The lysophosphatidic acid axis in fibrosis: Implications for glaucoma

Amy O'Regan MB, BCh BAo | Colm J. O’Brien MD, FRCS FARVO | Sarah B. Eivers PhD

Abstract

Glaucoma is a common progressive optic neuropathy that results in visual field defects and can lead to irreversible blindness. The pathophysiology of glaucoma involves dysregulated extracellular matrix remodelling in both the trabecular meshwork in the anterior chamber and in the lamina cribrosa of the optic nerve head. Fibrosis in these regions leads to raised intraocular pressure and retinal ganglion cell degeneration, respectively. Lysophosphatidic acid (LPA) is a bioactive lipid mediator which acts via six G-protein coupled receptors on the cell surface to activate intracellular pathways that promote cell proliferation, transcription and survival. LPA signalling has been implicated in both normal wound healing and pathological fibrosis. LPA enhances fibroblast proliferation, migration and contraction, and induces expression of pro-fibrotic mediators such as connective tissue growth factor. The LPA axis plays a major role in diseases such as idiopathic pulmonary fibrosis, where it has been identified as an important pharmacological target. In glaucoma, LPA is present in high levels in the aqueous humour, and its signalling has been found to increase resistance to aqueous humour outflow through altered trabecular meshwork cellular contraction and extracellular matrix deposition. LPA signalling may, therefore, also represent an attractive target for treatment of glaucoma. In this review we wish to describe the role of LPA and its related proteins in tissue fibrosis and glaucoma.

KEYWORDS
fibrosis, glaucoma, lysophosphatidic acid

1 | INTRODUCTION

Fibrosis is a dysregulated and persistent state of wound healing that results in deposition of extracellular matrix (ECM) components, tissue remodelling and increased tissue stiffness. This progressive remodelling compromises tissue structure over time, resulting in tissue dysfunction or organ failure.1 Glaucoma is a common progressive optic neuropathy characterized by retinal ganglion cell loss and...
cupping of the optic nerve head, leading to visual field defects. It is the second most common cause of blindness, estimated to affect 76 million people worldwide. The incidence of glaucoma increases with age, with 3.3% of those aged over 70 affected. Other than age, the primary risk factor for development of glaucoma is elevated intraocular pressure (IOP). IOP is generated in the anterior chamber of the eye by a balance of aqueous humour (AH) production by the ciliary epithelium, and AH drainage through the trabecular meshwork (TM) and Schlemm's canal (SC) (Figure 1). Glaucoma pathogenesis involves fibrosis in both the TM in the anterior chamber of the eye, as well as in the lamina cribrosa (LC) of the optic nerve head at the back of the eye (Figure 1). Fibrosis in these two regions of the eye in glaucoma result in elevated IOP and optic disc cupping, respectively.

The LC is a region of perforated fibroelastic plates through which retinal ganglion cell axons pass as they converge to become the optic nerve. In glaucoma, the ECM in the LC is markedly disturbed. It is within this region that retinal ganglion cell axons degenerate resulting in glaucomatous visual field defects. Optic nerve head cupping in glaucoma occurs due to a combination of pre-laminar thinning, as well as laminar deformation through posterior displacement, posterior curvature and thinning of the LC (Figure 2). The cells that populate the LC include astrocytes and LC cells. LC cells are localized exclusively to the LC region, situated between cribriform plates. Phenotypically, LC cells are similar to myofibroblasts, which are the key cellular mediators in wound healing and fibrosis. LC cells are flat and polygonal and constitutively express alpha smooth muscle actin (α-SMA), fibronectin and collagen type I. ECM components including collagen type I (encoded by COL1A1 and COL1A2), V (COL5A1) and XI (COL11A1) are typically overexpressed by LC cells from glaucoma patient donors compared with normal. Multiple factors such as cyclical stretch, hypoxia and transforming growth factor-β (TGF-β) have been shown to upregulate the expression of genes involved in ECM synthesis by LC cells. In conjunction with this, increased stiffness of the cell microenvironment also has been shown to induce a myofibroblastic phenotype in LC cells. In glaucoma, the architecture of the cribriform plates in the LC is disrupted through compression, stretching and remodelling. Eventually the LC becomes thinned and stiffer.

