Retina

The Role of ERK1/2 Activation in Sarpogrelate-Mediated Neuroprotection

Cristy A. Ku, Renee C. Ryals, Dan Jiang, Aaron S. Coyner, Kyle K. Weller, Wrik Sinha, Bryan M. Robb, Paul Yang, and Mark E. Pennesi

Casey Eye Institute, Oregon Health & Science University, Portland, Oregon, United States

Correspondence: Mark E. Pennesi, Casey Eye Institute, 3375 SW Terwilliger Boulevard, Portland, OR 97230, USA; pennesi@ohsu.edu.

Submitted: October 16, 2017
Accepted: December 17, 2017

Purpose. To characterize the mediators of 5-HT2A serotonin receptor-driven retinal neuroprotection.

Methods. Albino mice were treated intraperitoneally with saline or sarpogrelate, a 5-HT2A antagonist, immediately before light exposure (LE). Following LE, retinas were harvested for a high-throughput phosphorylation microarray to quantify activated phosphorylated proteins in G protein-coupled receptor (GPCR) signaling. To confirm microarray results and define temporal changes, Western blots of select GPCR signaling proteins were performed. Since both methodologies implicated MAPK/ERK activation, the functional significance of sarpogrelate-mediated ERK1/2 activation was examined by inhibition of ERK1/2 phosphorylation via pretreatment with the MEK inhibitor (MEKi) PD0325901. The degree of neuroprotection was evaluated with spectral-domain optical coherence tomography (SD-OCT) and electroretinography (ERG). To determine the effects of sarpogrelate on gene expression, a qPCR array measuring the expression of 84 genes involved in oxidative stress and cell death was performed 48 hours post LE.

Results. Sarpogrelate led an activation of the MAPK/ERK pathway. Temporal analysis further demonstrated a transient activation of ERK1/2, starting with an early inhibition 20 minutes into LE, a maximum activation at 3 hours post LE, and a return to baseline at 7 hours post LE. Inhibition of ERK1/2 with MEKi pretreatment led to attenuation of sarpogrelate-mediated neuroprotection. LE caused significant changes in the expression of genes involved in iron metabolism, oxidative stress, and apoptosis. These changes were prevented by sarpogrelate treatment.

Conclusions. Sarpogrelate-mediated retinal protection involves a transient activation of the MAPK/ERK pathway, although this pathway alone does not account for the full effect of neuroprotection.

Keywords: sarpogrelate, MAPK/ERK, retinal degeneration, light damage, 5-HT2A

Retinitis pigmentosa (RP), an inherited retinal dystrophy that results in debilitating vision loss, is genetically heterogeneous with mutations in over 60 genes that lead to progressive photoreceptor death. Despite the diverse function of various genes that cause RP, common pathways mediate photoreceptor cell death. Researchers have long sought gene-independent treatments for RP that can cease or slow photoreceptor cell death. One such approach, systems pharmacology, encompasses the use of a combination of two or more drugs to achieve a positive therapeutic effect by affecting downstream common effectors or signaling pathways. This strategy allows for a rational therapeutic design based off basic knowledge of signaling pathways. Since the pathways that cause photoreceptor cell death are complex and multifactorial, targeting multiple signaling pathways is a more effective approach for treating such a complex disease.

Increasing evidence suggests that multiple G protein (guanine nucleotide–binding protein)-coupled receptors (GPCRs) are expressed in the retina and modulation of these receptors can protect the retina from outer retinal degeneration. A retinal transcriptome analysis has demonstrated expression of serotonergic (5-HT), adrenergic, and dopaminergic GPCRs in the mammalian retina. Activation and inhibition of these common GPCRs are known to modulate diverse secondary pathways including adenylyl cyclase, small GTPase Ras homologs, phospholipase C (PLC), and regulation of ion channels, which are important for regulating cellular functions including metabolism, growth, proliferation, survival, transcription, and protein synthesis. However, it has yet to be determined if these pathways are activated/inhibited in the retina and account for the neuroprotection achieved with GPCR agonists/antagonists.

