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Astrocyte-derived lipoxins A<sub>4</sub> and B<sub>4</sub> promote neuroprotection from acute and chronic injury

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The authors have declared that no conflict of interest exists.

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Introduction

Astrocytes perform critical non–cell autonomous roles following CNS injury that involve either neurotoxic or neuroprotective effects. Yet the nature of potential prosurvival cues has remained unclear. In the current study, we utilized the close interaction between astrocytes and retinal ganglion cells (RGCs) in the eye to characterize a secreted neuroprotective signal present in retinal astrocyte conditioned medium (ACM). Rather than a conventional peptide neurotrophic factor, we identified a prominent lipid component of the neuroprotective signal through metabolomics screening. The lipoxins LXA<sub>4</sub> and LXB<sub>4</sub> are small lipid mediators that act locally to dampen inflammation, but they have not been linked directly to neuronal actions. Here, we determined that LXA<sub>4</sub> and LXB<sub>4</sub> are synthesized in the inner retina, but their levels are reduced following injury. Injection of either lipoxin was sufficient for neuroprotection following acute injury, while inhibition of key lipoxin pathway components exacerbated injury-induced damage. Although LXA<sub>4</sub> signaling has been extensively investigated, LXB<sub>4</sub>, the less studied lipoxin, emerged to be more potent in protection. Moreover, LXB<sub>4</sub> neuroprotection was different from that of established LXA<sub>4</sub> signaling, and therapeutic LXB<sub>4</sub> treatment was efficacious in a chronic model of the common neurodegenerative disease glaucoma. Together, these results identify a potential paracrine mechanism that coordinates neuronal homeostasis and inflammation in the CNS.

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Results

Transplanted RAs exhibit neuroprotective activity that is compromised by stress. We have previously extensively characterized astrocytes cultured from the adult retina and optic nerve (ON) head (27–29). These cells display typical astrocyte morphology and markers, and respond robustly to stress (27) (Supplemental Figure 1; supplemental material available online with this article; https://doi.org/10.1172/JCI77398DS1). We studied the effects of RAs on inner retinal neurons by transplanting them into recipient C57BL/6 eyes. After 16 days, transplants remained viable and showed minimal inflammatory infiltrates, cell death, or endogenous glial activation (Supplemental Figure 2). Transplant eyes were challenged after 16 days with injection of kainic acid (KA). Similar excitotoxic models of inner retinal metabolic injury have been previously demonstrated to be dependent on glial have direct neuronal actions. The activities of LXB₄ are distinct from those of LXA₄ and include potent effects on macrophages and nonphlogistic monocyte activation (25, 26). Yet LXB₄ has been far less studied than LXA₄ in part because reliable commercial sources have only recently become available. There is consequently little knowledge about LXB₄ functions in the CNS or roles in neurodegeneration.

