A GPR119 Signaling System in the Murine Eye Regulates Intraocular Pressure in a Sex-Dependent Manner

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Cannabinoid receptors are the endogenous target of the psychoactive ingredients of marijuana and hashish.1 The first cannabinoid receptor CB1,2 was cloned in the early 1990s. A few years thereafter, the lipid 2-arachidonoyl glycerol (2-AG) was found to bind and activate these receptors.3 However, the endogenous role of other structurally related acylglycerols, such as 2-oleoylglycerol (2-OG), long remained elusive. Although these lipids are present in the brain,4 they have been considered until recently “orphan” ligands, entourage products, or even by-products of lipid metabolism.5

The human GPR119 receptor was cloned in 2003,6 and was found to contain 335 residues encoded by a single-exon gene located on the X chromosome.6,7 GPR119 is part of the MECA (melanocortin, endothelial differentiation gene, cannabinoid, adenosine) receptor cluster. Cannabinoid receptors are among its closest relatives,6,8 accounting for interest in GPR119 as a potential orphan receptor for endogenous lipids with endocannabinoid-like structures. Initial studies of GPR119 distribution suggest a relatively limited expression pattern: mRNA transcripts of hGPR119 and rGPR119 are found chiefly in pancreas and intestinal tissues.9–11 although rGPR119 also has been detected in some brain regions.9,11–13 To our knowledge, there have been no studies of GPR119 expression in the eye.

Overexpression of GPR119 constituively increases intracellular cAMP consistent with Gs coupling.9 Candidate GPR119 agonists increase cAMP, stimulate adenylyl cyclase, and enhance protein kinase A activity in GPR119-expressing cells.9,10,12–14,15 GPR119-mediated responses also may involve ATP-sensitive K+ and voltage-dependent Ca2+ channels.16 Because of the phylogenetic relationship between GPR119 and cannabinoid receptors, lipids structurally related to endocannabinoids were tested early as potential GPR119 ligands. Overton and colleagues12 found that acylethanolamines induced responses in GPR119-overexpressing cells, with oleoylethanolamide (OEA) being the most potent, with EC50s (concentration that gives half-maximal response) in the low micromolar range, followed by palmitoyl-lethanolamide (PEA), and anandamide (AEA). OEA also stimulated cAMP production in cell lines expressing GPR119, but not in cells lacking GPR119.10,12 More recently, 2-OG also has been proposed as an endogenous ligand for GPR119.17

Elevated IOP is implicated in most forms of glaucoma, a disease responsible for millions of cases of blindness worldwide. Multiple treatments are available for glaucoma, but all patients are not responsive to these drugs, and may develop tolerance or side effects in the course of treatment. There is, therefore, a need for additional classes of drugs. We now report that a GPR119-based signaling system exists in the eye and that activation of this system lowers IOP in a sex-dependent manner.

METHODS

All procedures used in this study were approved by the Animal Care Committee of Indiana University and the National Cheng
Kung University and conform to the Guidelines of the National Institutes of Health on the Care and Use of Animals. Adult mice (>5 weeks, of either sex, from breeding colony) were housed under a 12/12-hour day/night cycle. GPR119 knockout animals were obtained from the University of California Davis Knockout mouse project. Animals were used in adherence to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Cow eyes were obtained from healthy animals of indeterminate sex from a local farm that also houses a slaughtering facility. Eyes were obtained within several hours of the slaughter of the animals.

**PCR and Quantitative PCR**

For polymerase chain reaction (PCR) experiments, we designed two different primers against mouse GPR119 gene (mGPR119a and mGPR119b) and one primer against bovine GPR119 gene (bGPR119) (see below). β-Actin is a housekeeping gene used as an internal control. Expression of mRNAs was measured as an internal control for each experimental condition. Total RNA was isolated from male and female anterior eyes or from bovine corneal epithelium, corneal endothelium, retina, and trabecular meshwork using Trizol reagent (Life Technologies, Grand Island, NY, USA) and RNeasy Kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions. RTPCR was carried out in two steps. The first strand DNA was made using High-Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA, USA) using 400 ng RNA in a 40-μL reaction. PCR was performed following the AmpliTaq 360 DNA Polymerase Protocol (Applied Biosystems). An amount of 1 μL respective mouse male/female anterior eye or bovine ocular tissue cDNA was added into a 25-μL PCR reaction that was processed through 40-cycle amplification. PCR products were examined on 1% agarose gel stained with ethidium bromide.

