

# The Effect of Aspartate-Lysine-Isoleucine and Aspartate-Arginine-Tyrosine Mutations on the Expression and Activity of Vasopressin V2 Receptor Gene

Hossein Najafzadeh<sup>1</sup>, Leila Safaeian<sup>\*2</sup>, Hamid Mirmohammad Sadeghi<sup>2</sup>, Mohammad Rabbani<sup>2</sup> and Abbas Jafarian<sup>3</sup>

<sup>1</sup>Dept. of Pharmacology and Toxicology, School of Veterinary Medicine, Shahid Chamran University, Ahvaz; <sup>2</sup>Dept. of Pharmacology and Toxicology, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan; <sup>3</sup>Isfahan Pharmaceutical Sciences Research Center, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran

Received 25 October 2009; revised 18 April 2010; accepted 19 April 2010

## ABSTRACT

**Background:** Vasopressin type 2 receptor (V2R) plays an important role in the water reabsorption in the kidney collecting ducts. V2R is a G protein coupled receptor (GPCR) and the triplet of amino acids aspartate-arginine-histidine (DRH) in this receptor might significantly influence its activity similar to other GPCR. However, the role of this motif has not been fully confirmed. Therefore, the present study attempted to shed some more light on the role of DRH motif in G protein coupling and V2R function with the use of site-directed mutagenesis. **Methods:** Nested PCR using specific primers was used to produce DNA fragments containing aspartate-lysine-isoleucine and aspartate-arginine-tyrosine mutations with replacements of the arginine to lysine and histidine to tyrosine, respectively. After digestion, these inserts were ligated into the pcDNA3 vector and transformation into *E. coli* HB101 was performed using heat shock method. The obtained colonies were analyzed for the presence and orientation of the inserts using proper restriction enzymes. After transient transfection of COS-7 cells using diethylaminoethyl-dextran method, the adenylyl cyclase activity assay was performed for functional study. The cell surface expression was analyzed by indirect ELISA method. **Results:** The functional assay indicated that none of these mutations significantly altered cAMP production and cell surface expression of V2R in these cells. **Conclusion:** Since some substitutions in arginine residue have shown to lead to the inactive V2 receptor, further studies are required to define the role of this residue more precisely. However, it seems that the role of the histidine residue is not critical in the V2 receptor function. *Iran. Biomed. J. 14 (1 & 2): 17-22, 2010*

**Keywords:** Mutation, Polymerase chain reaction, Vasopressin receptor

## INTRODUCTION

Vasopressin (8-arginine vasopressin, AVP) is a hypothalamic peptide hormone which acts on its target tissues via different G protein coupled receptors (GPCR) [1]. The vasopressin type 2 receptor (V2R) is positively coupled to adenylyl cyclase via G protein and its main function is anti-diuretic response by promoting water reabsorption in the kidney [2, 3]. A segment of GPCR in the second intracellular loop seems to

play an important role in the signal transduction pathways of these receptors [4, 5]. This is the highly conserved DRY (aspartate-arginine-tyrosine) motif (Asp/Glu-Arg-Tyr triplet) in GPCR which is DRH (Asp-Arg-His) in V2R [6, 7].

The results of various site-directed mutageneses have shown that this motif has an important role on cell surface delivery, internalization, phosphorylation, desensitization, ligand binding affinities and normal receptor function [8-11]. Diseases causing receptor mutations where these residues

\*Corresponding Author; Tel. (+98-311) 662 0406; Fax: (+98-311) 667 1780; E-mail: lsafaeian@yahoo.com; **Abbreviations:** vasopressin (AVP), vasopressin type 2 receptor (V2R), G protein coupled receptor (GPCR), aspartate-arginine-histidine (DRH), aspartate-arginine-tyrosine (DRY), nephrogenic diabetes insipidus (NDI)

have been replaced by other amino acids have been identified [12, 13]. One such mutation was identified in congenital nephrogenic diabetes insipidus (NDI) as R137H in this motif in V2R [14, 15]. This mutant receptor binds vasopressin with wild-type-like affinity but is unable to stimulate G protein activity.