Here, we report that LXA₄ and LXB₄ are highly enriched in ACM and demonstrate their direct neuroprotective activity. We characterize their biosynthesis and roles in inner retinal injury and discover unexpectedly higher activity for LXB₄, independent of LXA₄ signaling. Finally, we demonstrate that therapeutic LXB₄ administration is functionally and pathologically efficacious in a chronic glaucoma model, suggesting that strategies to rescue this astrocytic signal may be relevant to treatment of neurodegeneration.
RA neuroprotection is mediated through small secreted factors enriched in lipoxins. The protective activity produced by transplanted RAs could be driven by endogenous detoxifying mechanisms or secreted signals. To distinguish these possibilities, we collected ACM and tested whether it was sufficient to provide protection. Either ACM or cell-free control media incubated under identical conditions was injected intravitreally into C57BL/6 mice 24 hours prior to KA challenge. RGC survival was specifically assessed by probing for the marker RBPMS, along with complementary TUNEL staining and quantification. Significant rescue of RGCs was observed in eyes injected with ACM compared with control media ($P < 0.05$) (Figure 2, A and B), while TUNEL staining showed that ACM injection strongly reduced GCL death ($P < 0.01$) (Figure 2, C and D). To facilitate further study, we established an in vitro assay in which ACM was applied to the neuronal cell line HT22. Consistent with the in vivo results, ACM treatment function (12, 14, 30). Remarkably, we found that RA transplants strongly protected neurons in the ganglion cell layer (GCL) (Figure 1, A–C). In complementary studies, TUNEL staining found a marked reduction of GCL apoptosis in transplanted eyes compared with PBS controls (Figure 1, D–F). As further controls, RAs were first exposed to oxidative stress with the redox agent paraquat (PQ) (Figure 1G) (27) or heat-killed to control for generalized inflammatory effects (Figure 1H). Neither treatment rescued GCL neurons, nor did injection of immortalized astrocytic A7 cells or 661W neuronal cells (Figure 1, I and J). Complementary results were likewise obtained through TUNEL staining (Figure 1, K–N). Following quantification, RA-transplant eyes showed a significant ($P < 0.01$) 3-fold rescue of GCL neurons compared with PBS control, while the additional controls offered reduced or no protection (Figure 1, O and P). These experiments indicate that RAs actively provide neuroprotective support.
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The produced significant HT22 cell protection from glutamate challenge (P < 0.01) (Figure 2E). These data established a platform for future studies of the ACM activity. Surprisingly, in preliminary fractionation experiments, we discovered that a substantial portion of protective activity was contained in filtrate smaller than 3 kDa, ruling out most common neurotrophic factors (Figure 2F).

In order to identify small molecules in the ACM that might account for the protective activity, we performed metabolomic analysis. This approach allowed us to identify a specific group of metabolites that were upregulated in the ACM. These metabolites were further characterized using high-resolution mass spectrometry, revealing the presence of lipoxins (LXA₄ and LXB₄).

Figure 3. Lipoxins are regulated in the inner retina in response to acute injury. (A) qPCR of mouse retinal cDNAs shows significantly reduced expression of Alox5 and Fpr2, but not Alox15, 2 hours after KA insult compared with PBS controls (n = 3). (B) LXB₄ concentrations in total mouse retina are reduced at 6 hours following injury, while LXA₄ concentrations are reduced in the ON compared with PBS-injected controls (n = 10 retinas/aggregate group). (C) Confocal microscopy shows 5-LOX immunostaining in primary RAs. (D) Confocal imaging of 5-LOX immunostaining (green) shows accumulation in the GCL and NFL, with partial colocalization (yellow, arrows) in astrocytes (GFAP: red). (E) Signal for 5-LOX in the inner retina is reduced at 3 and 6 hours after injury (arrows). (F) FPR2 immunostaining (green) is prominent in cultured primary RGCs stained with β3-tubulin (red). (G) ALX/FPR2 immunostaining (green) is specific to the GCL and colocalizes (yellow, arrows) with RGCs (Brn3a: red). Scale bars: 20 μm (C and F); 10 μm (D and G). ***P < 0.005. Bars represent SEM. Statistical analyses were performed by 1-way ANOVA with TUKEY post-hoc test.
LXA₄ levels were low and did not change following insult. However, levels of LXB₄ were reduced by nearly 50% (Figure 3B). In addition to total retina concentrations, we also assessed lipoxin levels in pooled ONs, which are enriched in astrocytes. In these samples, LXA₄ levels were dramatically reduced by 6 hours after injury, with a small reduction of LXB₄ (Figure 3B).

As 5-LOX is the rate-limiting enzyme for LXA₄ and LXB₄ formation, we confirmed its presence in cultured RAs (Figure 3C). Localized 5-LOX immunoreactivity was also present in the inner retina in astrocytes and RGCs (Figure 3D). Consistent with our qPCR results, retinal insult reduced the 5-LOX signal by 3 and 6 hours (Figure 3E).

