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Regulation of intraocular pressure in mice: Structural analysis of dopaminergic and serotonergic systems in response to cabergoline

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ABSTRACT

Elevated intraocular pressure (IOP) is the main recognized risk factor of glaucoma. To investigate the contribution of dopaminergic and serotonergic systems in IOP regulation, we used cabergoline, a mixed dopamine and serotonin agonist, in C57BL/6J WT and dopamine D_3 receptor knock-out ($D_3R^{-/-}$) mice with normal eye pressure or steroid-induced ocular hypertension. Furthermore, we studied the structural basis of the cabergoline-mediated activation of the dopaminergic and serotonergic systems by molecular modeling. Topical application of cabergoline, significantly decreased, in a dose-dependent manner, the intraocular pressure in WT mice, both in an ocular normotensive group (−9, −5 and −2 mmHg with 5%, 1%, and 0.1%, respectively) and an ocular hypertensive group, with a prolonged effect in this latter group. No change of intraocular pressure was observed after topical application of cabergoline in $D_3R^{-/-}$ mice. We modeled and optimized, with molecular dynamics, structures of hD_3, $h5HT_{1A}$ and $h5HT_{2A-C}$ receptors; thereafter we carried out molecular docking of cabergoline. Docking revealed that binding of cabergoline into D_3 and $5HT_{1A}$ receptors is associated with a better desolvation energy in comparison to $5HT_{2A-C}$ binding. In conclusion, the present study support the hypothesis that dopaminergic system is pivotal to regulate IOP and that D_3R represents an intriguing target in the treatment of glaucoma. Furthermore, the structure-based computational approach adopted in this study is able to build and refine structure models of homologous dopaminergic and serotonergic receptors that may be of interest for structure-based drug discovery of ligands, with dopaminergic selectivity or with multi-pharmacological profile, potentially useful to treat optic neuropathies.

1. Introduction

Elevated intraocular pressure (IOP) is the main recognized risk factor of glaucoma, a progressive optic neuropathy, which is a prominent cause of blindness in industrialized countries. The increase of IOP is due to two principal impaired physiologic mechanisms: dysfunctional outflow of aqueous humor due to abnormalities of the drainage system of the anterior chamber angle of the eye and the limited access of aqueous humor to the drainage system. The first dysfunction leads to the primary open angle glaucoma (POAG), the second one is the cause of the angle closure glaucoma (ACN). POAG represents the most frequent form of glaucoma, and epidemiologic studies demonstrated that the risk of POAG increases by 12% for each increment of 1 mmHg in IOP [1]. National Institute for Health and Care Excellence (NICE, http://guidance.nice.org.uk/CG85/Guidance) recommends a stepwise treatment algorithm for glaucoma, where the initial step is the pharmacological reduction of IOP, followed by laser surgery of the trabecular meshwork and glaucoma-filtering surgery.

We know that several systems are involved in IOP regulation, including adrenergic, cholinergic, purinergic, serotonergic, and dopaminergic [2]. The role of this latter is still unclear, though it represents one of the most intriguing systems implicated in the modulation of IOP both in physiological and pathological conditions. The involvement of the serotonergic and dopaminergic systems in regulation of IOP has been recently investigated in more details using several pharmacological tools and new paradigms [3–5]. However, the precise role of the two systems, and in particular the relative magnitude of the effect of each single system in the regulation of IOP is still evanescent.

Some compounds, such as cabergoline, are serotonergic and dopaminergic ligands and are able to decrease IOP with a mechanism that remains uncertain. Cabergoline is an ergot derivative approved for hyperprolactinemia and Parkinson's disease; it is a potent dopamine receptor agonist on D_2 and D_3 subtypes, and it also possesses significant affinity for serotonin receptors such as $5HT_{1A}$, $5HT_{2A-B-C}$ [6,7]. Cabergoline has been

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shown to decrease IOP in different species [3]. To investigate the contribution of dopaminergic and serotonergic systems in ocular hypotensive mechanisms we used cabergoline in wild-type (WT) and dopamine D_3 receptor knock-out mice ($D_3R^{-/-}$) both ocular normotensive and ocular hypertensive. Furthermore, we studied the structural basis of the cabergoline-mediated activation of the dopaminergic and serotonergic systems, by molecular modeling, using the same approach that we previously applied in optimization and validation of structure models of D_3 and D_2 receptors [8].

2. Material and methods

2.1. In vivo *studies*

2.1.1. Animals

C57BL/6J $D_3R^{-/-}$ and WT littermates (male 8–12 weeks old) mice were used in this work. The animals were fed with standard laboratory food and were allowed free access to water in an air conditioned room with a 12-h light/12-h dark cycle. The experimental procedures were performed during the light cycle. $D_3R^{-/-}$ mice, used in these experiments, were 10th–12th generation of congenic C57BL/6J mice, and generated by a backcrossing strategy as reported by Accili et al. [9]. The genotypes of the dopamine D_3 receptor mutant and WT mice were identified by a PCR method with two pairs of primers flanking either exon 3 of the wild-type dopamine receptor D_3 or the phosphoglycerate kinase 1 gene promoter cassette of the mutated gene [9]. All the animals were treated according to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research, and the Directive 2010/63/EU of the European Parliament and of the Council.