To further our ability to rationally develop therapies for photoreceptor cell death, we sought to investigate the cell signaling associated with sarpogrelate-mediated neuroprotection. Sarpogrelate, marketed as ANPLAG, is a 5-HT2A antagonist that has been used to treat patients with peripheral arterial disease in Japan, China, and South Korea. We have recently characterized sarpogrelate’s neuroprotective effects in the retina and observed a dose-dependent protection against light-induced retinopathy. Many light-induced retinopathy methods have been established. Some include the use of fluorescent white light, broadband green light, or narrowband blue or green light. Depending on the intensity and duration of...
Mechanisms of Sarpogrelate-Mediated Neuroprotection

the varying light sources, degeneration rates and induction of cell death pathways can differ. For our studies we have consistently used an acute bright white light exposure, which has been well characterized as a model for studying apoptosis associated with retinal degeneration. In the current study, phosphorylation changes of proteins in two main GPCR pathways, mitogen-activated protein kinase/extracellular signal-related kinase (MAPK/ERK) and phosphoinositide 3-kinase/phosphoinositide-dependent kinase 1/protein kinase B (PI3K/PDK/AKT), were investigated to determine the signaling pathways playing a role in sarpogrelate-mediated neuroprotection.

METHODS

Animals

All experiments and animal handling procedures were approved by and performed in compliance with the policies of the Institutional Animal Care and Use Committee at Oregon Health & Science University and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Male albino BALB/c mice (The Jackson Laboratory, Bar Harbor, ME, USA) between 2 to 3 months of age were used. Mice were housed in a 12-hour alternating light/dark cycle room for future in vivo imaging and electrophysiology studies.

Injections

At 11:00 AM, 2 hours after the start of the dark cycle, mice were administered an intraperitoneal injection of 25 mg/kg sarpogrelate (dissolved in saline) (Tocris Bioscience, Bristol, UK) or equivalent volume of saline immediately before 1 hour of bright light exposure (LE). For MEK inhibitor experiments, mice were administered an intraperitoneal injection of 5 mg/kg PD0325901 (dissolved in distilled water with 1% dimethyl sulfoxide) (SelleckChem, Houston, TX, USA) or vehicle 15 minutes before sarpogrelate or saline injection. After LE, mice were returned to the dark until the appropriate time of retinal harvest, or returned back to the 12-hour alternating light/dark cycle room for future in vivo imaging and electrophysiology studies.

Light-Induced Retinopathy

A custom light box was built for the induction of retinopathy. It was able to hold up to 16 mice and produced a light having an emission spectrum similar to daylight. Bright light exposure (LE) was performed with two light sources, one of which was a grow light having an emission spectrum similar to daylight. For our experiments evaluating sarpogrelate without LE, retinas were collected at 5 minutes, 20 minutes, 30 minutes, 1 hour 20 minutes, 6 hours, 12 hours, 24 hours, and 48 hours post sarpogrelate or saline injection. For our experiments evaluating sarpogrelate-mediated neuroprotection with LE, retinas were collected immediately after 20 minutes of LE, 1 hour of LE; or at 20 minutes, 1 hour, 3 hours, 7 hours, 24 hours, 48 hours, or 96 hours post LE. Retinas were harvested and sonicated in phosphate-buffered saline (1X PBS) containing protease and phosphatase inhibitors (Thermo Scientific).

Phosphorylation Antibody Array

Western Blot

Temporal phosphorylation changes were assessed by Western blot. For our experiments evaluating sarpogrelate without LE, retinas were collected 5 minutes, 20 minutes, 30 minutes, 1 hour 20 minutes, 6 hours, 12 hours, 24 hours, and 48 hours post sarpogrelate or saline injection. For our experiments evaluating sarpogrelate-mediated neuroprotection with LE, retinas were collected immediately after 20 minutes of LE, 1 hour of LE; or at 20 minutes, 1 hour, 3 hours, 7 hours, 24 hours, 48 hours, or 96 hours post LE. Retinas were harvested and sonicated in phosphate-buffered saline (1X PBS) containing protease and phosphatase inhibitors (Thermo Scientific). Loaded samples (200 μg) were separated on a 12% acrylamide gel and transferred onto a membrane overnight at 4°C. The next day, membranes were incubated in Odyssey infrared imaging system blocking buffer (Li-Cor, Lincoln, NE, USA) for 1 hour. Blots were then labeled with the following rabbit polyclonal antibodies diluted at 1:1000: phospho-PKCβ Ser661 (A8169; Assay Biotechnology [AB], Sunnyvale, CA, USA), phospho-PKCα/βII Thr638/641 (9375; Cell Signaling Technology [CST], Danvers, MA, USA), phospho-MEK1/2 Ser217/221 (9121; CST), MEK1/2 (9122; CST), phospho-p44/42 MAPK (pERK1/2) Thr202/Tyr204 (9101; CST), p44/42 MAPK (ERK1/2) (9102; CST), phospho-PDK1 Ser424 (3438; CST), phospho-Akt Ser473 (9271; CST), Akt (9272; CST), phospho-CREB Ser133 (A7053; AB), B-cell lymphoma 2 (Bcl2, 2870; CST), and phospho-Fos Thr232 (A8226; AB). After overnight incubation in primary antibodies, blots were incubated in a goat anti-rabbit IRDye 680RD secondary antibody (Li-Cor) diluted at 1:15000 for 1 hour at room temperature. Finally, blots were imaged with an infrared imaging system (Odyssey, Odyssey 2.1 software; Li-Cor). As an internal control for protein loading, membranes were also probed with GAPDH (2118; CST) or eIF4E (9742; CST). Using optical density measurements obtained with Odyssey software, each sample was normalized to the loading control. Then experimental groups were compared to control groups to determine fold changes in phosphorylation due to LE or sarpogrelate treatment. The experiments were performed independently two times, three animals per group.