Although various mutation studies have been conducted in other GPCR, only limited findings have been reported about the DRH motif in V2R [16-18]. It seems that the arginine residue in this motif is a key amino acid in signal transduction because its replacement with histidine residue has been associated with receptor signaling impairment in NDI; however, other mutations in this residue have not been studied. On the other hand, the last residue in DRY motif is less conserved in GPCR and has not been extensively studied among DRH sequence and in V2R.

Therefore, this study sought to conduct experiments to further investigate the role of arginine and histidine residues in V2R function and expression by using site-directed mutagenesis as aspartate-lysine-isoleucine (DKH) and DRY.

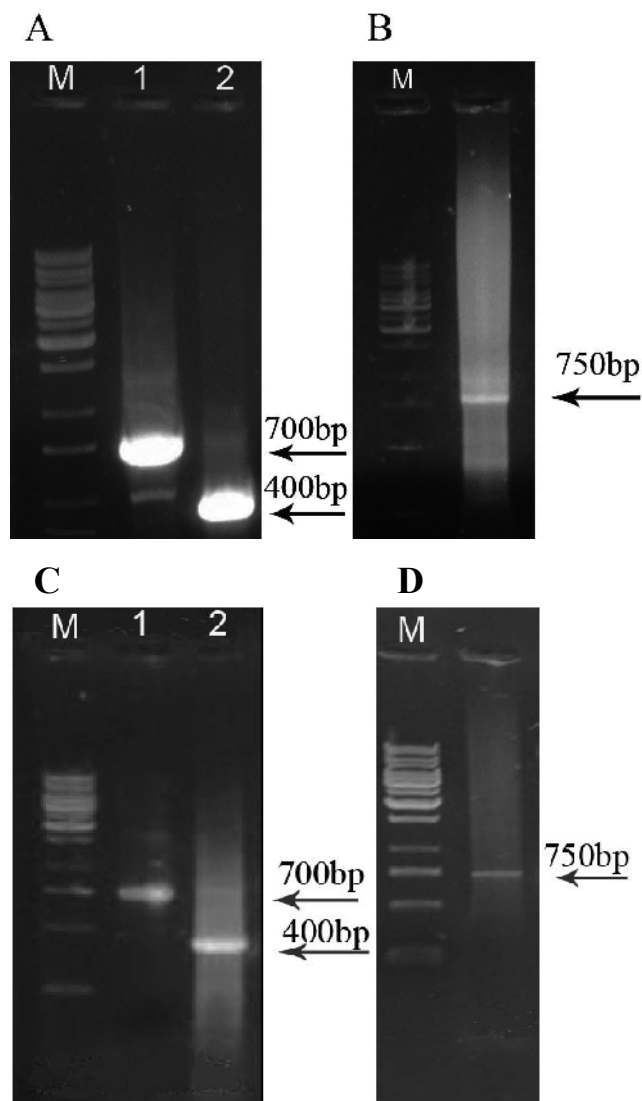
## MATERIALS AND METHODS

**Generation of plasmid constructs.** Using WDNASIS26 program, a pair of primers was designed for DKH and for DRY mutants. Two pairs of outer and inner sense and anti-sense primers in cDNA of V2R and pcDNA3 vector (Fermentas, Lithuania) were also designed. Primer sequences were designed as sense (5'-tgacaagcaccgtgccatctg-3') and anti-sense (5'-cagatggcagcgtgcttgc-3') for DKH mutant; sense (5'-gaccctaccgtgccatctgcc-3') and anti-sense (5'-ggcagatggcagcgttagcggtc-3') for DRY mutant; sense (ccagcaacagcagccag) and anti-sense (tcctcctcaca gtcttg) for inner in V2R; sense (ccaccactccgctgtgc) and anti-sense (accacagctcctcag) for outer in V2R; sense (gtggatagcggttgact) and anti-sense (ggccaaggaag gcacgg) for inner in pcDNA3; sense (tgggtggacta tttagc) and anti-sense (gttcttccgcctcaga) for outer in pcDNA3. In the first PCR, a mixture of 50 ng cDNA of V2R, which was prepared previously (as template) and 5 µl 10× PCR buffer containing 2 mM MgCl<sub>2</sub> (Biotools, B and M Labs., Spain), 0.5 mM dNTP (Eurobio Laboratories, France), 5 U DNA *Taq* polymerase (Biotools), 2.5 µM of each sense and anti-sense of DKH or DRY with outer primers in V2R in 2 steps (total volume of 50 µl) were set in a DNA thermocycler (Bio-Rad