The TM consists of beams and sheets of ECM through which aqueous humour passes into SC to exit the anterior chamber. In glaucoma, ECM alterations and increased tissue stiffness leads to increased resistance to aqueous humour outflow, and elevated IOP. The TM and the LC are biochemically comparable tissues, with similar cellular properties and gene expression. The alterations in ECM gene expression observed in the TM in primary open angle glaucoma (POAG) resemble those observed in the LC, therefore, it is hypothesized that the fibrotic changes affecting both of these regions in glaucoma may result from a common defect. Furthermore, simulation of raised IOP with mechanical stretch leads to similar

**FIGURE 1** Anatomy of the eye and aqueous humour circulation system: The eye is composed of an anterior chamber and a posterior chamber. The anterior chamber contains aqueous humour, which is produced by the epithelium on the ciliary processes. Aqueous humour flows around the iris and drains through the trabecular meshwork and Schlemm's canal. Intraocular pressure is maintained by a balance of aqueous humour production versus outflow. In glaucoma, fibrosis and increased stiffness in the trabecular meshwork and Schlemm's canal leads to increased resistance to aqueous humour outflow and resultant high intraocular pressure. The optic nerve head is damaged in glaucoma which results in irreversible visual field defects. The lamina cribrosa is a region of perforated fibroelastic plates through which retinal ganglion cell axons pass before they become the optic nerve. In glaucoma there is fibrosis in the lamina cribrosa which contributes to visual loss through retinal ganglion cell axonal degeneration.
upregulation of TGF-β1, α-SMA and collagen types I and IV in both regions. Human TM cells bear similarities to myofibroblasts, as well as possessing endothelial cell-like characteristics. A recent publication on single cell transcriptomics in the TM described both fibroblast-like and myofibroblast-like types of TM cells. TM cells have previously been described by their location within three distinct layers; the uveal, corneoscleral or juxtacanalicular cribriform layer. Uveal and corneoscleral TM cells behave like endothelial cells, whereas those located in the juxtacanalicular tissue adjacent to the endothelial cells of SC resemble myofibroblasts, expressing proteins such as α-SMA and myosin. TM cells are also found to express collagen types I, III, IV, V and VI, fibronectin and laminin. TM cells rapidly turnover these ECM proteins through matrix metalloproteinases which are constitutively expressed in this region. In POAG, ECM components accumulate in the TM which leads to increased AH outflow resistance. Glaucoma is also associated with altered contraction of TM cells and oxidative damage leading to tissue stiffness.

Biological factors such as TGF-β and cellular communication network factor 2 (CCN2) recruit, activate and promote survival of myofibroblasts in wound healing and fibrosis. These mediators are overexpressed in glaucoma, leading to ECM changes. Recently, the lipid mediator lysophosphatidic acid (LPA) has attracted much attention for its role in human pathology. Dysregulated signalling mediated by LPA has been shown to play an important role in many disease processes such as cancer, atherosclerosis, and fibrotic diseases including glaucoma. This review will focus on the role of the LPA axis in tissue fibrosis and glaucoma.

2 THE LYSOPHOSPHATIDIC ACID AXIS

LPA is a bioactive glycerophospholipid that is essential for a variety of cellular and developmental processes. It is structurally composed of a glycerol backbone, a single fatty acyl chain and a free phosphate group. LPA is abundant in serum, extracellular fluid, intracellular fluid and aqueous humour. LPA levels in serum are much higher than in plasma, due to release of its precursor lysophosphatidylcholine (LPC) from activated platelets.

Extracellular LPA binds to at least six different G-protein coupled receptors on the cell membrane (LPA1-6) to regulate intracellular signalling. LPA1-3 receptors are classed as Endothelial Differentiation Gene (Edg), while LPA4-6 are non-Edg receptors. The seven-transmembrane LPA receptors are coupled to the following Gα sub-units: Gα12/13, Gαq/11, Gαi/o and Gαs (Table 1). These G proteins activate a variety of intracellular pathways (Figure 3). Gα12/13 stimulates Rho/Rho kinase activity which leads to cytoskeletal rearrangements, promoting cell motility as well as focal adhesion formation. Gαq/11 regulates intracellular calcium levels through phospholipase C (PLC). Gαs activates adenylyl cyclase (AC) to increase cyclic adenosine monophosphate (cAMP) levels, which have been implicated in TM cell function.