qPCR Array

Changes in gene expression were assessed with a customized RT2 Profiler PCR Array (SABiosciences, Germantown, MD, USA) that consisted of 84 genes involved in oxidative stress and cell death (see Supplementary Table S2). Briefly, retinas were harvested at 48 hours post LE and RNA was extracted from retina by using an RNeasy Mini Kit (QIAGEN, Germantown, MD, USA). Total RNA (0.5 μg) was converted to cDNA by using the RT2 first strand kit (SABiosciences). All samples were checked for DNA contamination and impurities that would affect PCR amplification by using the RT2 RNA QC PCR array.
Mechanisms of Sarpogrelate-Mediated Neuroprotection

In Vivo Spectral-Domain Optical Coherence Tomography (SD-OCT)

Seven days following drug treatment and LE, in vivo SD-OCT imaging was performed as previously reported.\textsuperscript{10,11,21} Retinal layers were segmented for quantification of outer retinal thickness by using a custom-designed SD-OCT segmentation program built in IGOR Pro (IGOR Pro 6.37; WaveMetrics, Inc., Lake Oswego, OR, USA). Outer retinal thickness is defined as the thickness from Bruch’s membrane to the inner nuclear layer/outerciplexum layer interface.

In Vivo Electrophysiology

Two to 7 days after SD-OCT acquisition, ERGs were performed as previously reported.\textsuperscript{10,11} Light intensities ranged from \(-4.34\) to \(-3.55\) log cd/m\(^2\) and ERG traces were averaged and analyzed by using custom software.

Statistical Analysis

All temporal Western blot data were plotted in Prism (Prism 6.0; GraphPad Software, Inc., LaJolla, CA, USA). To determine significant differences between groups at different time points, a 2-way ANOVA multiple comparisons test was performed. Average outer retinal thickness (REC\(^+\)) was calculated for each animal and plotted in Prism as well. A 1-way ANOVA was used to compare REC\(^+\) in different treatment groups. For all analysis, a P value less than 0.05 was considered significant.

RESULTS

Phosphorylation Changes Associated With Sarpogrelate Treatment

We have previously demonstrated the neuroprotective effects of the 5-HT\(_{2A}\) antagonist, sarpogrelate, against bright light exposure.\textsuperscript{10} With this study, we sought to investigate the cell signaling associated with sarpogrelate-mediated neuroprotection (see Fig. 1 for experimental schematic). The 5-HT\(_{2A}\) receptor is known to modulate both the MAPK/ERK and the PI3K/PDK/AKT pathway, which play prominent roles in cell survival.\textsuperscript{22–27} To investigate if these complex signaling changes also occur in the retina in association to neuroprotection, a high-throughput phosphorylation antibody array was used to quantify 63 specific phosphorylation sites on proteins important to the activation and deactivation of common signaling pathways (Table; Supplementary Table S1). Retinas from three groups of mice, namely, (1) saline injected, unexposed to light (Saline\(+\)No LE), (2) saline injected, exposed to light (Saline\(+\)LE), and (3) sarpogrelate injected, exposed to light (Sarp\(+\)LE), were harvested 3 hours after light exposure (LE, \~10,000 lux, 1 hour) and used to calculate protein phosphorylation changes specifically due to LE and sarpogrelate treatment. At 3 hours post LE, sarpogrelate treatment led to an increase in phosphorylation of proteins critical for activation of the MAPK/ERK pathway, including PKCa2/βII, PKCβ, c-Raf, Raf1, MEK1, ERK1/2, and SHC-transforming protein 1 (Shc) (Table). Downstream transcription factors including CREB and serum response factor (SRF) also showed increased phosphorylation of their main activation sites at 3 hours post LE (Table), indicating activation of these transcription factors. In most cases, sarpogrelate treatment reversed phosphorylation events driven by LE (Supplementary Table S1).

