and placed into cold Krebs' solution. Aortic rings were mounted onto a multichannel isometric myograph system. The integrity and functionality of the aortic rings were tested by 124 mM K^{+}-containing Krebs'

solution which caused vasoconstriction. The relaxation ability of the vessels was tested with acetylcholine. Vasoconstrictor responses were calculated as percent values of the reference 1 μM phenylephrine-caused pre-contraction. Concentration-dependent vasoconstrictor response curves to agonists were obtained using parallel segments.

Data analysis and statistical evaluation

Vector NTI software was used for plasmid design. GraphPad Prism 5 was used for presentation of experimental results, non-linear regression of the dose-response curves and for statistical analysis. Statistical analysis was performed by one-sample t-test in the flow cytometry experiments. One-way ANOVA statistical analysis with the Tukey multiple comparison test was used for the analysis of pEC_{50} values (negative logarithm of the concentration that gives half-maximal response) of the agonists. The statistical analysis was carried out with two-way analysis of variance and Bonferroni's post hoc test in the BRET measurements.

Results

Identification of the N321K mutation

The male patient was born in 1984 with polyuria and polydipsia, and NDI was diagnosed at the age of 18 months. DNA sequencing of the *AVPR2* confirmed a missense mutation in the gene. The substitution results in an asparagine lysine change (N321K). Symptoms of DI were present in at least 3 generations of his family.

Cell surface expression of the N321K-V2R

Confocal microscopy revealed that the mutant N321K-V2R is localized in the plasma membrane of the transfected cells very similarly to that in the WT receptor. Immunostaining of permeabilized cells expressing either WT or mutant receptors showed marked intracellular fluorescence. These data show that the N321K-V2R can reach the plasma membrane of the cells. We did not detect any significant difference in flow cytometry experiments in the relative fluorescence intensities of the WT and N321K receptors on the cell surface, indicating that the plasma membrane expression of the mutant receptor is similar to that of the WT receptor.

Measurement of cAMP production of N321K-V2R

The basal BRET ratio was higher in the cells expressing the N321K-V2R than in the cells expressing the WT receptor using Epac-BRET probe. The mutant receptor was able to stimulate cAMP production upon AVP stimulus with amplitude very similar to that of the WT receptor, the kinetics of the activation was different. We also determined the dose-response curve of the mutant and the WT receptor upon AVP stimulus. The maximal BRET changes were similar for both receptors, but the potency of the N321K-V2R is dramatically decreased compared with that for the WT. For the N321K mutant receptor, we could not measure detectable cAMP production upon dDAVP stimulation.

Internalization properties of N321K-V2R

We investigated the β-arrestin2 binding of the receptors using the BRET technique. Association of the receptor with β-arrestin2 was detected as the BRET signal elevation after the AVP stimulus indicating the interaction of mVenus-tagged WT-V2R with β-arrestin2-Rluc. We were not able to detect β-arrestin2 binding for the N321K-V2R even at high, supraphysiological levels of AVP in dose-response study.

We also examined the internalization kinetics with BRET technique. The BRET ratio monitored the nonspecific resonance energy transfer, which is dependent on the distance between the receptor and plasma membrane. Stimulation of the WT-V2R with AVP reflected internalization of the cell surface–localized receptors into the endosomal compartments. The internalization of N321K-V2R was reduced compared with that for the WT receptor.

Examination of the agonist sensitivity of the N321K-V2R

We tested several commercially available peptides, which are known ligands of the V2R. The dose-response curves were measured in transiently transfected cells using our Epac-based cAMP-sensitive BRET probe. Of the peptides tested, the agonist dVDAVP had the highest potency to activate the mutant receptor. We have tested the effect of dVDAVP on V1R-initiated vasoconstriction of isolated mouse arteries by wire myography. The dVDAVP was not able to evoke vasoconstriction.

Determination of cellular localization of the I130N-V2R

We examined a newly identified mutation of the V2R. The mutant receptor showed similar intracellular fluorescence to

the wild-type receptor, but the plasma membrane localization was less pronounced with confocal microscopy. Cells expressing HA-I130N-V2R showed plasma membrane localization of the mutant receptor.

Investigation of the cAMP production of the I130N-V2R

The basal BRET ratio of the I130N-V2R was markedly decreased, indicating constitutive activity of this mutant receptor. Treatment of I130N receptor with the inverse agonist tolvaptan resulted in decreased cAMP concentration. 130N receptor stimulation with AVP resulted in a robust cAMP response.

Characterization of the plasma membrane expression of the I130N-V2R

Flow cytometry experiment showed decreased plasma membrane expression of I130N-V2R compared to that of the wild type.