**Table 1** G-proteins known to couple to each of the LPA receptors

| G-protein | Downstream effects |
|-----------|--------------------|
| LPA1      | Gα12/13, Gαq/11, Gαi/o | Activation of Rho, PLC, PI3K, Ras/MAPK |
|           |                     | Inhibition of AC |
| LPA2      | Gα12/13, Gαq/11, Gαi/o | Activation of Rho, PLC, PI3K, Ras/MAPK |
| LPA3      | Gαq/11, Gαi/o       | Activation of PLC, PI3K, Ras/MAPK |
| LPA4      | Gα12/13, Gαq/11, Gαi/o | Activation of Rho, PLC, PI3K, Ras/MAPK |
|           |                     | Activation or inhibition of AC |
| LPA5      | Gα12/13, Gαq/11, Gαi/o | Activation of Rho, PLC, PI3K, Ras/MAPK |
| LPA6      | Gαq/11              | Activation of Rho |

**Figure 2** Cupping of the optic nerve head in glaucoma. Retinal ganglion cell axons pass through a region of perforated fibroelastic plates known as the lamina cribrosa as they converge to become the optic nerve. In the normal optic nerve head, the lamina cribrosa functions to provide mechanical and biological support to these axons as they exit the eye. In glaucoma, cupping of the optic nerve head occurs due to both pre-laminar thinning of the retinal nerve fibre layer, and laminar deformation. Deformation of the lamina cribrosa involves posterior migration within the sclera, increased posterior curvature and laminar thinning.

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| LPA3      | Gαq/11, Gαi/o       | Activation of PLC, PI3K, Ras/MAPK |
| LPA4      | Gα12/13, Gαq/11, Gαi/o | Activation of Rho, PLC, PI3K, Ras/MAPK |
|           |                     | Activation or inhibition of AC |
| LPA5      | Gα12/13, Gαq/11, Gαi/o | Activation of Rho, PLC, PI3K, Ras/MAPK |
| LPA6      | Gαq/11              | Activation of Rho |
monophosphate (cAMP) concentration.\(^{52}\) The \(G_{\alpha_{i/o}}\) subunit is versatile, activating phosphatidylinositol-3-kinase (PI3K),\(^{53}\) PLC,\(^{54}\) Ras/Mitogen activated protein kinase (MAPK)\(^{55}\) and inhibiting AC leading to reduced cAMP levels.\(^{56}\) The effects of this include enhanced cell proliferation, suppressed apoptosis and enhanced cell migration.\(^{54}\)

LPA signalling is important in a wide range of biological processes, where it can promote cell proliferation, transcription, migration and survival.\(^{47}\) It is required for embryonic vasculogenesis\(^{57}\) and neural development.\(^{58}\) Significantly, LPA signalling is important for wound repair, which will be discussed further below. Dysregulated LPA signalling results in chronic inflammation\(^{59}\) and has a major role in cancer progression and metastases,\(^{47}\) as well as fibrosis.\(^{60}\)

The majority of extracellular LPA is produced by a secreted enzyme Autotaxin (ATX) in the extracellular space.\(^{61}\) ATX is a member of the ecto-nucleotide pyrophosphatase/phosphodiesterase (ENPP) family, a group of enzymes that degrade nucleotide phosphates. ATX, encoded by the \(ENPP2\) gene, is the only member of this family that possesses lysophospholipase-D activity capable of generating LPA.\(^{62}\) Alternative splicing results in five isoforms, denoted \(\alpha, \beta, \gamma, \delta\) and \(\varepsilon\), respectively.\(^{63-65}\) All five isoforms are catalytically active, with the \(\beta\) isoform the most prominent form.\(^{65}\) ATX converts lysophospholipids, predominantly LPC, to LPA (Figure 4). Serum ATX is thought to be secreted mostly by adipose tissue.\(^{66}\) ATX possesses two somatomedin B-like domains, a central phosphodiesterase domain where its catalytic site is, and a nuclease-like domain.\(^{67}\) The somatomedin B-like domains may allow binding of ATX to integrins, resulting in localized LPA production close to the receptors it will act on.\(^{68}\) ATX is essential in embryonic development for vasculogenesis. Of note, \(Enpp2\) knockout in mice is embryonically lethal.\(^{69}\)