**Immunohistochemistry**

For immunocytochemistry, after animals were euthanized, their eyes were removed, and the anterior or posterior eye section cut away, forming a posterior or anterior eyecup. For immunocytochemistry, the eyecup was fixed in 4% paraformaldehyde followed by a 30% sucrose immersion for 24 to 72 hours at 4°C. Tissue was then frozen in optimum cutting temperature compound and sectioned (20–50 μm) using a Leica CM1850 cryostat (Leica Microsystems, Wetzlar, Germany). Tissue sections were mounted onto Superfrost-plus slides, washed, preblocked with SEABLOCK (Thermo Fisher Scientific, Waltham, MA, USA), and treated with a detergent ( Triton X-100, 0.5% or saponin, 0.1%) followed by primary antibodies to 1 to 3 days at 4°C. Secondary antibodies (Alexa 405, 488, 594 or 647, 1:500; Invitrogen, Inc., Carlsbad, CA, USA) were subsequently applied at room temperature for 1.5 hours or at 4°C for 1 to 2 days. To enhance specificity of the antibodies, they were preincubated with GPR119 KO tissue for 24 hours at 4°C.

Images were acquired with a Leica TCS SP5 confocal microscope using Leica LAS AF software and a ×63 oil objective. Images were processed using ImageJ (http://im age.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA) and/or Photoshop (Adobe Systems, Inc., San Jose, CA, USA). Images were modified only in terms of brightness and contrast.

**Antibody Generation**

For the current study, we generated rabbit polyclonal antibodies for human GPR119 and characterized their specificity as described below. The antibody is directed to an epitope in the third intracellular loop of the receptor (amino acids: 200–225; residues: RKMEHAGAMA GAYRPPRSVN DFKAVR). For GPR119, a GST fusion protein expression construct was produced by inserting the DNA coding for the described region. Each fusion protein was purified from BL21 Escherichia coli lysates on a glutathione Sepharose column and the cocktail mixture was injected into two rabbits to generate antisera (Cocalico Biologicals, Inc., Reamstown, PA, USA) using standard approaches. The antisera was purified in two steps, first by exclusion on a GST column and then by binding to and elution from an affinity column made with the GPR119 fusion protein.

**IOP Measurements**

IOP was measured in mice by rebound tonometry, using a Tonolab (icare Finland Oy, Helsinki, Finland). This instrument uses a light plastic-tipped probe to briefly make contact with the cornea; after the probe hits the eye, the instrument measures the speed at which it rebounds to calculate IOP. To obtain reproducible IOP measurements, mice were anesthetized with isoflurane (5% induction). The anesthetized mouse was then placed on a platform in a prone position, where anesthesia was maintained with 3% isoflurane. Baseline IOP measurements are taken in both eyes. A “measurement” consisted of the average value of six readings. A minimum of three measurements were taken for each time point. One eye was then treated with drug and the other eye was treated with vehicle. We dissolved 2-OG to a 100-mM stock in ethanol, then diluted to 5 mM in Tocrisolve, a soya-based solvent. An amount of 5 μL 5 mM 2-OG was applied topically after which the drug was again anesthetized as above. IOP was then measured in the drug-treated and vehicle-treated contralateral eye. We typically used 8 to 12 animals per group. Importantly, we noted that for both 2-OG and MBX2982, effectiveness drops off once the drug is dissolved. We therefore used 2-OG and MBX2982 within a week of dilution.