In comparison, immunostaining demonstrated ALX/FPR2 protein in RGCs in vitro and in vivo (Figure 3, F and G). These data suggest that the lipoxin circuit, comprising biosynthetic enzymes and the LXA₄ receptor, is present in the inner retina and that its functional activity is compromised in response to injury.

LXA₄ and LXB₄ promote RGC survival following acute insult. We next asked whether treatment with LXA₄ or LXB₄ is sufficient to promote RGC protection in vivo. To test this point, 10 μM LXA₄ or LXB₄ was injected intravitreally 1 hour prior to retinal insult. Both LXA₄ and LXB₄ treatments significantly (P < 0.01) increased RGC survival, by 57% (±3.8) and 99% (±4.7), respectively, compared with vehicle (Figure 4, A and B). Unexpectedly, LXB₄ was

screening, including an assessment of SPMs, using liquid chromatography–tandem mass spectrometry (LC-MS/MS). Lipid mediators were quantified to generate a lipidomic profile of SPMs, including eicosanoids and PUFAs. Pathway markers for DHA-derived resolvins and protectins were detected (DHA, 17-HDHA), but not their direct formation. In contrast, lipidomic analyses revealed significant enrichment of the lipoxins LXA₄ and LXB₄ in ACM compared with control media (Figure 2G). DHA is highly enriched in the retina, but can be regulated by diet. Hence, we cannot exclude the possibility that the absence of resolvins and protectins in ACM reflects dietary DHA intake.

LXA₄ and LXB₄ biosynthesis and signaling are regulated in the inner retina. To corroborate the presence of lipoxin signaling in the mouse retina, we used quantitative reverse-transcription PCR (qPCR) to assess expression of Alox5 and Alox15 as well as the LXA₄ receptor Fpr2 (mouse orthologs of 5-LOX, 15-LOX-1, and FPR2, respectively). All 3 transcripts were detected, and expression of Alox5 and Fpr2, but not Alox15, was significantly reduced following retinal insult (P < 0.005) (Figure 3A). Due to their short half-life and rapid metabolic inactivation, endogenous lipoxins can be challenging to detect in small tissue samples. However, by pooling 10 retinas, we were able to quantify aggregate LXA₄ and LXB₄ concentrations in control eyes and at 6 hours following injury. In retina samples, baseline LXA₄ levels were low and did not change following insult. However, levels of LXB₄ were reduced by nearly 50% (Figure 3B). In addition to total retina concentrations, we also assessed lipoxin levels in pooled ONs, which are enriched in astrocytes. In these samples, LXA₄ levels were dramatically reduced by 6 hours after injury, with a small reduction of LXB₄ (Figure 3B).

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more effective than LXA₄. As an alternative approach, we blocked lipoxin biosynthesis by administering a selective 5-LOX inhibitor, zileuton. Intravitreal zileuton alone had no effect on RGC numbers (Supplemental Figure 3), but significantly exacerbated RGC loss in response to injury, by 60% (±1.2) (Figure 4, C and D). We also administered a selective ALX/FPR2 inhibitor, WRW4, which also had no effect on RGC survival alone (Supplemental Figure 3), but increased RGC loss by 67% (±1.8) (Figure 4, E and F). Together, these data provide evidence that lipoxins are protective to RGCs in vivo and that their endogenous biosynthesis and signaling are necessary for acute retinal injury responses.