Additionally, LPA can be synthesized intracellularly via a number of pathways (Figure 4). Phosphatidic acid (PA) can be converted to LPA by phospholipase A1 (PLA1) or phospholipase A2 (PLA2).\(^{47}\) LPA can also be synthesized de novo from glycerol 3 phosphate (G3P) through the action of glycerophosphate acyl transferase (GPAT).\(^{70}\) Moreover, phosphorylation of mono-acyl glycerol (MAG) by mono-acyl glycerol kinase (MAGK) produces LPA.\(^{71}\)
LPA is rapidly degraded by lipid phosphate phosphatases (LPPs). The LPPs consist of three integral membrane enzymes—LPP1 (and a splice variant LPP1a), LPP2 and LPP3, which are encoded by three separate genes PPAP2A, PPAP2C and PPAP2B, respectively. LPPs located in the plasma membrane possess six transmembrane domains with their amino and carboxy termini in the cytosol, and catalytic domains on the extracellular side. LPPs metabolize LPA and sphingosine-1-phosphate (S1P) outside the cell by dephosphorylation. This "ecto-activity" of LPPs terminates LPA signalling and its downstream effects. Extracellular LPA availability is, therefore, dependent on rate of synthesis by ATX, as well as rate of degradation by the LPPs. LPPs are also present inside the cell, on internal membranes such as in the endoplasmic reticulum, where their catalytic domains are presumed to face the luminal side. It has been suggested that intracellular LPPs may modify signal transduction inside the cell by regulating intracellular concentrations of various lipid phosphates, for example by converting phosphatidic acid to diacylglycerol. The LPPs have been found to have other functions outside of their catalytic activity. LPP3 possesses an arginine–glycine–aspartate sequence which can facilitate cell–cell interactions by binding to αvβ3 and αvβ1 integrins. Both of these integrins are involved in normal wound healing and fibrosis, including in fibroblast migration and differentiation into myofibroblasts. LPP3 has also been linked to signalling via the Wnt pathway and may regulate β-catenin stability.

3 | THE LYSOPHOSPHATIDIC ACID AXIS IN FIBROSIS

It has been established that pathways activated by LPA are involved in wound healing and chronic fibrosis. In response to inflammation post-injury, ATX is produced to promote wound repair. High levels of ATX and LPA have been shown to persist in chronic inflammatory settings, cancer states and fibrotic diseases.

3.1 | Lysophosphatidic acid axis in wound healing

Findings in human skin bullae support a role for the LPA axis in wound healing. LPA is found in high levels in skin bullae where there is also upregulation of LPA4 and ATX. Interestingly, topical application of LPA to animal wounds has been shown to accelerate wound healing. The stages of normal wound healing include hemostasis, inflammation, proliferation, remodelling and wound contraction. Activated platelets release LPA and LPC which can be converted to LPA by ATX. LPA stimulates proliferation of inflammatory cells and acts as a chemoattractant to recruit monocytes to injured tissues during the inflammatory phase of wound healing. Subsequently LPA regulates proliferation, migration, and contraction of fibroblasts. LPA treatment of fibroblasts causes immediate upregulation of transcription factors associated with cell cycle progression and growth. Of note, proliferation of fibroblasts is reduced LPA1 receptor null mice. As well as proliferation, LPA also has an important role in migration and contraction of fibroblasts by inducing cytoskeletal changes, permitting formation of actin stress fibres and focal adhesions. Cell migration induced by LPA involves activation of both RhoA and Rac GTPases via G12/13 and G13, respectively. Myofibroblast contraction in the later stages of wound healing involves LPA acting through G12/13. Rho and Rho associated protein kinase (ROCK). ROCK activity permits myosin light chain (MLC) phosphorylation by decreasing activity of MLC phosphatase. The Rho kinase inhibitor Y-27632, therefore, attenuates LPA mediated myofibroblast contraction. Actin stress fibre formation and cell contraction mediated by LPA stimulate fibronectin matrix assembly.

The main function of ATX in the postnatal organism appears to be in wound healing. Fibroblasts are among the cells that express the highest levels of ATX mRNA in the body. ATX is responsible for the majority of extracellular LPA production, which it generates from LPC, the most abundant phospholipid in plasma. ATX and LPA have been found to have other functions outside of their catalytic activity. LPP3 possesses an arginine–glycine–aspartate sequence which can facilitate cell–cell interactions by binding to αvβ3 and αvβ1 integrins. Both of these integrins are involved in normal wound healing and fibrosis, including in fibroblast migration and differentiation into myofibroblasts.

3.2 | Lysophosphatidic acid axis in pathological fibrosis

Dysregulated wound healing in response to chronic injury stimuli and inflammation is central to the development of fibrotic disease states. ATX and LPA are present at very high levels in both human and animal models of organ fibrosis. ATX expression is increased by inflammatory cytokines tumour necrosis factor (TNF) and interleukin-1β (IL-1β). These mediators have been shown to overcome the inhibition of ATX expression by LPA and S1P signaling, resulting in further synthesis of LPA. LPA enhances expression of inflammatory cytokines IL-1β, IL-8 and monocyte chemoattractant protein-1 (MCP-1) in endothelial cells via LPA1 and LPA3. In the setting of pathological fibrosis, LPA promotes recruitment and survival of fibroblasts resulting in excess deposition of ECM proteins.