IOP measurements following drug administration were analyzed by paired t-tests comparing the drug-treated eye with the vehicle-treated eye of the same animal.
Lipid Extraction

Mice were killed via cervical dislocation and both eyes were immediately removed and placed in an Eppendorf tube on dry ice. The eyes were then stored at −80°C. To begin the lipid extraction, samples were shock frozen in liquid nitrogen, which allowed them to be easily removed from the Eppendorf tube and weighed before being transferred to a 15-mL centrifuge tube. The mass of the largest sample was multiplied by 50 to determine how many milliliters of HPLC-grade methanol (Avantor Performance Materials, Inc., Center Valley, PA, USA) was to be added to the centrifuge tube. Then, 5 μL vortexed 1 μM deuterium-labeled N-arachidonoyl glycine (d8NAGly) (Cayman Chemical, Ann Arbor, MI, USA) was added to each test tube to serve as an internal standard. The spiked tubes were covered with Parafilm and were allowed to sit in the covered ice bucket for 2 hours. The eyes were then briefly homogenized using a sonicator (VirTis, Gardiner, NY, USA). Then, samples were spun in a centrifuge at 19,000 g for 20 minutes at 20°C.

After centrifugation, supernatant was poured from the centrifuge tubes into 15-mL polypropylene tubes. Enough HPLC H2O (EMD Milipore Corporation, Billerica, MA, USA) to make a 75:25 water-to-organic solution was added to the supernatant. To partially purify the supernatant/water solution, solid-phase extraction columns were used. One solid-phase 500-mg C18 extraction cartridge (Agilent Technologies, Lake Forest, CA, USA) for each tube of extract was inserted into a Preppy vacuum manifold apparatus located in a fume hood. To activate the hydrophobic carbon chains in the column, 5 mL HPLC methanol was added to each column. When the methanol almost reached the bottom of the columns, 2.5 mL HPLC H2O was added to the columns to activate the polar silica in the columns. When the water had run through the column, the supernatant/water solution was added and allowed to slowly drip through the column. After the solution had eluted, another 2.5 mL HPLC H2O was added to the columns to wash off impurities. Then, 1.5 mL 40% methanol was added to the column to wash off more impurities. The 40% methanol was allowed to completely elute and any eluate in the collector vials was discarded. The collector vials were then replaced with labeled autosampler vials (Perkin Elmer, Waltham, MA, USA) that corresponded to each sample. A series of four elutions with 1.5 mL 60%, 75%, 85%, and 100% methanol as the eluting solvent was performed to partially purify the lipids being measured. More polar lipids, such as PGE2 or PGF2α, were purified in the 60% and 75% elutions. On the other hand, lipids such as 2-AG and AEA were purified in the 85% elution. Vials of eluants were stored in the −80°C freezer until they were ready for analysis.

HPLC/Mass Spectrometry/Mass Spectrometry

Methods for HPLC/mass spectrometry/mass spectrometry (HPLC/MS/MS) have been previously validated, including in murine eye tissue. Samples were analyzed using an Applied Biosystems API 3000 triple quadrupole mass spectrometer (Applied Biosystems Sciex, Framingham, MA, USA) with electrospray ionization. Levels of each compound were determined by running each sample using a multiple reactions monitoring method tailored for each amide family of compounds. To chromatograph the samples, an XDB-C18 (Agilent Technologies) reversed-phase HPLC analytical column was used, which was kept at 40°C by a column oven (HP, Palo Alto, CA, USA). Two different types of mobile phase were used. Mobile phase A consisted of 20%/80% (vol/vol) methanol/water and 1 mM ammonium acetate (Sigma-Aldrich Corp., St. Louis, MO, USA). Mobile phase B instead contained 100% methanol with 1 mM ammonium acetate. Every method run began with 0% mobile phase B, reached a state of 100% mobile phase B flowing at 0.2 mL per minute, and gradually returned to 0% mobile phase B. Before running batches of samples, the ionization source was allowed to reach its operating temperature of 500°C and every vial warmed to room temperature and was vortexed for approximately 30 seconds.