LXA₄ and LXB₄ provide direct neuroprotection. To determine whether the protective activity we observed was direct, we treated neuronal cells with LXA₄ or LXB₄ in our established in vitro assay. Increasing concentrations of LXA₄ or LXB₄ produced increased viability of glutamate-challenged HT22 cells (Figure 5A). Consistent with our in vivo results, LXB₄ appeared more potent than LXA₄, with significant activity at 50 nM versus 500 nM, respectively (Figure 5A). A subsequent dose-response curve generated an EC₅₀ for LXB₄ of 39.2 nM and for LXA₄ of 631.0 nM, indicating a 16-fold increase in potency, though with similar efficacy (Supplemental Figure 4). In contrast, neither the lipoxin precursor 15-HETE nor
the structurally related trihydroxy SPM RvD2 had any protective activity up to 1 μM (Figure 5B). There is no established receptor for LXB4. However, LXA4 and RvD2 signal via the ALX/FPR2 and GPR18 receptors (31), respectively. Therefore, we assessed whether ALX/FPR2 or GPR18 antagonists could block LXB4-mediated protection. Increasing concentrations of WRW4 (ALX/FPR2 antagonist; IC50 = 0.23 μM) or O-1918 (GPR18 antagonist; IC50 = 5.3 μM) did not block LXB4-protective activity (Figure 5C). To further clarify the LXB4 effect on cell viability, we also assessed mitochondrial membrane potential. LXB4 treatment had no effect on membrane potential alone, but strongly protected against glutamate-induced activity compared with vehicle (Figure 5D and Supplemental Figure 5). These data suggest that LXB4 neuroprotection is mediated through a distinct signaling mechanism influencing mitochondrial activity independently of LXA4 or RvD2.

To confirm this activity in primary neurons, we tested the ability of LXA4 or LXB4 to protect RGCs. RGCs were isolated by immunopanning and challenged by PQ-induced oxidative stress. Cell viability was assessed by measuring mitochondrial membrane potential. LXB4 treatment had no effect on membrane potential alone, but strongly protected against glutamate-induced activity compared with vehicle (Figure 5D and Supplemental Figure 5). These data suggest that LXB4 neuroprotection is mediated through a distinct signaling mechanism influencing mitochondrial activity independently of LXA4 or RvD2.
and neurite survival were then quantified in a series of β3-tubulin–stained images for each group. Under normal culture conditions, the RGCs extended an extensive network of neurites (Figure 5E). However, after 24 hours of PQ exposure, both neurite and cell numbers of RGCs extended an extensive network of neurites (Figure 5E). How-
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ature and function. We focused on LXB4 for this challenging study
for primary cortical neurons (Figure 5H) or when RGCs were
able to be consistently maintained over 1.5 weeks (32, 33), resulting in compromised RGC function, reduced retinal nerve fiber layer (RNFL) thickness, and RGC death (32). As RGC functional deficits are reversible for up to 8 weeks (33), we chose this time point to initiate therapeutic treatment of LXB4 or
vehicle (Figure 6A). Electroretinogram (ERG) and optical coherence
tomography (OCT) were measured monthly to assess retinal struc-
tural Figure 8). To confirm these results, animals were euthanized
those in the vehicle group, eliminating a potential indirect effect
(Supplemental Figure 6). Importantly, IOP levels in the LXB4 group did not differ from
in either parameter (Figure 5, E–G). Similar protective LXB4 activity was observed
for primary cortical neurons (Figure 5H) or when RGCs were challenged with serum deprivation (SD) (Supplemental Figure 6). Therefore, LXB4 demonstrates broad and distinct neuroprotective activity that is independent of established LXA4, signaling. Therapeutic LXB4 treatment rescues RGC function and survival in a chronic glaucoma model. To assess whether lipoxin neuroprotective actions extend to chronic neurodegenerative injury, we investigated a rat circumscribed status model of glaucoma. In this assay, sustained intraocular pressure (IOP) elevation can be consistently maintained over 15 weeks (32, 33), resulting in compromised RGC function, reduced retinal nerve fiber layer (RNFL) thickness, and RGC death (32). As RGC functional deficits are reversible for up to 8 weeks (33), we chose this time point to initiate therapeutic treatment of LXB4 or
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ture and function. We focused on LXB4 for this challenging study
due to its consistently higher potency than LXA4 in our acute assays.