TGF-β is a major regulator of fibroblast activity in disease. In both human bronchial epithelial cells and mouse proximal tubule cells, LPA was shown to induce αvβ6 integrin-mediated TGF-β activation via its LPA2 receptor. TGF-β has been shown to recruit fibroblasts, induce their differentiation into myofibroblasts, promote ECM deposition and myofibroblast survival. LPA also increases the expression of CCN2, another key player in tissue remodelling and organ fibrosis. CCN2, which was previously known as connective tissue growth factor, promotes differentiation of recruited fibroblasts and epithelial cells into myofibroblasts and mediates TGF-β induced synthesis of the ECM components fibronectin and collagen. LPA treatment of renal fibroblasts was shown to upregulate CCN2 expression via G12/13 and Rho proteins. In myofibroblasts, CCN2 upregulation by LPA required transactivation of TGF-β receptors and the presence of Smad-2/3. Furthermore, LPA induction of actin stress fibre formation, but not proliferation, required transactivation of TGF-β receptors. Treatment with LPA and TGF-
β1 generated an additive effect on the expression of CCN2 in renal fibroblasts.104

The LPA axis has a major role in idiopathic pulmonary fibrosis (IPF). In a mouse model of pulmonary fibrosis, LPA was significantly elevated in bronchoalveolar lavage fluid after bleomycin injury.60 Moreover, mice lacking Lpa1 exhibited less fibrosis and reduced collagen expression following bleomycin challenge, where LPA was found to mediate fibroblast recruitment and vascular leak through the LPA1 receptor.60 In primary mouse lung fibroblasts, LPA acting via LPA1 receptors promoted resistance to apoptosis.97 In line with these discoveries in mice, patients with IPF have also been reported to have higher LPA in bronchoalveolar lavage fluid compared with normal controls.60 ATX is known to be overexpressed in IPF and fibrotic non-specific interstitial pneumonia patients compared with other interstitial lung diseases and patients controls.105 Genetic deletion of ATX from bronchiolar epithelial cells in bleomycin treated mice (Enpp2−/−) lead to reduced collagen production and reduced infiltration of inflammatory cells.105 Pharmacological inhibition of ATX with GWJ-A-23 in bleomycin challenged mice resulted in decreased expression of TGF-β.105

Dysregulation of the LPA axis has also been reported in liver fibrosis. Hepatic stellate cells are activated in liver fibrosis and transform into myofibroblasts.106 LPA treatment of hepatic stellate cells was shown to enhance cell proliferation,106 and cellular contraction.107 ATX has been used as a biomarker for liver fibrosis in patients with hepatitis C infection.108 Serum levels of ATX were found to correlate with stage of liver cirrhosis.109 Liver specimens from infants with biliary atresia were also shown to express higher levels of ATX compared with normal.110 Higher ATX levels were correlated with stage of fibrosis and poorer prognosis in these patients.110 Interestingly, patients with later stage biliary atresia have reduced methylation of the ATX promoter, resulting in ATX over-expression.111 In a rat model of cirrhosis, treatment with either the ATX inhibitor AM063 or the LPA1 inhibitor AM095 in vivo lead to attenuated liver fibrosis associated with significantly reduced expression of Ccn2, a-Sma and Col1a1.112

LPA has also been shown to have a role in other forms of pathological fibrosis. In a mouse model of renal fibrosis, both LPA and Lpa2 receptor expression were increased.95 Both knockout of Lpa2 in mice and pharmacological inhibition of LPA2 receptors with Ki16425 attenuated hallmarks of fibrosis, with reduced Col2a1 and α-SMA expression.113 Ki16425 has additionally shown beneficial results in a mouse model of scleroderma, reducing myofibroblast accumulation and the expression of fibrogenic cytokines and Col1a1.114 Expression of the LPA2 receptor was upregulated after rat renal ischemia-reperfusion injury in vivo.110

There is less work published on LPPs in fibrosis. Lpp3 is upregulated in a state of vascular injury.115 In human aortic endothelial cells, LPP3 silencing lead to increased expression of pro-inflammatory mediators IL1β, IL-6, IL-8 and MCP-1.116 It has, therefore, been suggested that LPP3 attenuates vascular inflammation.116,117 LPPs have been studied extensively in the setting of cancer however, which is commonly described as a “wound that does not heal.” In many cancers, expression of the LPPs is low,118 which allows LPA to act for longer. In ovarian cancer for example, very high levels of LPA are present in peritoneal fluid,119 and LPP1 is consistently underexpressed.118 Overexpression of LPP1 and LPP3 in ovarian cancer cells inhibits proliferation and markedly increases apoptosis.120,121 In contrast to this, LPP expression has been found to be increased in some other cancers.118,119 In glioblastoma, LPP3 was shown to be required for tumor adaptation, with LPP3 knockdown leading to decreased cell proliferation and migration mediated through β-catenin/cyclin D1 cell cycle controls.177 LPP2 was upregulated in bladder and cervical cancer but downregulated in CNS cancers and sarcoma.118 The role of LPPs in pathophysiology is, therefore, not limited to their degradation of LPA and appears to be more complex.