Laboratories, USA). The first cycle of PCR was carried out at 94°C for 5 min and the next 33 cycles at 94°C for 1 min, 55°C for 2 min and 72°C for 2 min, and the last cycle at 72°C for 20 min. The PCR products were electrophoresed on agarose gel (0.7%) and the size of bands were determined. In the second (nested) PCR, a mixture of the first PCR products was used as template with each sense and anti-sense of inner primers in V2R as the same condition [19]. Nested PCR products were gel electrophoresed and confirmed by ethidium bromide using an UV transilluminator and the size of bands was determined. The products containing the desired mutation were digested by *NheI*, *BbrPI*, *EcoRI* and *XbaI* restriction enzymes (Fermentas, Lithuania). The advantage of using *NheI* and *BbrPI* enzymes was that they would produce a minimal length for the mutant inserts to be put into the vector. This would minimize the risk of having random mutations created by PCR procedure into the V2 receptor cDNA. DNA was extracted from the agarose gel by using QIAquick kit (Qiagen, CA, USA). The insert of each DKH or DRY mutant was ligated to pcDNA3 vector with a molar ratio of 3/1 (insert to vector). To have reliable constructs, some of the samples were sent to Kawsar Biotech Company (Iran) for sequencing. Transformation was performed using *E. coli* HB101 competent cells (Cinnagen, Iran) with heat shock method and plasmid was prepared by alkaline lysis procedure [20, 21].

**Cell culture and Transfection.** African green monkey kidney fibroblast cell line (COS-7 cells, Iranian National Cell Bank, Pasteur Institute of Iran, Tehran) were grown in DMEM containing 10% fetal bovine serum (Gibco, BRL Life Technologies, Scotland), penicillin (50 U/ml), and streptomycin (50 µg/ml) in an atmosphere containing 95% air and 5% CO<sub>2</sub> at 37°C. The cells were transiently transfected using the modified Luthman and Magnusson method as previously described [13].

**ELISA.** After transfection of COS-7 cells with the mutants or wild-type V2R containing human influenza hemagglutinin epitope at the C or N terminus, receptor expression was evaluated [16]. ELISA was performed using the 12CA5 monoclonal antibody (Sigma, USA) against N-terminal epitope of V2R as described [13]. Cells that were transfected without DNA and cells without transfection were used as negative controls. The optical density was



**Fig. 1.** First and nested PCR of DKH (A and B, respectively) and DRY (C and D, respectively) mutants at V2 receptor. Products of PCR were electrophoresed on 0.7% agarose gel. M, molecular weight marker; 250 bp unit.

measured at 492 nm in a microplate reader (Stat Fax, USA).

**Adenylyl cyclase activity assay.** The cellular cAMP was measured using a cAMP (direct) enzyme immunoassay kit (Assay Designs, USA). Forty eight hours after transfection, the cells were exposed to 100 nM vasopressin (AVP, Sigma), 2 mM isobutylmethyl-xanthine (Sigma) or 100  $\mu$ M forskolin (Sigma) for 20 min at 37°C [13, 15]. After rinsing with Hanks buffer and lysis by 0.1 M HCl, the cAMP was measured according to the manufacture's instruction by using a polyclonal antibody against cAMP. The generated yellow color was read on a microplate reader at 405 nm.

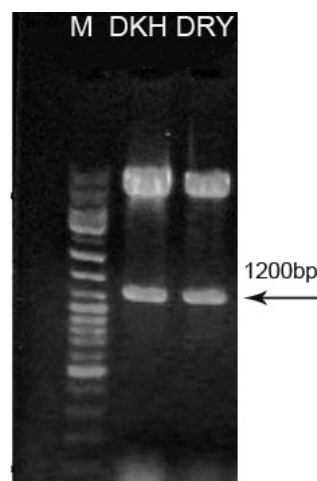
**Statistical analysis.** The data were presented as the mean  $\pm$  SEM. Statistical analysis was performed using Student's *t*-test or one-way analysis of variance (ANOVA) followed by Dunnett's test. *P* value < 0.05 was considered statistically significant.