To confirm findings from the phosphorylation array and to assess temporal changes in cell signaling, Western blots for...
Table. Phosphorylation Changes Mediated by Sarpogrelate at 3 Hours Post Light Exposure

<table>
<thead>
<tr>
<th>Name</th>
<th>Ratio Change Sarp+LE/(Saline+LE)</th>
<th>Function of Phosphorylation Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAPK/ERK pathway</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PKCβ/II (Thr638)</td>
<td>1.54↑</td>
<td>Unspecified</td>
</tr>
<tr>
<td>PKCβ (Ser661)</td>
<td>1.83↑</td>
<td>Unspecified</td>
</tr>
<tr>
<td>c-Raf (Ser43)</td>
<td>1.81↑</td>
<td>Feedback P from MEK/ERK</td>
</tr>
<tr>
<td>c-Raf (Ser296)</td>
<td>1.68↑</td>
<td>Feedback P from MEK/ERK</td>
</tr>
<tr>
<td>Raf1 (Ser358)</td>
<td>1.63↑</td>
<td>Main activation sites for kinase activity</td>
</tr>
<tr>
<td>Raf1(Tyr541)</td>
<td>1.49↑</td>
<td>Required for phosphorylation of MEK</td>
</tr>
<tr>
<td>Raf1(Ser259)</td>
<td>0.47↓</td>
<td>Feedback P from AKT/PKA; stabilizes inactive form</td>
</tr>
<tr>
<td>MEK1 (Ser217)</td>
<td>1.59↑</td>
<td>Main activation site</td>
</tr>
<tr>
<td>MEK1 (Ser221)</td>
<td>1.59↑</td>
<td>Main activation site</td>
</tr>
<tr>
<td>ERK1/2 (Thr202)</td>
<td>1.26↑</td>
<td>Main activation site</td>
</tr>
<tr>
<td>ERK1/2 (Tyr204)</td>
<td>1.35↑</td>
<td>Main activation site</td>
</tr>
<tr>
<td>Shc (Tyr419)</td>
<td>1.89↑</td>
<td>Stress-induced Ras activation</td>
</tr>
<tr>
<td>Shc (Tyr427)</td>
<td>1.91↑</td>
<td>Stress-induced Ras activation</td>
</tr>
<tr>
<td>Gene transcription factors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CREB (Ser133)</td>
<td>1.75↑</td>
<td>Recruitment of CREB-binding protein and gene regulation</td>
</tr>
<tr>
<td>CREB (Thr100)</td>
<td>1.66↑</td>
<td>Enhances CREB-binding protein binding affinity</td>
</tr>
<tr>
<td>CREB (Ser121)</td>
<td>1.69↑</td>
<td>Enhances CREB-binding protein binding affinity</td>
</tr>
<tr>
<td>CREB (Ser142)</td>
<td>1.40↑</td>
<td>Entrainment of the mammalian clock</td>
</tr>
<tr>
<td>SRF (Ser77)</td>
<td>1.66↑</td>
<td>Binds SRE; immediate-early gene transcription</td>
</tr>
<tr>
<td>SRF (Ser99)</td>
<td>1.51↑</td>
<td>Binds SRE; immediate-early gene transcription</td>
</tr>
</tbody>
</table>

Fold changes in phosphorylation at specific phosphorylation sites, mediated by sarpogrelate treatment. Fold changes were quantified in two independent studies, n = 3 animals per group. Sarp, 25 mg/kg sarpogrelate; up arrow, increase; down arrow, decrease; PKCβ/II, protein kinase C α/β II; Thr, threonine; PKCβ, protein kinase C; Ser, serine; c-Raf, RAF proto-oncogene serine/threonine protein kinase; Raf1, RAF proto-oncogene serine/threonine protein kinase; Tyr, tyrosine; MEK, mitogen-activated protein kinase; SRF, serum response factor; P, phosphorylation; PKA, protein kinase A; SRE, serum response element.