ECM deposition and fibrosis are major features of diabetic nephropathy.122 In a rat model of type 2 diabetes mellitus, reduced expression of Lpp3 and increased deposition of collagen was observed.123 Furthermore, transfection of rat proximal tubule NRK-52E cells with micro (mi)RNA 184 caused downregulation of Lpp3 and produced a profibrotic phenotype, increasing Ccn2 expression.123 Over-expression of Lpp1 lead to inhibition of LPA-mediated migration in Rat2 fibroblasts but had no effect on platelet derived growth factor or endothelin-induced fibroblast migration.124 This action, however, was not explained by the ecto-activity of LPP1, where fibroblast migration was not dependent on extracellular LPA degradation. The authors, therefore, concluded that the enzyme also had an effect on LPA signalling downstream of its receptor activation.124

3.3 Therapeutic strategies in fibrosis

The LPA signalling pathway has been targeted in humans for treatment of pathological fibrosis. Inhibitors of both ATX and LPA receptors have been developed. As mentioned previously, the LPA1 receptor was shown to mediate fibroblast recruitment and survival resulting in lung fibrosis in bleomycin treated mice.60 These findings lead to the development of a LPA1 receptor antagonist, BMS-986020, for treatment of IPF in humans. BMS-986020 underwent a phase II clinical trial for IPF,125 but unfortunately the trial was terminated early due to an adverse event of cholecystitis occurring in three of the patients treated. A small molecule ATX inhibitor GLPG1690 is currently in phase III clinical trials for IPF,126 having shown promising results on the preservation of forced vital capacity of the lungs in phase II trials.127 BBT-877 is another ATX inhibitor that has been developed for IPF.128 It underwent phase I trials, demonstrating safety and tolerability as well as an 80% reduction in plasma LPA levels.128 In addition to IPF, the LPA axis has been targeted for treating liver and skin fibrosis. BLD-0409 is an ATX inhibitor undergoing phase I clinical trials for use in non-alcoholic steatohepatitis and chronic liver fibrosis.129 A specific antagonist of the LPA1 receptor, SAR100842, underwent phase II clinical trials for systemic sclerosis and demonstrated an improvement in Modified Rodnan Skin thickness Score.130 Further development of this drug, however, was terminated for undisclosed reasons.131
THE LYPHOSPHATIDIC ACID AXIS IN GLAUCOMA

There is increasing evidence that the LPA axis may play a significant role in the pathogenesis of glaucoma. As previously mentioned, pathogenesis of glaucoma involves fibrosis in both the anterior chamber and in the optic nerve head. This section will examine the role of the LPA axis in both of these regions, as well as in tenons fibroblasts which are of relevance in fibrosis after glaucoma drainage surgery.

4.1 Lysophosphatidic acid and autotaxin in aqueous humour

The level of LPA and ATX in the AH in glaucoma patients strongly supports a role for the LPA axis in glaucoma pathogenesis. Glaucoma patients were shown to have significantly higher levels of LPA, its precursor LPC, and ATX in the AH, compared with those without glaucoma. This was particularly significant in those with secondary open angle glaucoma including pseudoexfoliation glaucoma, a form of glaucoma caused by the accumulation of white, flaky, pseudoexfoliative material in the trabecular meshwork. Levels of ATX in the AH were positively correlated with higher IOP readings in patients with glaucoma, where ATX inhibition with topical application of the small molecule inhibitor S32826 reduced IOP. The profibrotic mediator CCN2 was also significantly increased in the AH of glaucoma patients, and these levels positively correlated with ATX levels in the AH. It has been postulated that the levels of ATX and TGF-β1, TGF-β2, and TGF-β3 in the AH could be used as biomarkers for differentiating glaucoma subtypes. A recent study showed that ATX and TGF-β1 and β3 levels were higher in pseudoexfoliation glaucoma and other forms of secondary open angle glaucoma than in POAG or normal patients. Conversely, levels of TGF-β2 were highest in AH from POAG patients. Levels of ATX and TGF-β1 were significantly associated with each other in AH taken from patients with secondary open angle glaucoma, leading the authors to speculate that there is crosstalk between ATX and other pro-fibrotic mediators in glaucoma.