2.1.2. IOP measurement

IOP (mmHg) was measured before and after the drug treatment using a Tono-Lab tonometer (Icare, Espoo, Finland). IOP was measured both in the treated and the contralateral eye. Three baseline readings were taken 60, 30 and 0 min before the drug administration. IOP determination was made 30, 60, 120, 180 and 240 min after the topical application of the drug in the right eye. IOPs of animals were measured between 10 a.m. and 12.30 p.m. All measurements, under the same environmental conditions, were made by the same operator blind to treatment.

2.1.3. Treatments

Cabergoline hydrochloride, U99194A maleate, hydroxypropylmethylcellulose (HPMC; viscosity 80–120 cP), and polysorbate 80 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Pharmacological treatments were performed after a baseline IOP measurement, defined as three baseline readings. Fresh aqueous formulation of carbergoline (0.01, 0.1, 1 and 5% w/v) containing 0.5% HPMC, 0.05% polysorbate 80, 0.2% PBS, and 0.75% NaCl, was prepared (pH 7.2) in order to optimize the drug's residence time [10]. Cabergoline formulations were unilaterally instilled in the right eye in a volume of 10 μl. In each experimental group, animals received either drug or the appropriate vehicle. The animals were randomly assigned to treatment groups ($n = 6–7$) and were used only once.

2.1.4. Animal model of steroid-induced ocular hypertension

Steroid-induced hypertension was done as previously described by Bucolo et al. [5]. Briefly, Alzet micro-osmotic pumps (Model 1004, DURECT Corp., Cupertino, CA, USA) were filled with water-soluble dexamethasone (drug/cyclodextrin complex; cod. n. D2915 Sigma-Aldrich) in sterile PBS or with PBS alone (sham). Dexamethasone (DEX) was formulated at a concentration of 34.5 mg/ml. The flow rate for the micro-osmotic pumps was 0.11 μl per h, which delivers 0.09 mg of DEX per day. Animals were anesthetized with tiletamine hydrochloride and zolazepan hydrochloride (Zoletil 100®, Virbac, Milan, Italy), a small incision was made midline at the base of the scapula to obtain a small subcutaneous pocket along the side of the animal; pumps were placed into the pocket with the flow moderator pointed posterior to the surgical site. Tissue bond adhesive was placed on the surgical wound and allowed to dry. Mice were then single housed and placed on a heating pad to recover. Three baseline readings of IOP were taken in a slot between 8 and 11 a.m. one day prior to the pump implantation surgery, and the day of implantation. Weekly IOP measurements, at the same day and time, were carried out to assess the onset of DEX-induced elevation in IOP. The final IOP was obtained at comparable times during the fourth week following surgery.

2.2. In silico *studies*

2.2.1. Protein sequence alignment

We retrieved full sequences of the human serotonergic receptors from the Protein-NCBI database (www.ncbi.nlm.nih.gov/protein/), the accession numbers of the analyzed human serotonergic receptors are respectively: $5HT_{1A}$ [NP_000515.2]; $5HT_{1B}$ [NP_000854.1] $5HT_{2A}$ [NP_000612.1]; $5HT_{2B}$ [NP_000858.3] and $5HT_{2C}$ [NP_000859.1]. The accession number of human D_3 receptor is NP_000787.2. Multiple sequence alignment of receptors were carried out with CLUSTAL W [11] using the BLOSUM62 matrix, the alignments were further analyzed with JALVIEW v2.7 [12].

2.2.2. Structure modeling of receptors

The structure models of the human serotonergic receptors were obtained by a threading modeling approach. We used the GPCRRD-ITASSER modeling facility [13] (http://zhanglab.ccmb.med.umich.edu/GPCRRD/) through which the threading method ITASSER is guided by the experimental restrains of GPCR Restrain Database (GPCRRD). The GPCRRD-ITASSER automatic pipeline uses at first the LOMETS threading program in order to identify the putative related template structures in the Protein Data Bank. When significant template alignments are not found, an *ab initio* transmembrane helix folding program is used to build the seven transmembrane bundle from scratch. Meanwhile, GPCRRD is searched for experimental restraints. Then a replica exchange Monte Carlo simulation is carried out to search the conformation space restricted by all the previous steps. The final atomic structure model is rebuilt by fragment guided-molecular dynamics (FG-MD). The output of GPCRRD-ITASSER provides six models per each analyzed protein; we selected the model with the best Ramachandran plot for further refinements. We carried out an *in vacuo* energy minimization (1000 steps of steepest descent algorithm, using NAMD with CHARMM27 force field) of output models in order to model canonical and "accessory" disulfide bridges. The accessory disulfide bridge of serotonergic receptors was individuated by sequence alignment. We created two sets of models, termed: 1_disu receptors with the canonical disulfide bridge, and 2_disu receptors with both canonical and accessory bridges. These models were then optimized by molecular dynamics simulation in a water-membrane environment.

2.2.3. Molecular dynamics refinement

Structure models of receptors were embedded in a palmitoyl-oleoyl phosphatidyl choline (POPC) bilayer, the orientation of each protein was guided by the output of the Orientations of Proteins in Membranes (OPM) database (http://opm.phar.umich.edu/). The systems were hydrated with TIP3P water molecules,