B.E. Tunçdemir et al. / Hacettepe J. Biol. & Chem., 2022, 50 (1), 37-43

Research Article

Hacettepe Journal of Biology and Chemistry

journal homepage: www.hjbc.hacettepe.edu.tr

Chemical Chaperone Effects on Arginine Vasopressin Receptor 2 Mutants

Kimyasal Şaperonların Arjinin Vazopressin Reseptör 2 Mutantları Üzerine Etkisi

Beril Erdem Tunçdemir[1], Tuğçe Karaduman[2], Merve Özcan Türkmen[3], Dilara Şahin[1], Hatice Mergen[1], Emel Sağlar Özer[1*]

[1]Department of Biology, Molecular Biology Section, Hacettepe University, Ankara, Turkey.

[2]Biotechnology and Molecular Biology Department, Aksaray University, Aksaray, Turkey.

[3]Molecular Biology and Genetics Section, Department of Molecular Biology and Genetics. Necmettin Erbakan University, Konya, Turkey.

ABSTRACT

Improper folding of the mutant proteins may finally cause several conformational diseases such as Nephrogenic Diabetes Insipidus (NDI). In recent years, as a therapeutic strategy, chaperone treatment for such diseases is among current issues. In our study, we aimed to analyze the effect of several chemical chaperones on mutant V2 receptors which cause NDI. V2R mutant constructs were introduced into the pLV2R. Mutants were transiently expressed in COS-7 cells. After MTT analyses, cell surface and sandwich ELISA experiments was performed for understanding the rescue potential of the chaperones of the mutated proteins. As a result, we analyzed that rescue potential of a chemical chaperone depends on both chemical compound and the mutation type. We may conclude that such chaperone treatment studies are valuable for development of the therapeutic strategies.

Key Words

Chaperone, AVPR2, NDI.

ÖZ

Mutant proteinlerin yanlış katlanması, Nefrojenik Diabetes İnsipidus gibi bazı konformasyonel hastalıklara sebep olabilmektedir. Son yıllarda, terapötik bir strateji olarak, şaperon tedavisi bu tür hastalıklar için güncel konular arasındadır. Bu çalışmanın amacı, bazı kimyasal şaperonların Nefrojenik Diabetes İnsipidus'a neden olan V2 reseptör mutantları üzerine etkisinin araştırılmasıdır. V2R mutant örnekleri pLV2R vektöründe oluşturulmuştur. Mutant örnekler geçici trasfeksiyon ile COS-7 hücrelerinde ifade edilmiştir. MTT analizinden sonra, mutant proteinler üzerinde şaperonların kurtarma potansiyellerinin analizi için hücre yüzey ve sandviç ELİZA deneyleri uygulanmıştır. Sonuç olarak, kimyasal şaperonların kurtarma potansiyellerinin hem kullanılan kimyasal bileşiğe hem de mutasyonun tipine göre etki ettiği gözlenmiştir. Bu ve bunun gibi şaperon uygulamalarının terapötik stratejilerin geliştirilmesi açısından değerli olduğu sonucuna varılmıştır.

Anahtar Kelimeler

Şaperon, AVPR2, NDI.

Article History: Received: Jan 14, 2020; Revised: Apr 14, 2021; Accepted: Apr 14, 2021; Available Online: Apr 14, 2021.

DOI: https://doi.org/10.15671/hjbc.669854

Correspondence to: E. Saglar Özer, Department of Biology, Hacettepe University, Ankara, Turkey.