ATX levels in the AH have been positively correlated to post-operative corticosteroid use after microhook ab interno trabeculotomy for POAG. Trabeculotomy is a procedure which involves cleaving the TM and inner wall of SC to reduce resistance to AH outflow in glaucoma patients. Post-operative corticosteroids are associated with IOP spikes, and therefore their use post microinvasive glaucoma surgery is controversial. Patients who did not receive post-operative corticosteroids demonstrated significantly lower ATX levels in the AH 1 week post-operatively and required significantly less IOP-lowering medications at 3 months versus those who did received steroids. Post-operative topical corticosteroids are, therefore, associated with reduced success of microhook ab interno trabeculotomy, likely due to ATX-mediated fibrosis in the TM.

4.2 Lysophosphatidic acid signalling in the trabecular meshwork and Schlemm's canal

As stated previously, the TM region undergoes significant ECM remodelling/fibrosis in glaucoma, resulting in reduced AH drainage through the TM and SC. Human TM and SC cells express LPA receptors LPA1 and LPA2. Under cyclic mechanical stretch, which can mimic fluctuations in IOP, the expression of LPA1, LPA3, LPA4, LPP1 and ATX in TM cells was significantly increased. LPA treatment of TM cells caused significant upregulation of LPA1 and LPA4 and downregulation of LPA2 and LPA4. Moreover, treatment of TM cells with IL-6 significantly increased LPA4.

Elevated IOP in glaucoma occurs as a result of increased resistance to AH outflow through the conventional pathway. The regulation of AH outflow is complex, but involves mechanical stretch, cytoskeletal changes, the ECM, and locally acting mediators including LPA (Figure 5). Perfusion of enucleated eyes with LPA decreased AH outflow by 37% from baseline. Treatment of TM cells with LPA lead to increased contractile activity through Rho GTPase activation and MLC phosphorylation, which likely contributes to the reduction in AH outflow in response to LPA. Rho GTPase promoted actin stress fibre formation and focal adhesion formation as well as MLC phosphorylation in TM cells, and these actions of LPA were blocked significantly by Y-27632, a Rho kinase inhibitor. MLC phosphorylation in response to LPA could also be promoted through an increase in intracellular calcium, which was
consistently observed when cells were treated with LPA. MLC phosphorylation was suppressed in TM cells treated with a combined LPA1/LPA3 receptor inhibitor Ki16425. Similar induction of actin cytoskeletal rearrangements and focal adhesion formation via Rho GTPase were observed when SC cells were treated with LPA, resulting in decreased outflow facility. The micro-RNA, miR-200c has been shown to downregulate LPA1 expression in TM and inhibits LPA mediated TM cell contraction. Delivery of miR-200c to the anterior chamber of rat eyes has also been shown to decrease IOP.

S1P, the sphingolipid analogue of LPA, also influences outflow facility in anterior chamber. S1P1 and S1P3 receptors are expressed in the TM and SC. As well as LPA, S1P has been shown to induce cytoskeletal changes, stimulate Rho and Rac GTPases and increase cell adhesive interactions in the TM and SC. In one study, S1P treatment decreased AH outflow facility by 36% in enucleated human eyes, although no morphological changes were observed in the SC that could explain this. Moreover, both S1P and LPA have been found to increase SC cell stiffness.

As well as inducing cytoskeletal changes, Rho GTPase activation by LPA leads to increased synthesis of ECM proteins in TM cells. ECM proteins are excessively deposited in the TM in glaucoma which contributes to increased resistance to AH outflow. Treatment of human TM cells with LPA increased expression of α-SMA, fibronectin and laminin in a Rho-dependent fashion. LPA induced α-SMA and fibronectin expression was also found to require ERK activation.

Mechano-transducing factors YAP and TAZ may also link contractility of TM cells to the expression of pro-fibrotic matricellular and ECM proteins. YAP and TAZ are important transcriptional coactivators in the Hippo pathway, which regulates cell growth and proliferation. Deyphosphorylated YAP and TAZ translocate to the nucleus and induce gene transcription by associating with transcriptional enhanced associate domain (TEAD) transcription factors. LPA treatment of TM cells activated YAP and TAZ via the LPA1 and LPA3 receptors, which in turn increased levels of CCN2, CYR61 and ECM proteins fibronectin, laminin, collagen I and α-SMA. LPA, IL-6 or a combination of LPA and IL-6 all elevated YAP and TAZ levels in TM cells. Pan-TEAD was markedly increased by a combination of LPA and IL-6, neither of which had an effect alone. This suggests that there is crosstalk between the respective signalling pathways of LPA and IL-6. Furthermore, LPA treatment upregulated signal transducer and activator of transcription 3 which is involved in IL-6 signaling. Consistent with previous studies, genes involved in cytoskeletal rearrangements MLC and ROCK1 were upregulated in LPA treated cells. In the presence of the YAP inhibitor verteporfin, upregulation of MLC and ROCK1 by LPA was blocked, and conversely these were downregulated. Verteporfin also blocked the upregulation of ECM proteins, CCN2 and CYR61 in response to LPA and/or IL-6. YAP and TAZ are, therefore, key downstream mediators of the ocular hypertensive phenotype induced by LPA. These results indicate that the combination of increased trabecular tone and ECM deposition mediated by LPA act synergistically to elevate intraocular pressure in glaucoma.