selected phosphorylated proteins were performed (Fig. 2, Supplementary Fig. S1A). In addition, effects of sarpogrelate were assessed in mice not exposed to light (Fig. 2A, Supplementary Fig. S1A) and in mice that were exposed to light (Fig. 2B, Supplementary Fig. S1B) to determine if sarpogrelate-mediated cell signaling changes were affected by stress. In the absence of LE, sarpogrelate treatment caused an increase in phosphorylation of PKCβ Ser661 at 5 minutes post injection, an increase in phosphorylation of MEK Ser217/222 at 5 minutes, 20 minutes, and 1 hour 20 minutes post injection, and an increase in phosphorylation of ERK1/2 Thr202/Tyr204 at 1 hour 20 minutes post injection (P < 0.05; Fig. 2A). Further downstream, phosphorylation of CREB Ser133 was significantly increased at 24 hours post injection and total Bcl2 protein was increased at 48 hours post injection (P < 0.05; Fig. 2A).

Temporal effects of sarpogrelate differed with LE (Fig. 2B). Sarpogrelate treatment with LE did not show the immediate increase in phosphorylation of PKCβ Ser661, but rather a late increase at 7 hours post LE (P < 0.05; Fig. 2B). In addition, sarpogrelate treatment led to significantly decreased phosphorylation of MEK Ser217/222 at 20 minutes and 1 hour into LE, followed by a peak increase at 3 hours post LE (P < 0.05; Fig. 2B). Peak phosphorylated MEK levels returned to Saline+No LE baseline control levels by 7 hours through 96 hours post LE. Phosphorylation of ERK1/2 Thr202/Tyr204 followed the same temporal pattern as phosphorylation of MEK Ser217/222 with early inhibition, peak activation at 3 hours post LE, followed by return to baseline after 7 hours post LE (Fig. 2B). LE alone increased phosphorylation of ERK1/2 Thr202/Tyr204 at 3, 24, and 48 hours post LE. At 24 hours post LE, sarpogrelate treatment significantly reduced this increased phosphorylation of ERK1/2, restoring pERK1/2 back to control levels (P < 0.05; Fig. 2B). Further downstream, sarpogrelate treatment led to increased phosphorylated CREB Ser133 as compared to both Saline+LE mice and Saline+No LE mice at 3 hours and 96 hours post LE (P < 0.05; Fig. 2B). At the 48-hour time point, LE alone caused a significant decrease in phosphorylation of CREB Ser133, but sarpogrelate treatment increased phosphorylation, restoring pCREB levels to those observed in normal, nondamaged mice (P < 0.05; Fig. 2B).

Data from the phosphorylation antibody array suggested that PI3K and Akt were activated at 3 hours post LE (Supplementary Table S1). However, temporal Western blot analysis did not confirm these findings (Supplementary Fig. S2). For the most part, phosphorylated PDK1 Ser241 and AKT Ser473 levels stayed consistent with controls over time (Supplementary Fig. S2). Owing to this discrepancy, we cannot confirm activation of the PI3K/PDK/AKT pathway.

Functional Significance of the MAPK/ERK Pathway

To determine the neuroprotective role of the observed transient MAPK/ERK activation, ERK1/2 phosphorylation was blocked with a MEK inhibitor (MEKi), PD0325901 (Fig. 3). Pretreatment with MEKi effectively reduced levels of phosphorylated ERK1/2 by approximately 80% at 3 hours post LE, the time at which peak levels of phosphorylated ERK1/2 were observed (Fig. 3A). MEKi treatment did not affect phosphorylation of other signaling proteins such as protein kinase A regulatory subunit R2B (PKA-R2B) and Akt (Fig. 3A). Pretreatment with the MEKi (MEKi+Sarp+LE) led to a decrease in the neuroprotection achieved with sarpogrelate treatment (Vehicle+Sarp+LE) as assessed by measurements of outer retinal thickness, particularly evident in the superior and temporal quadrants of the retina (P < 0.05; Figs. 3B, 3C). The statistically significant reduction in retinal layer preservation, however, did not lead to a significant decrease in retinal function as measured by ERGs in MEKi-pretreated mice (Fig. 3D).
FIGURE 2. Sarpogrelate treatment results in transient MAPK/ERK activation. Line graphs showing average fold change in phosphorylation of key proteins in the MAPK/ERK pathway at multiple times post injection (A) and post light exposure (B). Data points are presented as mean ± standard error. A 2-way ANOVA multiple comparisons test was used to compare fold changes between Sarp+No LE and Saline+No LE groups in (A) and also used to compare fold change difference between Sarp+LE and Saline+LE in (B). *P ≤ 0.05. n = 3 to 5 animals per group. Sarp, 25 mg/kg sarpogrelate; p, phosphorylated; PKC, protein kinase C; MEK, mitogen-activated protein kinase kinase; ERK1/2, extracellular signal-related kinase 1/2.
Gene Expression Changes Associated With Sarpogrelate Treatment