Importantly, IOP levels in the LXB4 group did not differ from
in the vehicle group, eliminating a potential indirect effect
(Supplemental Figure 7). However, by week 12, the positive
scotopic threshold response (pSTR, an RGC-dependent ERG signal) showed significant preservation in RGC function in the LXB4 group compared with vehicle. This protection continued to
week 15, while the vehicle group continued to decline (P < 0.05)
(Figure 6, B and C). Similarly, OCT measurement of RNFL thickness reflecting axonal loss was significantly rescued in the
LXB4-treatment group compared with the vehicle group by week
15 (P < 0.05) (Figure 6, D and E). These results were specific to
inner retinal injury, as there was minimal effect on other ERG parameters and no decline in total retinal thickness (Supplemen-
tal Figure 8). To confirm these results, animals were euthanized at week 15 and flat-mounted retinas were stained for BRN3a. LXB4-treated retinas presented a significant rescue of RGCs in both peripheral and central retinal regions compared with vehicle (P < 0.005) (Figure 6, F and G, and Supplemental Figure 9), although there was still cell loss in both groups. Together, these data demonstrate that therapeutic LXB4 administration provides substantial protective effect in RGC function and survival in a model of chronic glaucoma.

Discussion
Neurodegeneration in response to stress or injury has been associ-
at with both the induction of neuroinflammatory signals (34–37) and a corresponding loss of homeostatic prosurvival cues (38–41). However, the mechanisms by which these activities are coordinated in the CNS remain unclear. This study provides the first evidence, to our knowledge, that astrocyte–released LXA4 and LXB4 have direct protective neuronal actions that are compromised following neuronal injury in addition to their proresolving roles. Under normal conditions, astrocytes maintain a host of homeo-
static support functions, but they transition to parainflammatory reactive states that can have both positive and negative non–cell autonomous influences on neighboring neurons (6, 42). In particu-
lar, Sun et al. have recently demonstrated beneficial effects of astrocyte remodeling in the ONH, but did not describe an under-
lying molecular mechanism (4). Our data suggest that, together, astrocyte–derived LXA4 and LXB4 help coordinate a protective signal in the retina and ONH, while simultaneously dampening neuroinflammation. This mechanism would complement a classi-
ﬁcation proposed by Zamanian et al., in which reactive astrocytes can transition from homeostatic quiescent to either neurotoxic A1 or protective A2 states (3, 5), although we cannot rule out potential autocrine contributions from neurons as well. Thus, regulation of lipoxin levels may help coordinate between paracrine neuroin-
flamatory and neuroprotective processes.

SPM dysregulation has been implicated in neuroinflammation related to Alzheimer’s disease, stroke, and age-related macular
degeneration (17, 18). Additionally, mice deficient in ALOX5 or
ALOX15 have increased vulnerability in models of multiple sclerosis (MS) and Huntington’s disease (43, 44). Yet most SPM actions have been linked to their established antiinflammatory and pro-
resolving activities. To our knowledge, only the structurally distinct DHA derivative, NPD1, has been linked directly to neuroprotec-
tive actions (45, 46). The lipoxins were the first identified and remain the only AA-derived SPMs. AA is the most abundant substrate for lipooxygenase enzymes in most tissues, and LXA4 has been detected throughout the CNS (24, 47) and in human vitreous (48). Addi-
tionally, endogenous and aspirin-triggered lipoxins have been shown to reduce inflammatory pain processing through effects on astrocytes (49). However, the generation of lipoxins by astrocytes and their direct neuroprotective actions have not been reported, and the present findings would likely not have been detected in a more conventional genomic or proteomic screen.