Analysis of the HPLC/MS/MS data was performed using Analyst software (Applied Biosystems Sciex). Chromatograms were generated by determining the retention time of analytes with a [M−1] or [M+1] parent peak and a fragmentation peak corresponding to the programmed values. The retention time was then compared with the retention time of a standard for the suspected compound. If the retention times matched, then the concentration of the compound was determined by...
calculating the area under the curve for the unknown and comparing it with the calibration curve obtained from the standards. Extraction efficiency was calculated with the d8NAGly spiked recovery vial as a standard. Concentrations in moles per gram adjusted for percent recovery from the knockout animals were compared with wild-type concentrations using a 1-way ANOVA. All statistical tests were carried out using SPSS Statistics 20 (IBM, Armonk, NY, USA). Statistical significance was defined as $P \leq 0.05$ and a trending effect was defined as $0.05 < P \leq 0.10$.

**Drugs**

The 2-OG was obtained from Cayman Chemical (Ann Arbor, MI, USA); OEA and Tocrisolve were obtained from Tocris (Ellisville, MO, USA), and MBX2982 and APD668 were obtained from Medchemexpress (Monmouth Junction, NJ, USA).

**RESULTS**

**GPR119 Receptor Message Is Detected in Anterior Murine Eye**

We tested for GPR119 mRNA expression using RT-PCR in murine anterior eye. We detected expression of message for this X-linked gene (Fig. 1A) and found using qPCR that it was more pronounced in females than in males (Fig. 1B; in males [double delta cycle threshold [CT] ± SEM]: $1.96 ± 0.03$; in
females: 2.09 ± 0.03, n = 4, P < 0.01 by unpaired t-test).

Because the mouse eye is too small to reliably excise specific tissues of interest, such as trabecular meshwork, we additionally tested in bovine tissue to obtain tissue-specific samples. Testing in bovine retina, corneal epithelium, corneal endothelium, and trabecular meshwork, we detected expression of GPR119 in each tissue type (Fig. 1B).

**GPR119 Protein Is Associated With Corneal Endothelium and Ciliary Body**

We developed an antibody against GPR119 to test for protein expression in murine anterior eye. Using GPR119 knockout tissue as a control, we determined that the antibody stains several structures of interest, such as trabecular meshwork, we additionally tested in bovine tissue to obtain tissue-specific samples. Testing in bovine retina, corneal epithelium, corneal endothelium, and trabecular meshwork, we detected expression of GPR119 in each tissue type (Fig. 1B).

**Lipid Species Vary by Genotype but Only 2-OG Varies by Sex in GPR119 Knockouts**

In a separate series of experiments, we tested for alterations in levels of a panel of endocannabinoid-related lipids. Although most attention has focused on the canonical cannabinoids, the body produces a range of related lipids. What function, if any, these might have is still largely an open question. We tested two sets of conditions: wild-type female versus GPR119 knockout female and GPR119 knockout female versus GPR119 knockout male. We examined a panel of 32 lipids listed in Supplementary Table S1; 24 members of this panel included the oleoyl-, arachidonoyl-, palmitoyl-, stearoyl-, linolenoyl-, and docosahexaenoyl-based acylethanolamines, acylGABAs, acylglycines, and acylserines. The list additionally included linoleic and arachidonic free fatty acids, three acylglycerols (2-AG and 2-OG as well as 2-linolenoyl-sn-glycerol [2-LG]), the prostaglandin metabolites PGE2 and PGF2α, and N-arachidonoyl taurine. For these experiments, we used whole murine eye to enhance reproducibility for this larger lipid panel. We found that levels of only one lipid changed measurably in female versus male knockouts. Interestingly that lipid, with a 2.7-fold elevation in females, was 2-OG, which has also been proposed to serve as an endogenous agonist at GPR119.17 The lipid profile for GPR119 knockouts differed