Since pretreatment with the MEKi decreased sarpogrelate’s neuroprotective effects, we hypothesized that MEKi treatment may also alter gene expression associated with cell death, linking upstream phosphorylation changes with downstream gene expression. Thus, a qPCR array was used to capture the expression of genes involved in oxidative stress, iron metabolism, apoptosis, autophagy, and necrosis at 48 hours post LE (Fig. 4; Supplementary Table S2). Pretreatment with MEKi alone did not alter gene expression (Supplementary Table S2). However, LE significantly altered the regulation of several genes associated with oxidative stress (Aox1, Cypb, Mb, Tpo, Ucp3), apoptosis (Bcl2, Bcl2a1a, Casp1, Casp7, Tnfrs1a, Xiap, Parp2), and iron metabolism (CP, Lcn2, Ppef2, Steap4, Trf) (*P < 0.05, fold change > 2; Fig. 4). Treatment with sarpogrelate before LE reversed gene expression changes caused by LE (*P < 0.05, fold change > 2; Fig. 4).

**DISCUSSION**

We have previously demonstrated the neuroprotective benefits of sarpogrelate in protecting against light-induced retinopathy. To further the development of such therapies for retinal degeneration, it is important to understand the neuroprotective mechanisms mediating cell survival. In this study, we examined the GPCR signaling pathways activated by sarpogrelate in a light-induced retinopathy model. Results from both phosphorylation antibody arrays and temporal Western blots demonstrated that sarpogrelate treatment results in a transient activation of multiple proteins in the MAPK/ERK pathway and consistent activation of CREB, a converging effector transcription factor highly relevant in neuronal survival and plasticity. Inhibition of ERK1/2 activation reduced but did not eliminate sarpogrelate’s retinal neuroprotective effects. This suggests that the MAPK/ERK pathway plays an important role in neuroprotection, but that this pathway alone does not account for the full protective effect.

Previous studies have demonstrated that activation of 5-HT2A receptors with serotonin or a 5-HT2A agonist leads to activation of the MAPK/ERK pathway (Fig. 5). One study has evaluated signaling pathways in human embryonic kidney cells stably expressing 5-HT2A receptors. Upon activation with 5-HT2A receptor agonists, a sustained phosphorylation of ERK1/2 is observed, but when cells are treated with a 5-HT2A antagonist, phosphorylation of ERK1/2 becomes transient. In our in vivo studies, treatment with sarpogrelate, a 5-HT2A antagonist, also resulted in transient MAPK/ERK activation. Temporal dynamics following delivery of 25 mg/kg sarpogrelate before LE demonstrated a biphasic pattern of MEK/ERK1/2 phosphorylation (pMEK/pERK1/2). There was an initial reduction in pMEK/pERK1/2 at 20 minutes into LE, followed by a reversal with maximum phosphorylation at 3 hours post LE. By 7 hours post LE, pMEK/pERK1/2 returned to control levels (Fig. 2B).

Activation of ERK1/2 has been extensively studied for its role in neuronal cell death and survival. Ultimately, studies suggest that the magnitude and the duration of ERK1/2 activation determines cellular outcomes. In our study, sarpogrelate-treated mice showed a transient ERK1/2 activation with onset at approximately 3 hours post LE and return to basal levels at 7 hours post LE, whereas LE alone showed a low magnitude, long duration of ERK1/2 activation with onset approximately 3 hours post LE and return to baseline at 96 hours post LE. We hypothesize that this sarpogrelate-mediated...
transient ERK1/2 activation plays a role in retinal neuroprotection, altering the ERK1/2 signaling that occurs in response to stress alone. In addition to our data, studies have shown that transient ERK1/2 activation reflects a prosurvival response. For example, in cultured hippocampal neurons, brain-derived neurotrophic factor–mediated neuroprotection involves increased phosphorylation of ERK1/2 from 2.5 minutes to 1 hour post administration and a decrease in ERK1/2 phosphorylation to basal levels 6 to 24 hours post administration.