E-Mail: esaglar@hacettepe.edu.tr

INTRODUCTION

Function of a protein is connected with its proper three-dimensional structure. Generally mutated proteins are misfolded and retained in Endoplasmic reticulum (ER) or Golgi apparatus of the cells and after that follow the degradation processes [1]. The consequences of the misfolding of a protein may change according to the variation of the related gene [2]. Mutations may affect the function of the related protein and may lead to accumulation of aggregated forms of the protein in the cell which is finally toxic for cell or they are degraded by the quality control mechanism of the cell [3]. These possibilities may cause many conformational diseases, which are the consequences of improper trafficking of the mutated and misfolded proteins [3]. Nephrogenic Diabetes Insipidus (NDI) is such a rare disease and classified among these conformational diseases [3]. Most of the missense arginine-vasopressin receptor 2 (*AVPR2*) gene mutations known to cause NDI disease which is characterized by polyuria, hypoosmolar urine and hypernatremia [4]. Almost 90% of the NDI cases are related to *AVPR2* gene mutations and seen in a X-linked recessive form [5]. *AVPR2* is located on Xq28 and encodes 371 amino acid-long vasopressin type 2 receptor (V2R), which belongs to G protein-coupled receptor (GPCR) family [6]. In healthy subjects, decrease in the blood pressure leads to releasing of arginine vasopressin (AVP) hormone from hypothalamus into the blood stream which finally binds to AVPR2 receptor proteins within the distal convoluted tubules of the kidney. By this binding, secondary messenger cAMP increase in the cell and leads to protein kinase A (PKA) activation. PKA activation lead to phosphorylation of water channel aquaporin 2 (AQP2) which finally reabsorbs the water into the collecting duct cells [7, 8]. In this flow of the reabsorption of free water from urine, V2R receptor proteins play important roles. Localization and the type of the mutations on the *AVPR2* gene affect the course of the disease. As it is well studied, some small molecules called as chaperones may lead to help folding of these mutant proteins. Several chemical chaperones such as dimethyl sulfoxide (DMSO), which is a well-known cryoprotectant, Trimethylamine N-oxide (TMAO) and glycerol play roles as osmolytes and correct the folding defects by the way of modifying the environment of the mutant protein [9]. However, chemical chaperones may not bind the protein [10]. Some chemical chaperones such as thapsigargin and curcumin, which are classified as SERCA pump inhibitors, were reported to induce the membrane trafficking [10]. Another type of chaperones is called as pharmacological chaperones such as VPA-985, SR121463B and SR49059, OPC31260, OPC41061 [11, 12]. Pharmacological chaperones are non-peptide and cell-permeable ligands and specifically bind to the related target proteins unlike chemical chaperones [10]. We have previously identified several mutations on *AVPR2* gene, causing the NDI disease in Turkish families and we also studied functional characterization of these mutations [7, 8]. In this study we aimed to determine the rescue potentials of these chemical chaperones on mutant proteins. By this way, possible therapeutic effects of these chemical chaperones on mutant AVPR2s were studied and differences between treated and untreated mutant AVPR2s were examined in terms of therapeutic effects of chemical chaperones.

MATERIALS and METHODS

Expression Constructs and Cell Culture Studies

Using the site directed mutagenesis method as described in our previous studies, R68W, ΔR67_G69/G107W, ΔR67_G69, G107W, V162A, T273M, V88M, R106C, G12E mutations were introduced into the pLV2R plasmid [8]. All mutant constructs were confirmed by DNA sequencing.

COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) High Glucose with stable Glutamine and sodium pyruvate (Biowest SAS France facility) supplemented with 10% fetal bovine serum (FBS) South America originated, 100 U/ml penicillin and 10 µg/ml streptomycin, in 5% CO_2 in air, at 37°C. For transient transfection studies, cells were seeded 96-well plates for MTT-analysis and 48-well plates for cell surface ELISA, and were transfected by using TurboFect™ Transfection Reagent (Thermo Fisher Scientific) according to the manufacturer's protocol.

Chaperones and Chaperone Treatments

DMSO was from AppliChem (Cell culture grade, AppliChem GmbH). Glycerol and Curcumin (SIGMA-ALDRICH, USA) 1 mM stocks were prepared in cell culture grade DMSO. For all experiments, cells were grown in 5% CO_2 in air, at 37°C and 48 h after transfection cells were treated with chemical chaperones.

MTT Analysis

It was necessary to arrange the optimal treatment concentrations of the chaperones. For this purpose, we

performed the MTT assay and treated the transfected cells in the different concentrations of each chemical chaperones (concentrations can be seen in Table 1). All the options also carried out for 16h and 18h. After MTT assay, plates were read at 570 nm and reference wavelength of 630 nm using an ELISA reader (EZ Read 400 Micro-plate Reader, Biochrom).

ELISA Studies

AVPR2s were double tagged with N-terminal HA-tag and C-terminal FLAG-tag. Therefore, monoclonal anti-HA High Affinity, HRP-conjugated (Roche Applied Science, Mannheim, Germany) were used for the ELISA experiments. The protocol was described in our previous studies [8].

Statistical Analysis

Compared percentages of cell surface expressions of mutant proteins according to the wild type protein between treated and untreated groups are presented as mean ± SEM in the Figure 1. Differences between the mean of treated and untreated groups were analyzed using Mann-Whitney U test and the level of significance was taken as $p<0.05$ in all instances. For statistical analysis GraphPad Prism 5.01 for Windows (GraphPad Software) was used.