Surprisingly, LXB4 was consistently more potent in our in vitro and in vivo experiments than LXA4, with a significant rescue of neurite survival and altered mitochondrial activity. Yet the 2 molecules are structural isomers and are inactivated by the same enzyme pathways (50). Our evidence demonstrates a complete lipoxin synthetic and signaling circuit in astrocytes and the inner retina, while ALX/FPR2 inhibition suggests that LXA4 signaling plays a role in acute injury. In comparison, the formation and bio-
actions of LXB4 have not been fully investigated, but it has been shown to block TNF-α secretion by activated T cells (51), and its production is regulated by NLRP3 inflammasome activity (52). We observed that inhibition of the LXA4 receptor (ALX/FPR2) or RvD2 receptor (GPR18) was insufficient to block protective LXB4 activity. Likewise, neither 15-HETE nor RvD2 showed any direct neuroprotection, suggesting that LXB4 signaling is specific and distinct from LXA4 and the structurally related DHA series resol-
vin RvD2 (31). Hence, LXB4 signaling warrants further investiga-
tion with respect to its neuroprotective role.

Glaucoma is a common neurodegenerative disease and a leading cause of vision loss and blindness worldwide, projected to affect 76 million people by 2020 (53, 54). Elevated IOP is the most established, and only treatable, glaucoma risk factor. However,
many patients do not exhibit elevated IOP, and nearly all patients eventually progress (8, 53, 55). In this respect, glaucoma shares the complex etiology common to related neurodegenerative processes throughout the CNS (7, 8, 10) and may involve both non-cell-autonomous and cell-autonomous RGC death mechanisms (10, 56). Yet no neuroprotective treatment is available, representing a major health challenge (57). In our experiments, therapeutic LXB₄ treatment was efficacious in clinically relevant measures of ERG (RGC function) and OCT (axonal loss) and in reducing RGC death. However, LXB₄ had no effect on IOP. Taken together, our results strikingly suggest LXB₄ mediates neuroprotection of RGC function and survival in a therapeutic context under conditions of chronic IOP-induced injury. These promising findings support further research into the role and mechanisms of lipoxin signaling during neuronal injury and exploration of their therapeutic potential in the context of neurodegeneration.

Methods

**Mouse acute retinal insult model.** Male C57BL/6 mice were anesthetized by i.p. injection of ketamine/xylazine. Intravitreal injections with 10 mM KA were performed as previously described (58). Similar excitotoxic damage models have been extensively used by ourselves and others to study the influence of retinal glia on neuronal survival and function, providing a consistent and accurate assessment of retinal neuropathology (12, 14, 30, 58–60). Briefly, a 30-gauge needle was inserted tangentially into the vitreous and replaced with a Hamilton syringe to inject a volume of 2 μl, followed by application of ophthalmic antibiotic ointment (BNP, Vetoquinol). In some experiments, 100,000 RAs were first injected 16 days prior to KA challenge. Alternatively 10× concentrated medium (ACM) or test compounds were injected intravitreally 24 hours or 1 hour, respectively, prior to the KA injection in the following concentrations: 10 μM LXA₃ and LXB₄, 2 μg/μl zileuton (a selective 5-LOX inhibitor that has been used clinically; refs. 61, 62), and 15 μM WRW4 (a selective ALX/FPR2 inhibitor; refs. 62–64), all dissolved in PBS. Mice were euthanized by CO₂ asphyxiation 18 hours after KA treatment, and the eyes were fixed in 4% paraformaldehyde. For RNA and lipodikin analyses, retinas and ONs were dissected and snap-frozen on dry ice. In all experiments, n refers to the number of animals tested.

**Astrocyte cultures.** Primary RAs were isolated and cultured as previously described (14, 27–29, 58). These cells display typical astrocyte morphology and a variety of appropriate markers, including GFAP, vimentin, Pax-2, GS, and S100A. They also respond robustly to oxidative and metabolic stress with changes in activation markers, secreted cytokines, and antioxidants (27, 58) (Supplemental Figure 1). Briefly, eyes were dissected out of adult Wistar rats and placed in ice-cold MEM-H17 with 10% FBS/1% penicillin/streptomycin. Isolated retinas were digested by shaking in MEM-H17 containing papain and DNase, followed by trituration to disperse cell aggregates. When cultures reached confluence, the cells were placed on a rotating shaker for 6 to 8 hours to remove microglia and then replated. An astrocyte-expression profile was confirmed by probing the cultures with a panel of glial and neuronal markers (27). Note that efforts to generate a serum-free defined media resulted in cellular stress that compromised the protective activity. Therefore, conditioned media experiments contained serum, although control media exhibited no activity. RA conditioned medium (ACM) or cell-free control medium was harvested after 24 hours incubation and stored at -80°C.