## RESULTS

The products of the first and second (nested) PCR of DKH mutant are shown in Figure 1A and 1B. By using the sense primer of DKH and anti-sense of the outer primer in V2R, a sharp and dense band of approximately 700 bp was produced. In another step, by using anti-sense primer of DKH and sense outer primer in V2R, a sharp and dense band of 400 bp was observed (Fig. 1A). In the nested PCR, by using the above mentioned products and sense and anti-sense of inner primer in V2R, a band of 750 bp was detected (Fig. 1B). In addition, the same results were obtained when DRY was used (Fig. 1C and 1D).

To obtain the insert for ligation to pcDNA3 vector, the nested PCR was digested with *Xba*I and *Eco*RI enzymes. The band of approximately 1200 bp was isolated and used as insert. After plasmid preparation, pcDNA3 containing the wild-type V2R was digested by *Eco*RI and *Xba*I enzymes which removed the V2 receptor DNA (1200 bp) out of the pcDNA3 plasmid (5400 bp).

DKH and DRY inserts were ligated to the pcDNA3 vector and the products of ligation were transformed into HB101 cells. The colonies of bacteria were observed after an overnight culture on



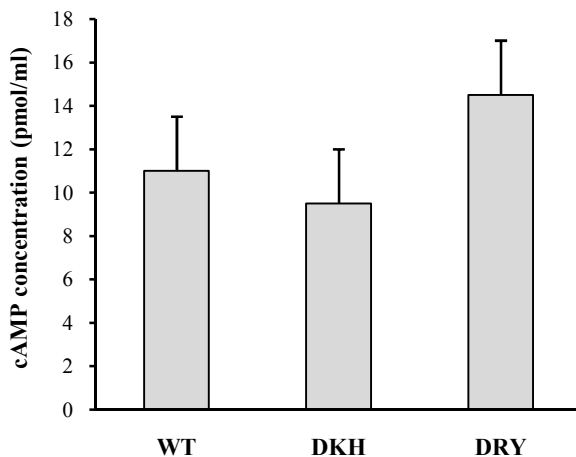
**Fig. 2.** Digestion of pcDNA3-DKH and -DRY plasmids by *Xba*I-*Eco*RI enzymes. Products of digestion were electrophoresed on 0.7% agarose gel. M, molecular weight marker, 100 bp unit.

an agar plate. The clones that were supposed to have uncut pcDNA3 containing DKH and DRY mutants were used for plasmid preparation. These plasmids were digested by *EcoRI* and *XbaI* enzymes and a 1200 bp band of V2 receptor cDNA was obtained (Fig. 2).

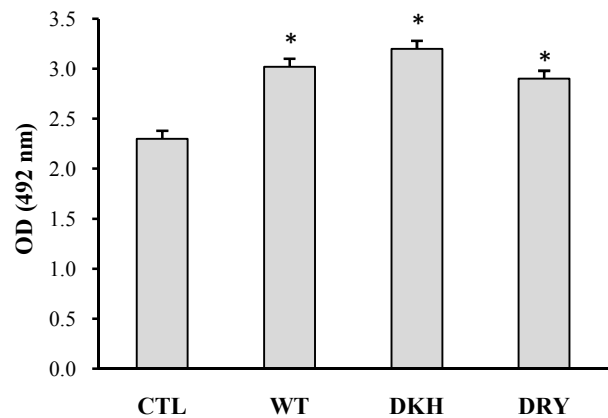
Functional characterization of DKH and DRY mutations on V2R signaling was evaluated by the adenylyl cyclase activity assay. The standard curve was generated using serial dilutions of standard solutions. The amount of cAMP was inversely related to optical absorbance. Production of cAMP was measured in COS-7 cells transfected with the wild-type and mutant V2R. There was a significant forskolin-induced production of cAMP in cells transfected with the wild-type receptor compared with untransfected cells ( $P > 0.05$ ).

However, the intracellular production of cAMP in cells transfected with the wild-type and mutant receptors was increased in the presence of vasopressin and no significant difference was observed among these groups (Fig. 3).