<table>
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<tr>
<th>Lipid Species</th>
<th>Δ in GPR119 KO relative to WT</th>
<th>Δ in GPR119 KO female relative to GPR119 KO male</th>
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<td>N-acyl ethanolamine</td>
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<td>PGE2</td>
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**Figure 4.** Cannabinoid-related lipid profile in wild-type versus GPR119 knockout mice and by sex. *Left column* lists cannabinoid-related lipids out of a panel of lipids that were seen to change in GPR119 knockout versus wild-type mice. *Right column* shows that 2-OG levels were higher in female versus male GPR119 knockout mice.
much more from wild type. Those lipids that were altered were, with one exception, elevated in the GPR119 knockout mice. Figure 4 shows only those lipids of which the levels were altered. The most pronounced difference was for 2-OG, but most classes of oleoyl-based compounds were elevated. Most acylethanolamines were increased, as was the prostaglandin PGE2. The only lipid that declined was N-oleoyl serine and only modestly so.

GPR119 Activation Sex-Dependently Reduces IOP in a Mouse Model

The third component of a bona fide signaling system is function. We tested for a potential role for 2-OG in regulation of IOP using rebound tonometry in a normotensive mouse model. The mouse has been developed as a model system for the study of IOP,24,25 offering among other things access to assorted genetic mutants; in our case, mutants associated with cannabinoid-related signaling and metabolism. We have made use of this system for several studies of the ocular endocannabinoid signaling system in relation to ocular pressure.23,26

We found that 2-OG applied topically at 5 mM in female mice yielded a statistically significant decrease in IOP at 1 hour and 4 hours (Figs. 5A, 5B). Significantly, however, this effect was seen only in female mice, not in males (Figs. 5C, 5D); 2-OG did not lower IOP in female GPR119 knockout mice (Figs. 5E, 5F). The effect of 2-OG did not persist at 8 hours (vehicle versus 2-OG [5 mM] at 8 hours: 17.0 ± 0.8, 16.3 ± 0.8; n = 8, NS by paired t-test).

Because OEA has also been proposed as a GPR119 ligand,12 we also tested OEA in our model but found that it did not lower IOP (mm Hg ± SEM at 1 hour for vehicle: 17.2 ± 1.2, versus OEA: 17.0 ± 0.8; n = 10, NS by paired t-test). We additionally tested several synthetic GPR119 ligands with mixed results. Of MBX2982, APD668,28 and AS1265974, only the first, MBX2982, lowered IOP. MBX2982 lowered IOP by 22%, but the duration of effect was not as long as the endogenous ligand (Fig. 6A). Curiously, in female GPR119 knockouts, MBX2982 raised IOP at 1 hour (Fig. 6B), but not at 4 hours (IOP [mm Hg ± SEM] at 4 hours for vehicle: 18.3 ± 1.1, versus MBX2982: 18.7 ± 0.9; n = 9). This suggests that MBX2982 has an additional target in the eye. In contrast, APD668 (3 mM) did not lower IOP at 1 hour (IOP [mm Hg ± SEM] at

![Image](https://via.placeholder.com/150)

**Figure 5.** Topical 2-OG lowers IOP in female but not male mice. IOP was measured in mice following topical corneal application of 2-OG (5 mM). IOP in female mice was reliably reduced by 2-OG at 1 and 4 hours (A, B) but not male mice (C, D); (E, F) 2-OG does not lower IOP in female GPR119 knockout mice. *P < 0.05, **P < 0.01 by paired t-test.
a blocker of monoacylglycerol lipase (MAGL), an enzyme that metabolizes endocannabinoids. However, it may prove possible to enhance the effect through optimization of agonist/penetration properties or the development of stable analogues. There may be species-dependent differences in the responses, although of course there is the possibility that the system will not even be preserved in humans. It is also possible that 2-OG will work cooperatively with another existing therapy. These are all areas suitable for further study.