**Neuronal cultures.** HT22 neuronal cells (65, 66) were cultured in high-glucose DMEM with 10% FBS/1% penicillin/streptomycin. For neuroprotection experiments, injury was induced by incubation with 5 mM glutamate. Cell viability was assessed by XTT assay and absorbance was measured at 490 nm, according to the manufacturer’s directions (Roche). MitoTracker Red (Life Technologies) was used according to the manufacturer’s instructions, with intensity per cell calculated for 42 cells for each well. RGCs were purified using magnetic MicroBeads (Miltenyi Biotec) according to the manufacturer’s protocol. Briefly, retinal cell suspensions were prepared from 8- to 10-day-old rats and incubated with CD90.1 microbeads and biotinylated depletion antibodies against microglia and endothelial cells. Cells were gently centrifuged and washed with DPBS/BSA buffer. Resuspended cells were incubated with magnetic Anti-Biotin MACs MicroBeads (Miltenyi Biotec) and applied to a MACs separation magnet. The preenriched population was applied to a MACs MS separation column twice to enrich for CD90.1-bound RGCs. RGCs were plated in poly-d-lysine–coated wells and cultured in Neurobasal-A media (GIBCO; Thermo Fisher Scientific) supplemented with 2% calf serum, 2% B27 supplement, 1 mM L-glutamine, 50 ng/ml BDNF, 50 ng/ml CNTF, 5 μM forskolin, and 1% penicillin/streptomycin. To assess RGC survival, cultures were challenged with serum and supplement deprivation or 30 μM PQ, fixed, probed for β3-tubulin, and imaged with a Nikon confocal microscope. The number of cell bodies and ratio of neurites/cell was established for each acquired frame, with 32 frames scored from each experimental group. Primary cortical neurons were cultured essentially as described (67). Briefly, cortices were dissected from E16 mouse embryos on ice. The tissue was homogenized and dissociated with papain and DNase, triturated, and strained to remove clumps. Cortical neurons were seeded onto poly-d-lysine–coated wells with Neurobasal-A media with L-glutamine and B27 supplement. All data represent at least 3 independent experiments for each treatment.

**LC-MS/MS.** Eicosanoids and PUFA in the conditioned media were quantified via LC-MS/MS according to our published protocol (61, 68–70). Briefly, class-specific deuterated internal standards (PGE2-d4, LTB4-d4, 15-HETE-d8, LXA₃-d5, DHA-d5, AA-d8) were used to calculate extraction recovery on an LC-MS/MS system consisting of an Agilent 1200 Series HPLC, Kinetic C18 minibore column (Phenomenex), and AB Sciex QTRAP 4500 system. Analysis was carried out in negative ion mode, and eicosanoids and PUFA were quantitated using scheduled multiple reaction monitoring (MRM) using from 4 to 6 specific transition ions for each analyte. We used specific retention time and established prominent fragment ions for identification and quantification of LXB₄ and LXA₃ (52, 71, 72). Prominent fragment ions for identification of LXB₄, (351.2 m/z, retention time 8.9 minutes) included 115.1, 189.2, 221.1, 217.3, 251.1, and for LXA₃ (351.2 m/z, retention time 10.5 minutes) fragment ions included 115.1, 135.1, 189.2, 217.3, and 235.3. Calibration curves were established with synthetic standards (Cayman Chemicals).