The results of ELISA for receptor expression assay are shown in Figure 4. There was no significant difference in the amount of receptor expression between the wild-type and mutant receptors.



**Fig. 3.** The effects of DKH and DRY mutations in V2 receptor on cAMP concentration (pmol/ml) in COS-7 cells. The cells were transfected with a plasmid containing wild-type cDNA of V2R (Wild-type; WT) or containing DKH and DRY mutants cDNA of V2R. All groups were treated with methylxanthine (2 mM) and vasopressin (100 nM) at 37°C for 20 min. Cells were placed at a density of  $5 \times 10^5$  cells per well in a 24-well plate and DNA amount of plasmids was 1000 ng. Data represent the mean  $\pm$  SEM (n = 20).



**Fig. 4.** The effects of DKH and DRY mutations in V2 receptor expression in COS-7 cells by ELISA assay. The cells were transfected without any plasmid (Control; CTL) or with a plasmid containing wild-type cDNA of V2R (Wild-type; WT) or containing DKH and DRY mutants cDNA of V2R. Cells were placed at a density of  $5 \times 10^5$  cells per well in a 96-well plate and DNA amount of plasmids was 1000 ng. Data represent the optical density as mean  $\pm$  SEM (n = 20), \* $P < 0.05$  vs. control.

## DISCUSSION

In this study, the DKH and DRY mutations were cloned into the pcDNA3 vector and functional assays showed no difference in the amount of cAMP production between the wild-type and the mutant V2 receptors. The V2 receptor expression assay by ELISA method, which allows quantification of receptors at the cell surface [10], also showed no changes in the presence of these mutations.

Recent studies suggest that DRY motif is an important determinant in the signal transduction of GPCR [22]. However, various site-directed mutageneses in this region have not revealed a universal role for this motif and its role in GPCR activation and signaling may be receptor and subtype specific [10]. In addition, much less is known about the role of this motif in V2 receptor.

The results of V2R studies have shown that mutation of aspartate residue (D136A) in DRH motif has increased the cAMP production by 5-folds. This mutation has lead to agonist-independent activation of the V2 receptor [16].

Although the function and expression of V2R have not changed by substitution of arginine residue with lysine in this study, there are different results in other researches. Mutations in arginine residue of the V2R have resulted in a decreased expression and loss of function and produce a constitutively

desensitized phenotype [17]. The R137H mutant which was present in a patient with congenital NDI binds vasopressin with the same affinity of wild-type receptor but fails to activate G protein [23]. The mutant R137W of V2 receptor is also unable to stimulate adenylyl cyclase activity [18].

It is important that mutations in arginine residue have been associated with different receptor activities. Unlike loss of function in R137H mutant in NDI patient, the replacement of arginine 131 by histidine in beta 2-adrenergic receptor has resulted in an increased agonist binding affinity preserving wild-type-like affinity [23]. The R131A mutant of CB2 cannabinoid receptor has been only partially reduced adenylyl cyclase activity [24]. It is possible that arginine could be substituted by other amino acids without loss of coupling to Gs demonstrating an unexpected lack of selectivity in some receptors [23].

The tyrosine residue is less conserved in the rhodopsin-like GPCR and has not been extensively studied among the DRY sequence [6]. Some studies have reported that variation of the Tyr residue in the DRY motif appears to be functionally tolerated [25-27]. In chemokine type 3 receptor, tyrosine 131 in DRY motif has been particularly sensitive to mutation as both Y131F and Y131S mutants were poorly expressed and were chemotactically inactive [28]. Mutations of Tyr124 in M1 muscarinic receptors have diminished the expression of antagonist binding sites [29]. Y143A mutant in AT2 angiotensin receptor has decreased binding to antagonist not to agonist [30]. Replacements of tyrosine (Y150A) in vasopressin V1a receptor has led to decreased signal transduction with little effect on ligand binding, signaling, and receptor internalization [10].

Functional relevance of these residues within DRY motif seems to be receptor and context-specific [7]; therefore, further studies are needed in each specific receptor to determine the role of individual residue.