The mechanism and site of action for 2-OG/GPR119 remain uncertain. Using an antibody with knockout controls, it was possible to identify sites of protein expression. Of particular interest for the current study was prominent expression in channel-like structures associated with the angle and the trabecular meshwork as well as staining in the iris and the corneal epithelium. Thus, GPR119 expression is most strongly associated with sites of outflow. Moreover, the trabecular meshwork remains a possible site of action.

Until recently, GPR119 was considered an "orphan" GPCR for which no dedicated ligand had been identified. Evidence was initially offered that OEA might serve this role, but the matter is not settled; 2-OG was proposed more recently and appears to be a more likely candidate in the eye. Also, 2-OG was found to increase pressure in mice lacking GPR119 receptor in vivo. It is also possible that OEA is rapidly metabolized into an inactive compound during transit across the murine cornea. We tested three commercially available synthetic GPR119 ligands, MBX2982, APD6608, and AS1265974, but found that only MBX2982 was able to lower pressure in our model. At 22%, the maximal effect was stronger than for the endogenous compound, suggesting that there is some downward room to lower pressure using a synthetic compound versus the natural ligand, but the effect did not last as long. Moreover, in GPR119 knockouts, MBX2982 actually raised pressure at 1 hour. Although the increase was modest, this suggests that MBX2982 has a second target that is coupled to an increase in pressure, not unlike our findings for WIN55212 and CB1. The other two compounds may have suffered from limited penetration of the cornea, an issue we encountered for MAGL blockers.

Our finding that GPR119 expression and function are enhanced in female mice is surprising and significant. The gene is expressed on the X chromosome of which females have two copies, but as a rule one copy is inactivated. Our RT-PCR results suggest that the difference occurs at the mRNA level and may be explained by greater levels of message (and presumably protein) rather than a difference in functionality of the protein. We have not noted any other reference to sex-dependence of GPR119 in the literature and the implications for sex-specific function of GPR119 are considerable. Two important questions are whether and to what extent this carries over to other species, including humans, and tissues. As noted above, GPR119 has potential as a clinical target for diabetes therapy. If the diabetes-related potential of GPR119 is borne out, potential sex differences should be taken into consideration. Women are not at greater risk of open angle glaucoma. Women outnumber men in cases of glaucoma (although likely substantially because women outlive men), meaning that there is a greater need for glaucoma medication among women than men, and the longevity of women leaves them at greater risk of developing tolerance to one or more of the available drugs.

Several enzymes are capable of metabolizing acylglycerols (reviewed in Ref. 31), but the strongest candidate is MAGL. We recently examined the ocular expression of MAGL as well as the therapeutic potential for blockers of this enzyme for...
lowering IOP. The observed effect (an approximately 30% drop in IOP) was absent in CB1 knockout mice, suggesting that the effect was due to action at CB1. However, the lipid profile in MAGL knockout mice showed substantially higher levels of all three acylglycerols tested: 2-OG, 2-AG, mentioned previously, and also 2LG. We therefore have a disconnect between an enzyme that clearly plays a role in metabolism of 2-OG but blockade of which appears to lower IOP via another receptor. It is possible that we encountered a floor effect with a 50% drop in IOP. Alternatively, this may be due to a difference in protein expression because the MAGL protein is restricted to pigmented ciliary epithelium and iris. However, the question of the relationship between the enzyme that metabolizes both 2-AG and 2-OG and the receptors CB1 and GPR119 invites further study.

In summary, we offer evidence that activation of a GPR119-based signaling system in the mammalian eye lowers IOP. It does so selectively in females of the species tested and acts through an unknown pathway, but protein expression suggests that this will involve outflow of aqueous humor. Because glaucoma remains a major cause of blindness, with a substantial number of patients unresponsive to conventional treatments, the identification of a novel mechanism to lower IOP is therefore of great therapeutic interest. Our results suggest that GPR119 may serve as a desirable target for development of novel ocular hypotensive medications.

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Indiana University has filed a provisional patent relating to the work described in this article. Patent application number PCT/US2017/035799.

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