5-HT$_2$A receptors are expressed in a variety of cell types. Thus, multiple studies have attempted to map 5-HT$_2$A signaling in these various cell types including the muscular/vascular system, neuronal tissue, and kidney tissue with differences observed in varying cells. These studies have found at least five different 5-HT$_2$A modalities to activate MAPK/ERK, which are demonstrated in Figure 5: (1) PLC/PKC-dependent raf activation, (2) PLC/Ca$^{2+}$-dependent ERK activation, (3) ROS-dependent MEK activation, (4) direct G protein activation of ras, and (5) tyrosine kinase (src/shc)–dependent activation of ras.

The classical 5-HT$_2$A activation of MAPK/ERK initiates through Gaq activation of PLC, which then hydrolyzes phosphatidylinositol 4,5-biphosphate (PIP2) to inositol triphosphate (IP3) and diacylglycerol (DAG), ultimately resulting in a release of intracellular calcium stores. The released calcium binds PKC, allowing for PKC docking at DAG and activation of raf. Activation of raf subsequently activates MEK1/2 and ERK1/2 (Fig. 5). Our phosphorylation array and Western blot data demonstrated activation of PKC$\beta$ and CamK2, CamK4, and CamK$\alpha$ in the PLC/PKC-dependent manner (Fig. 2; Table). Our phosphorylation array data also showed activation of CamK2, CamK4, and
Mechanisms of Sarpogrelate-Mediated Neuroprotection

**A. Oxidative Stress Gene Expression Changes**

![Graph showing oxidative stress gene expression changes]

**B. Apoptosis Gene Expression Changes**

![Graph showing apoptosis gene expression changes]

**C. Iron Metabolism Gene Expression Changes**

![Graph showing iron metabolism gene expression changes]

Figure 4. Sarpogrelate treatment reverses gene expression changes associated with light exposure. Bar graphs reporting retinal oxidative stress (A), apoptosis (B), and iron metabolism (C) gene expression changes caused by light exposure and sarpogrelate treatment. The comparative Ct method was used to determine gene expression changes associated with light exposure by comparing data from Vehicle+Saline-No LE and Vehicle+Saline+LE groups (black bar). To determine gene expression changes associated with sarpogrelate treatment, data from the Vehicle+Saline+LE and Vehicle+Sarp+LE groups were compared (striped bar). QIAGEN GeneGlobe data analysis center-calculated fold-regulation changes. Fold regulation greater than 2 represents a biologically significant change in gene expression. n = 6 animals per group. Sarp, 25 mg/kg sarpogrelate; Aox1, aldehyde oxidase 1; Cybb, cytochrome b-245 heavy chain; Mb, myoglobin; Tpo, thyroid peroxidase; Ucp3, mitochondrial uncoupling protein 3; Bcl2a1a, B-cell lymphoma 2-related protein a1; Casp1, caspase 1; Casp7, caspase 7; Tnfrsf1a, tumor necrosis factor receptor superfamily member 1a; Xiap, X-linked inhibitor of apoptosis protein; Parp2, poly (ADP-ribose) polymerase 2; CP, ceruloplasmin; Lcn2, lipocalin-2; Ppef2, protein phosphatase with EF-hand domain 2; Steap4, six-transmembrane epithelial antigen of prostate 4; Trf, transferrin.