In conclusion, the highly conserved DRY motif plays an important role in expression and function of GPCR, but may have different effects among various receptors. In V2 receptor, although substitution of the arginine residue with lysine did not alter the receptor function and expression, some substitutions have led to the inactive V2 receptor. Therefore, further studies are required to define the role of this residue more precisely. Regarding the histidine residue, its role is not critical by itself and its mutation did not affect the V2 receptor function and expression.

## ACKNOWLEDGMENTS

This study was financially supported by a research project No. 82262 from Isfahan University of Medical Sciences. We would like to thank Mrs. Moazen for her assistance in this project.

## REFERENCES

1. Birnbaumer, M. (2001) The V2 vasopressin receptor mutations and fluid homeostasis. *Cardiovasc. Res.* 51 (3): 409-415.
2. Schulein, R., Rutz, C. and Rosenthal, W. (1996) Membrane targeting and determination of transmembrane topology of the human vasopressin V2 receptor. *J. Biol. Chem.* 271 (46): 28844-28852.
3. Birnbaumer, M. (1999) Vasopressin receptor mutations and nephrogenic diabetes insipidus. *Arch. Med. Res.* 30 (99): 465-474.
4. Erlenbach, I., Kostenis, E., Schmidt, C., Serradeil-Le Gal, C., Raufaste, D., Dumont, ME., Pausch, MH. and Wess, J. (2001) Single amino acid substitutions and deletions that alter the G protein coupling properties of the V2 vasopressin receptor identified in yeast by receptor random mutagenesis. *J. Biol. Chem.* 276 (31): 29382-29392.
5. Aizaki, Y., Maruyama, K., Nakano-Tetsuka, M. and Saito, Y. (2009) Distinct roles of the DRY motif in rat melanin-concentrating hormone receptor 1 in signaling control. *Peptides* 30 (5): 974-981.
6. Rovati, G.E., Capra, V. and Neubig, R.R. (2007) The highly conserved DRY motif of class A G protein-coupled receptors: beyond the ground state. *Mol. Pharmacol.* 71 (4): 959-964.
7. Römpler, H., Yu, H.T., Arnold, A., Orth, A. and Schöneberg, T. (2006) Functional consequences of naturally occurring DRY motif variants in the mammalian chemoattractant receptor GPR33. *Genomics* 87 (6): 724-732.
8. Bowen-Pidgeon, D., Innamorati, G., Sadeghi, H.M. and Birnbaumer, M. (2001) Arrestin effects on internalization of vasopressin receptors. *Mol. Pharmacol.* 59 (6): 1395-1401.
9. Wilbanks, A.M., Laporte, S.A., Bohn, L.M., Barak, L.S. and Caron, M.G. (2002) Apparent loss-of-function mutant GPCRs revealed as constitutively desensitized receptors. *Biochemistry* 41 (40): 11981-11989.
10. Hawtin, S.R. (2005) Charged residues of the conserved DRY triplet of the vasopressin V1a receptor provide molecular determinants for cell surface delivery and internalization. *Mol. Pharmacol.* 68 (4): 1172-1182.
11. Kim, J.H., Cho, E.Y., Min, C., Park1, J.H. and Kim, K.M. (2008) Characterization of functional roles of DRY motif in the 2<sup>nd</sup> intracellular loop of dopamine