RESULTS

R68W, ΔR67_G69/G107W, ΔR67_G69, G107W, V162A, T273M, V88M, R106C, G12E mutations were introduced into pLV2R with a PCR-based site directed mutagenesis and restriction fragment replacement method in our previous studies [8]. Functional analyses studies of some of these mutant AVPR2s were published by our group [8]. Treatment concentration of the chaperones and the cell viability were analyzed by MTT assay. According to MTT analyses, for curcumin, 10^{-5} M; for DMSO, 0.5% and for glycerol, 2.5% treatment concentrations were decided to use for 16 h (Table 1). Results from 18 h treatment were not shown because cell death was obviously seen after the treatment.

Cell surface ELISA results were shown in Figure 1. According to cell surface ELISA results of our study, rescue potential of a chemical chaperone seems as both chemical compound and mutant specific. When we compare the results with untreated samples, we can see that for the mutants of ΔR67_G69, V162A, R106C and G12E, all chemical chaperones improved the mutant proteins cell surface expression by assisting the membrane trafficking. However, for the mutants of R68W, G107W, V88M we observed that only curcumin improved the cell surface activity. As it is seen in Figure 1, T273M is the worst mutant for the functionality and none of the chemical chaperones could assist the rescuing the mutant. ΔR67_G69/G107W is a compound hemizygous mutant and we can also observe that as T273M mutant, chemical chaperones did not work for this mutant. After chemical chaperone treatment, total expression of mutants was measured by sandwich ELISA and there was not a significantly difference between treated and untreated mutants for all chemical chaperones (Table 2).

We compared the treated samples with untreated samples for each chaperone. Within sight of Figure 1, we can say some chemical chaperones are successful to rescue of mutants but according to statistical analyses, only curcumin treatment with ΔR67_G69/G107W mutant showed a significantly difference from untreated samples (p: 0.0167).

DISCUSSION

Most of the cellular proteins gain their functions after the folding process and some molecules, called as molecular chaperones, are known to assist these processes. Generally, misfolded proteins are captured by protein quality control system in cells and eliminated by the system, since improper folding of a protein may lead to formation of protein aggregates which may toxic effects for the cell [2]. In this study, we analyzed chaperone response of several AVPR2 mutant proteins which functionality were determined in our previous studies [8]. As it is mentioned before, mutant AVPR2 proteins lead to Nephrogenic Diabetes Insipidus disease. Functionality of mutant V2 receptors at different levels affect the course of the disease. Several different chaperones were recently used to treat the conformational diseases such as NDI. In our study, we performed the DMSO, glycerol and curcumin. DMSO and glycerol are among the compounds which are known as osmolytes [9, 13]. Osmolytes may correct the folding defects by hydrating the unfolded proteins and affect the mutant protein's conformation either stabilizing it or creating a stress response which finally increase the amounts of molecular chaperones [14, 15]. These low-molecular-weight compounds were also reported as they have a healing potential for the conformational diseases by rescuing the function of mutant protein [9]. Ever since the

discovery the role of these kind of osmolytes, researchers were interested in the treatment of these compounds for conformational diseases. In a study about the amyloid-β assembly, TMAO and glycerol was found to be affected the amyloid formation [14]. In another study, glycerol treatment for a mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) were reported as stabilized immature mutant molecules [16]. After DMSO treatment, the LLC-PK1 cell line which express the CFTR, mutant CFTR were reported as increased as an amount and membrane-localized mutant CFTR is observed [17]. Treatment with chemical chaperones of V2R and AQP2 mutant proteins were also performed in several studies [15, 18-21]. In one of these studies, AQP2 mutants were shown to be properly targeted after treatment with glycerol [20]. Cheong and colleagues were observed that after treatment of the cells with DMSO and TMAO cell surface expression were restored for the mutant V2R proteins [19]. Also, Robben and colleagues were performed several chemical chaperones such as glycerol, DMSO, TMAO, thapsigargin, curcumin or ionomycin and they observed that rescue of a mutant protein is mutant specific [15]. Within the scope of curcumin acting mechanism, the exact mechanism of rescue potential of ER-retained mutant receptors has not been known yet. However, Robben and colleagues pointed out that curcumin could alter intracellular calcium levels which can cause inducing ER chaperones [15, 22], In this way, ER chaperones may act on ER-retained mutant AVPR2s to rescue. It is known that most of the ER chaperone polypeptides are proteins that bind calcium [23, 24]. Therefore, compounds like curcumin may affect the concentration of calcium in the ER. Then, calcium-dependent chaperoning can recognize misfolded protein. However, in another study, it was concluded that curcumin can bind mutant protein directly (because of its structure) instead of effecting calcium concentration and stabilize its three conformational structure to escape from ER quality control mechanism [25].