**qPCR.** Mouse retinal mRNA was isolated using RNasey Isolation Kit (QIAGEN), quantified using NanoDrop, and mRNA reverse transcribed with the High Capacity cDNA Kit (Applied Biosystems). qPCR was performed with SYBR Green Master Mix (Applied Biosystems) using the ΔΔCt method in the StepOnePlus qPCR System (Applied Biosystems). β-Actin was used as the reference gene. The primers used were as follows: β-actin: forward, AGGGCGAGGTCTACCATTTG, reverse, AGGGCCCGACTCATGTA; 5-LOX: forward,
ACTACATCTACCTCAGCCTCATT, reverse, GGGACAGTCTGAGACGTCAC; 12/15-LOX: forward, GCGGCGTGGCCCAATCTGTAATC, reverse, ATATGGCCACCGTGTGTTCAC; FPR2 (ALX): forward, GCCAGAACCTTCTGAGGAGAT, reverse, GATGAACGTGGACCTGATACACT.

Immunofluorescence microscopy. Following fixation, eyes were equilibrated in 30% sucrose, embedded in optimal cutting temperature compound, and cryosectioned. Sections were blocked and probed with primary antibodies to GFAP (Sigma-Aldrich), CD68, GR-1 and F4/80 (BioLegend), FPR2 (Abnova), RBPMS (PhosphoSolutions), BRN3a (Santa Cruz Biotechnology Inc.), and 5-LOX (Millipore) according to standard protocols. Following PBS-Tween washes, sections were incubated with fluorescent-conjugated secondary antibodies (Molecular Probes) and mounted with medium containing DAPI and a Nikon Eclipse-Ti confocal microscope.

Quantification of RGC survival. For in vivo experiments, RBPMS-positive cells in the GCL were counted and expressed as a fraction of total GCL nuclei. For each eye, at least 5 central retinal sections were analyzed at the level of the ON to the periphery and the results averaged, as previously described (14). In parallel, TUNEL-positive GCL nuclei were counted and expressed as a fraction of the total GCL nuclei (58, 73–76). For in vitro experiments, RGCs were isolated, treated, and probed with an antibody to β3-tubulin and imaged as above. A total of 32 frames from each experimental group were scored for the number of cell bodies and intact neurites. The ratio of neurites to RGC cell bodies was established for each acquired frame.

Rat chronic IOP-elevation model. A method for reliably inducing sustained IOP elevation in the rodent eye was recently developed by Liu et al. (32, 77). In this minimally invasive approach, a circumlimbal suture is placed to induce mild chronic ocular hypertension for up to 15 weeks (32). Elevated IOP is the primary clinical glaucoma risk factor, and this model results in similar compromised RGC function, reduced RNFL thickness, and death of RGCs by week 15 (32). Therefore, this model was used to measure the impact of elevated chronic IOP (32, 81). In addition, RNFL and total retinal thickness were quantified once a month using OCT. After 15 weeks, animals were euthanized and RGC numbers were counted on central (2000 μm from ON head) and peripheral (4000 μm from ON head) regions of retinal flatmounts following immunofluorescent staining for BRN3a as above.

Statistics. For all experiments, n refers to the number of animals or biological replicates. Dose-response data for LXA4 and LXB4 were fitted to logistic curves, using SigmaPlot 11.0 (Systat Software Inc.) and used to determine Emax (efficacy) and EC50 values. For TUNEL staining and RGC counts, statistical analyses were performed by t test or 1-way ANOVA with Tukey’s post hoc analyses. ERG and OCT results were analyzed by 2-way ANOVA with Bonferroni’s post hoc test.

Study approval. All experimental protocols were approved by the University Health Network and University of California at Berkeley’s ACUCs in accordance with applicable regulations.

Author contributions. ILB conceived, performed, and analyzed in vitro and in vivo experiments, prepared figures, and helped write the manuscript. JW conceived and performed lipidomic analyses and PCR and prepared figures. HHL performed and analyzed the chronic IOP model and prepared figures. SA, AT, and GJW conceived and performed in vitro experiments. KG and JGF conceived experiments, analyzed data, and helped with manuscript editing. JMS conceived experiments, analyzed data, prepared figures, and wrote the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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