- D2 and D3 receptors. *Arch. Pharm. Res.* 31 (4): 474-481.
12. Birnbaumer, M. (1995) Mutations and diseases of G protein coupled receptors. *J. Recept. Signal Transduct. Res.* 15 (1-4): 131-160.
  13. Sadeghi, H.M., Innamorati, G. and Birnbaumer, M. (1997) An X-linked NDI mutation reveals a requirement for cell surface V2R expression. *Mol. Endocrinol.* 11 (6): 706-713.
  14. Rosenthal, W., Antaramian, A., Gilbert, S. and Birnbaumer, M. (1993) Nephrogenic diabetes insipidus. A V2 vasopressin receptor unable to stimulate adenylyl cyclase. *J. Biol. Chem.* 268 (18): 13030-13033.
  15. Innamorati, G., Sadeghi, H., Eberle, A.N. and Birnbaumer, M. (1997) Phosphorylation of the V2 vasopressin receptor. *J. Biol. Chem.* 272 (4): 2486-2492.
  16. Morin, D., Cotte, N., Balestre, M.N., Mouillac, B., Manning, M., Breton, C. and Barberis, C. (1998) The D136A mutation of the V2 vasopressin receptor induces a constitutive activity which permits discrimination between antagonists with partial agonist and inverse agonist activities. *FEBS Lett.* 441 (3): 470-475.
  17. Barak, L.S., Oakley, R.H., Laporte, S.A. and Caron, M.G. (2001) Constitutive arrestin-mediated desensitization of a human vasopressin receptor mutant associated with nephrogenic diabetes insipidus. *Proc. Natl. Acad. Sci. USA* 98 (1): 93-98.
  18. Oksche, A., Dickson, J., Schulein, R., Seyberth, H.W., Muller, M., Rascher, W., Birnbaumer, M. and Rosenthal, W. (1994) Two novel mutations in the vasopressin V2 receptor gene in patients with congenital nephrogenic diabetes insipidus. *Biochem. Biophys. Res. Commun.* 205 (1): 552-557.
  19. Sadeghi, H.M., Rabbani, M., Jafarian, A., Ghafghazi, T. and Najafzadeh, H. (2005) Site directed mutagenesis of V2 vasopressin receptor and its cloning using PGEM3Z vector. *Biotechnology* 4 (4): 284-287.
  20. Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1999) Short protocols in molecular biology. 4<sup>th</sup> ed., John Wiley & Sons Inc, New York, USA.
  21. Sambrook, J. and Russel, D.W. (2001) Molecular cloning (a laboratory manual). 3<sup>rd</sup> ed., Cold Spring Harbor Laboratory Press, Plainview, New York, USA.
  22. Alewijnse, A.E., Timmerman, H., Jacobs, E.H., Smit, M.J., Roovers, E., Cotecchia, S. and Leurs, R. (2000) The effect of mutations in the DRY motif on the constitutive activity and structural instability of the histamine H2 receptor. *Mol. Pharmacol.* 57 (5): 890-898.
  23. Seibold, A., Dagarag, M. and Birnbaumer, M. (1998) Mutations of the DRY motif that preserve beta 2-adrenoceptor coupling. *Recept. Channels* 5 (6): 375-385.
  24. Rhee, M.H., Nevo, I., Levy, R. and Vogel, Z. (2000) Role of the highly conserved Asp-Arg-Tyr motif in signal transduction of the CB2 cannabinoid receptor. *FEBS Lett.* 466 (2-3): 300-304.
  25. Wess, J. (1998) Molecular basis of receptor/G-protein-coupling selectivity. *Pharmacol. Ther.* 80 (3): 231-264.
  26. Ohyama, K., Yamano, Y., Sano, T., Nakagomi, Y., Wada, M. and Inagami, T. (2002) Role of the conserved DRY motif on G protein activation of rat angiotensin II receptor type 1A. *Biochem. Biophys. Res. Commun.* 292 (2): 362-367.
  27. Gaborik, Z., Jagadeesh, G., Zhang, M., Spat, A., Catt, K.J. and Hunyady, L. (2003) The role of a conserved region of the second intracellular loop in AT1 angiotensin receptor activation and signaling. *Endocrinology* 144 (6): 2220-2228.
  28. Auger, G.A., Pease, J.E., Shen, X., Xanthou, G. and Barker, M.D. (2002) Alanine scanning mutagenesis of CCR3 reveals that the three intracellular loops are essential for functional receptor expression. *Eur. J. Immunol.* 32 (4): 1052-1058.
  29. Lu, Z.L., Curtis, C.A., Jones, P.G., Pavia, J. and Hulme, E.C. (1997) The role of the aspartate-arginine-tyrosine triad in the m1 muscarinic receptor: mutations of aspartate 122 and tyrosine 124 decrease receptor expression but do not abolish signaling. *Mol. Pharmacol.* 51 (2): 234-241.
  30. Moore, S.A., Patel, A.S., Huang, N., Lavin, B.C., Grammatopoulos, T.N., Andres, R.D. and Weyhenmeyer, J.A. (2002) Effects of mutations in the highly conserved DRY motif on binding affinity, expression, and G-protein recruitment of the human angiotensin II type-2 receptor. *Mol. Brain Res.* 109 (1-2): 161-167.