In our study, we also analyzed that mutant proteins are affected in different degrees and those effects depend on the mutation type. But we may conclude that in the mutants of ΔR67_G69, V162A, R106C and G12E, all three chemical chaperones were seemed to help the mutant protein to reach the plasma membrane and enable the functionality of the mutant proteins (Figure 1). As we discussed before, chemical chaperones may not bind the proteins. This explanation may be the reason of variability for the rescuing potential of the chaperones. In the mutant of T273M, which is the worst mutant protein for the functionality, we observed that none of the chaperones worked. Finally, according to our observations, curcumin seems as the most effective chaperone in terms of rescue potential for the mutant proteins in this study. The reason is could be because curcumin may bind directly to the mutant proteins instead of acting like other chemical chaperones. Curcumin could be better to rescue because both it may bind specifically to mutants and may affect ER chaperone proteins via calcium concentration. We believed that in addition to this study, different chaperone experiments of different kind of mutant receptors may help the therapeutic strategies for the conformational disease. In conclusion, we think that a chaperone could be successful about rescuing ER-retained mutant receptors if it could bind specifically to the mutant protein. Therefore, our future study will focus on understanding functional effects of different pharmacological chaperones on different mutants and showing their functionality via tracking their intracellular trafficking.

ACKNOWLEDGEMENTS

Plasmids of mutants and wild type AVPR2 were constructed through the experiments which were funded by TÜBİTAK SBAG112S513 and 216S304. MTT and ELISA experiments were performed with chemicals which were funded by TÜBİTAK SBAG 216S304. pLV2R plasmid is a kind gift from Dr. Angela Schulz, Rudolf Schönheimer Institute of Biochemistry, Faculty of Medicine, Leipzig University, Germany. There is no conflict of interest.

Table 1. MTT results of different concentrations of chemical chaperones. Results from three independent experiments (also, all independent experiments were performed triplicate) were given as mean±S.D. S.D: Standard deviation, conc: concentration, *: the chosen concentration.

Curcumin conc.	*10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M	10^{-9} M	0
Mean	99.35	97.05	95.42	94.61	91.01	100
S.D.	6.99	8.38	10.62	6.56	5.56	

DMSO conc.	0.01%	0.1%	0.25%	*0.5%	0.75%	1%	1.5%	2%	0
Mean	93.43	94.49	86.21	99.77	82.27	83.87	78.22	84.04	100
S.D.	26.68	8.09	11.17	19.41	12.00	12.13	12.76	4.93	

Glycerol conc.	0.04%	0.4%	1%	1.5%	2%	*2.5%	3%	3.5%	4%	4.5%	5%	0
Mean	65.00	69.37	72.37	66.80	73.47	74.90	61.06	61.59	48.44	74.05	56.86	100
S.D.	3.42	0.97	9.78	3.41	3.61	9.58	1.3	5.58	3.29	3.28	1.67	