Sarpogrelate, which support calmodulin- or tyrosine kinase-dependent activation of MAPK/ERK (Supplementary Table S1). These data are consistent with a study that has demonstrated PKC-independent and calmodulin- and tyrosine kinase-dependent MAPK/ERK activation in a well-characterized neuronal cell line.28

Once activated, potentially through multiple pathways, ERK1/2 may control cell survival or apoptosis by regulating the activity of anti- and pro-apoptotic transcription factors.26 ERK1/2-activated p90 ribosomal S6 kinase (p90RSK) phosphorylates CREB Ser133, which strongly enhances CREB-dependent transcription. Activated CREB can promote cell survival, whereas inhibition of CREB phosphorylation at Ser133 triggers neuronal apoptosis.29 We demonstrated that sarpogrelate significantly increases phosphorylation of CREB Ser133 at 3 hours and 96 hours post LE, indicating sustained CREB activation (Fig. 2B). Additionally, at 48 hours post LE, Western blot analysis indicated that light exposure inhibits phosphorylation of CREB Ser133, which could trigger apoptosis. Sarpogrelate treatment reverted phosphorylation of CREB levels back to control levels, which could prevent apoptotic signaling (Fig. 2B). Our data suggest that activation of CREB plays an important role in sarpogrelate-mediated neuroprotection.

CREB is a converging effector transcription factor that can be activated from multiple GPCR signaling pathways. In our study, when ERK1/2 activation was inhibited with MEK inhibitor PD0325901, sarpogrelate’s neuroprotective effects were reduced, but not completely suppressed, suggesting that either (1) phosphorylation of CREB may occur through both ERK1/2-dependent and ERK1/2-independent pathways, or (2) additional important neuroprotective pathways may occur independently from ERK1/2 and CREB activation. We chose a second-generation PD0325901 inhibitor, which reduced ERK1/2 activation by 80%, significantly more potent than first-generation inhibitors.29 Although ERK1/2 activation was significantly reduced, outer retinal degeneration decreased by only 8%. We hypothesize that additional pathways are playing a role in neuroprotection. However, it is also possible that the remaining 20% of ERK1/2 activation was sufficient to elicit neuroprotective effects. Further inhibition of ERK1/2 was not possible without causing retinal toxicity (data not shown).

CREB Ser133 activation is further supported by downstream gene expression changes. At 24 hours post treatment and LE, genes regulated by CREB or that contain multiple CREB binding sites, including Bcl2, X-linked inhibitor of apoptosis protein (XIAP), and mitochondrial uncoupling protein 3 (UCP3), were significantly increased with sarpogrelate treatment (Fig. 4).29,31

While there are differences in cell death signaling and apoptotic pathways between light-induced retinopathy models depending on the quality, duration, and intensity of light and the animal model,4,16 our findings resulting from acute bright light exposure have been implicated in other light-induced retinopathy and inherited retinal degeneration studies. Bcl2 and XIAP both play important roles in inhibiting apoptosis, whereas UCP3 protects the mitochondria from induced oxidative stress. Bcl2, which localizes to the outer membrane of mitochondria, binds and inhibits proapoptotic proteins Bax and Bak, which usually promote apoptosis by altering mitochondrial functions and activating the release of downstream apoptogenic factors.24 Treatment with Bcl2 has been shown to protect mice from light-induced retinopathy and slows retinal degeneration in rd1, rd2, and S354ter RP mouse models. XIAP binds and inhibits caspases 3, 7, and 9 and further possesses E3 ubiquitin ligase activity that can promote the ubiquitination and degradation of caspases.25 Treatment with XIAP has been able to protect photoreceptors in both
In summary, we showed that sarpogrelate-mediated neuroprotection involves a transient activation of the MAPK/ERK pathway and sustained activation of CREB, leading to transcription of antiapoptotic genes Bcl2, Xiap, and Ucp3.

Future studies will continue to map changes in signaling proteins with GPCR activation/inhibition and downstream gene expression changes. This method will not only provide specific and novel therapeutic targets for retinal cell death, but also aid in developing systems pharmacology therapeutics for retinal degeneration.
Acknowledgments

Supported by an unrestricted departmental funding from Research to Prevent Blindness (New York, NY, USA), Grant P30 EY010572 from the National Institutes of Health (Bethesda, MD, USA), K08 Career Development Award (K08 EY021180, MEP), Alcon Young Investigator Award (MEP), Foundation Fighting Blindness Enhanced Research and Clinical Training Award (CD-NMT-0914-0659-OHSU, MEP), Career Development Award from Research to Prevent Blindness (MEP), and Knights Templar Eye Foundation Career Starter Grant (RCR).

Disclosure: C.A. Ku, None; R.C. Ryals, None; D. Jiang, None; A.S. Coyner, None; K.K. Weller, None; W. Sinha, None; B.M. Robb, None; P. Yang, None; M.E. Pennesi, None.

References