We examined the plasma membrane presence of the mutant receptor with BRET technique. The BRET ratio monitored the nonspecific resonance energy transfer, which is dependent on the distance between the receptor and plasma membrane. The basal BRET ratio of the I130N receptor

expressing cells indicated decreased plasma membrane presence of the mutant. Tolvaptan could evoke a significant increase in the BRET ratio of the I130N-V2R. In response to AVP treatment, I130N mutant receptor expressing cells showed a robust fall in the BRET ratio.

Internalization properties of I130N-V2R

We examined the basal and agonist-dependent β-arrestin2 binding of the I130N mutant receptor with the BRET technique. There was no difference in the basal BRET ratios. The β-arrestin2 binding of the mutant was reduced upon AVP treatment compared with that of WT-V2R.

We investigated the effect of dominant-negative dynamins in order to characterize the internalization properties of the I130N-V2R. Cells expressing the dominant-negative dynamin1 (DNdyn1) showed an increased BRET ratio between the I130N-V2R-Sluc and the plasma membrane marker, compared with the effect of wild-type dynamin1 (dyn1). Tolvaptan treatment could increase the plasma membrane presence of I130N-V2R however, this elevation was less than that of dyn1 expressing cells.

We examined the effect of DNdyn1 on cAMP production of I130N-V2R. Cells expressing Epac-BRET,

I130N-V2R and DNdyn1 showed decreased basal BRET ratio than in control cells. Treating the cells with tolvaptan and AVP indicated similar changes as in cells without transfection of dynamin plasmids.

Conclusions

We identified a not characterized mutation in our patient. We demonstrated that N321K-V2R can reach the plasma membrane of the cells. The mutant receptor showed decreased potency and unchanged efficacy for AVP. The N321K-V2R showed impaired internalization and we could not detect β-arrestin binding. The mutant receptor had different sensitivity for agonists. The receptor function may be rescued with the administration of dDAVP without detectible side effects on V1R.

We characterized a newly identified gain-of-function mutation of the V2R. The conformation of the I130N receptor leads to selective G-protein activation and cAMP production without β-arrestin binding. The biased receptor showed constitutive internalization, which process was dynamin dependent. The activity of the I130N-V2R can be blocked with tolvaptan and the inverse agonist also leads to increased cell

surface expression of the receptor. According to our data, tolvaptan could be the treatment for patients carrying the I130N mutation.

List of publications

Publications related directly to the thesis

Az értekezés alapjául szolgáló közlemények

Erdélyi LS, Mann WA, Morris-Rosendahl DJ, Groß U, Nagel M, Várnai P, Balla A, Hunyady L. Mutation in the V2 vasopressin receptor gene, AVPR2, causes nephrogenic syndrome of inappropriate diuresis KIDNEY INTERNATIONAL Article in Press: doi: 10.1038/ki.2015.181. (2015) **IF: 8,563** *(first two authors contributed equally to this work)*

Erdelyi LS, Balla A, Patocs A, Toth M, Varnai P, Hunyady L. Altered agonist sensitivity of a mutant V2 receptor suggests a novel therapeutic strategy for nephrogenic diabetes insipidus. MOLECULAR ENDOCRINOLOGY 28:(5) pp. 634-643. (2014**) IF: 4,022**

Publications related indirectly to the thesis

Szakadati G, Toth AD, Olah I, **Erdelyi LS**, Balla T, Varnai P, Hunyady L, Balla A. Investigation of the fate of type I angiotensin receptor after biased activation MOLECULAR PHARMACOLOGY 87:(6) pp. 972-981. (2015**) IF: 4,128**

Szalai B, Hoffmann P, Prokop S, **Erdélyi LS**, Várnai P, Hunyady L. Improved methodical approach for quantitative BRET analysis of G protein coupled receptor dimerization PLOS ONE 9:(10) Paper e109503. (2014**) IF: 3,234**

Balla A, Toth D, Soltesz-Katona E, Szakadati G, **Erdelyi LS**, Varnai P, Hunyady L. Mapping of the localization of type I angiotensin receptor in membrane microdomains using bioluminescence resonance energy transfer-based sensors. JOURNAL OF BIOLOGICAL CHEMISTRY 287:(12) pp. 9090-9099. (2012) **IF: 4,651**

Balla A , **Erdelyi LS**, Soltesz-Katona E, Balla T, Varnai P, Hunyady L. Demonstration of angiotensin II-induced Ras activation in the trans-Golgi network and the endoplasmic reticulum using BRET-based biosensors. JOURNAL OF BIOLOGICAL CHEMISTRY 286:(7) pp. 5319-5327. (2011) **IF: 4,773**