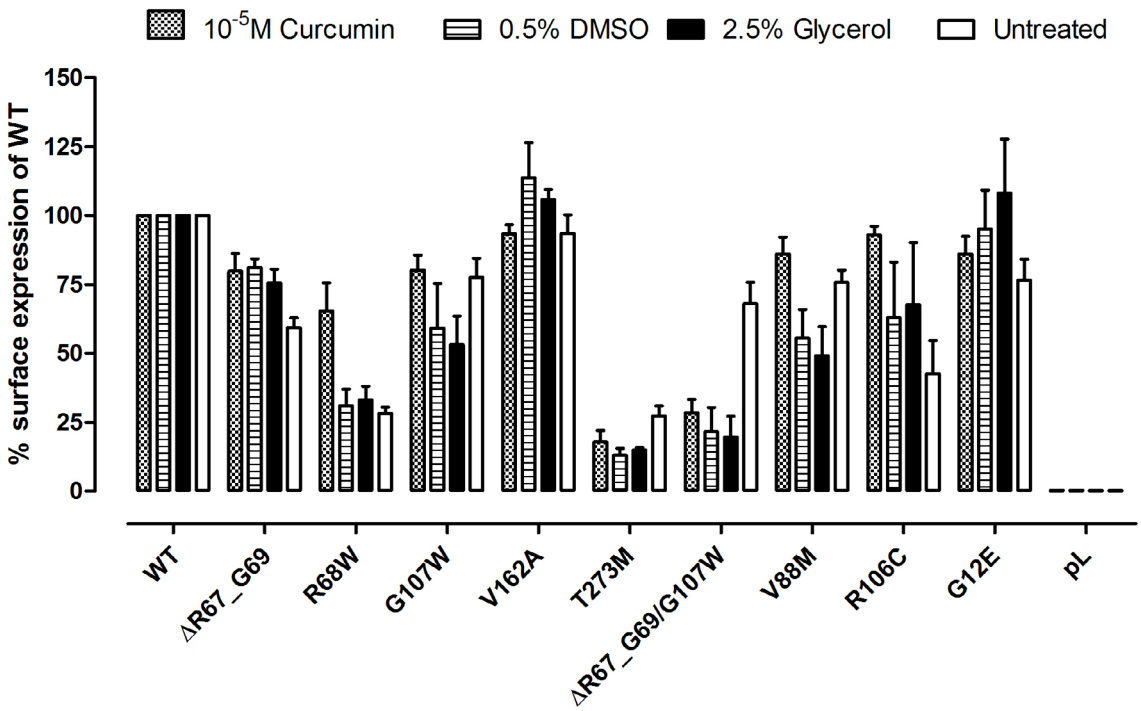

Figure 1. Cell surface expression of mutated proteins. WT: Wild type, pL: Plasmid without AVPR2 gene.

Table 2. Cell surface and sandwich ELISA were performed described as in Materials and Methods. For all experiments, the WT was set to 100% as a reference.The numbers in the parentheses indicate independent experiments. WT: Wild type, S.D.: Standard deviation, * Results are from our previous study (Erdem Tuncdemir et al., 2019).

	Cell Surface Expression (WT %)				Total Expression (WT %)			
	10^{-5} M Curcumin	0.5% DMSO	2.5% Glycerol	Untreated*	10 M Curcumin	0.5% DMSO	2.5% Glycerol	Untreated*
	Mean±S.D.	Mean±S.D.	Mean±S.D.	Mean±S.D.	Mean±S.D.	Mean±S.D.	Mean±S.D.	Mean±S.D.
WT	100 (3)	100 (3)	100 (3)	100 (4)	100 (3)	100 (3)	100 (3)	100 (5)
ΔR67_G69	79.9±11.1 (3)	81.2±5.5 (3)	75.6±8.8 (3)	59.3±9.4 (7)	89.4±3.2 (3)	74.2±5.2 (3)	94.9±16.8 (3)	82.0±23.2 (5)
R68W	65.3±17.9 (3)	30.9±10.5 (3)	33.2±8.6 (3)	28.3±6.0 (7)	85.6±4.3 (3)	79.8±4.9 (3)	99.0±10.7 (3)	85.6±25.4 (5)
G107W	80.2±9.4 (3)	59.1±28.2 (3)	53.2±18.0 (3)	77.6±18.6 (7)	92.8±11.3 (3)	79.9±5.8 (3)	94.8±9.8 (3)	90.4±11.0 (5)
V162A	93.3±5.8 (3)	113.8±22.0 (3)	105.8±6.3 (3)	93.6±17.9 (7)	86.2±3.7 (3)	92.4±9.3 (3)	117.2±22.8 (3)	88.4±20.6 (5)
T273M	17.8±7.3 (3)	13.1±4.1 (3)	15.0±1.4 (3)	27.3±9.5 (7)	90.9±5.4 (3)	80.8±4.1 (3)	105.1±16.7 (3)	91.6±16.3 (5)
ΔR67_G69/ G107W	28.4±8.5 (3)	21.6±15.3 (3)	19.6±13.4 (3)	68.0±20.5 (7)	98.9±2.4 (3)	71.9±2.7 (3)	78.8±4.6 (3)	94.0±15.4 (4)
V88M	86.1±10.7 (3)	55.6±17.9 (3)	49.2±18.2 (3)	75.7±7.8 (3)	85.8±5.4 (3)	90.2±3.1 (3)	110.9±26.2 (3)	123.0±21.5 (7)
R106C	92.9±5.6 (3)	62.9±35.2 (3)	67.7±39.0 (3)	42.7±24.0 (4)	103.5±4.6 (3)	107.2±8.2 (3)	130.8±33.1 (3)	95.4±19.2 (7)
G12E	86.2±11.0 (3)	95.3±24.2 (3)	108.2±33.9 (3)	76.6±15.3 (4)	80.0±3.4 (3)	82.5±4.6 (3)	103.5±14.5 (3)	84.8±16.7 (7)

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