4.3 | Autotaxin in the trabecular meshwork

ATX in combination with its substrate LPC have been shown to decrease AH outflow in enucleated mouse eyes. ATX induces cytoskeletal reorganization in TM cells and plays a direct role in fibrogenic changes of the TM. Moreover, increased ATX expression was observed in TM specimens from glaucoma patients compared with controls using immunohistochemical methods. In an ATX-dependent manner, LPC treatment led to increased expression of CCN2, COL1A1 and FN in TM cells. ATX has also been implicated in the pathogenesis of Posner–Schlossman syndrome (PSS). PSS is a type of inflammatory glaucoma that is associated with cytomegalovirus (CMV) infection. Patients with PSS have high levels of ATX and TGF-β1 in the AH. CMV infection of cultured TM cells upregulated levels of both ATX and TGF-β1, and induced fibrotic responses which were blocked by inhibitors of ATX or LPA1 and LPA3. Furthermore, cyclic mechanical stretch induced upregulation of ATX in TM cells.

Corticosteroids are known to induce ocular hypertension and glaucoma through ECM deposition and cytoskeletal changes in the TM. The fibrogenic phenotype produced by dexamethasone in the TM has been shown to involve ATX and LPA receptor activation. Inhibition of ATX with S32826 significantly attenuated dexamethasone induced MLC phosphorylation and resultant cytoskeletal rearrangements, as well as inhibiting dexamethasone induced COL1A1 and COL4A1 overexpression. Treatment of human TM cells with dexamethasone, TNF or IL-1β produced a significant increase in ATX levels. This effect of TNF and dexamethasone was blocked by pre-treatment with an NF-Kβ inhibitor or ERK inhibitor, suggesting that they play a role in transcriptional regulation of ATX. This line with this, silencing of ATX resulted in attenuated fibrotic response to dexamethasone, TNF and IL-1β in the TM.

4.4 | Lysophosphatidic acid signalling in tenons fibroblasts

Trabeculectomy is a glaucoma drainage procedure that creates a passage from the anterior chamber of the eye through the sclera to the sub-tenons space. This allows aqueous humour to flow from the anterior chamber into a filtration “bleb.” An antimetabolite is usually used during the procedure to prevent bleb scarring and fibrosis which is mediated by tenons fibroblasts. LPA treatment of tenons fibroblasts resulted in actin cytoskeletal changes and increased α-SMA expression. The receptors LPA1 and LPA3 are abundantly expressed in human tenons fibroblasts. Inhibition of LPA signalling with the LPA1/LPA3 inhibitor Ki16425 attenuated the effect of TGF-β1 on human tenons fibroblasts, reducing proliferation and migration of these cells, and preventing their conversion to myofibroblasts. The authors hypothesized that this effect was due to suppression of Smad 2/3 activation, but not p38MAPK and ERK1/2 activation. Collagen matrix contraction was also decreased in the presence of an LPA1/LPA3 inhibitor. Expression of FN and COL1A1 in human tenons fibroblasts were upregulated by ATX, and this effect was
blocked with an ATX-inhibitor.\textsuperscript{156} The requirement for bleb needling with an anti-metabolite post trabeculectomy were significantly positively correlated to ATX levels in the AH of donors, indicating the prognostic value of ATX in determining fibrotic responses in the TM following trabeculectomy.\textsuperscript{156}

### 4.5 Lysophosphatidic acid signalling in the optic nerve head

There is less published on the LPA axis in the optic nerve head in glaucoma. However, increased signalling via the LPA axis has been shown to play a role in neuronal death in cerebral ischemia–reperfusion injuries\textsuperscript{157} and models of neurodegenerative diseases.\textsuperscript{158} ATX expression was found to be increased 13-fold in glaucomatous versus normal astrocytes.\textsuperscript{159} It has been suggested that ATX inhibitors PF-8380 and ATX-R are protective against retinal ganglion cell axonal degeneration in an autoimmune model of glaucoma in rats.\textsuperscript{160} In contrast to this, however, a group who examined lysosphospholipids in the cadaveric human optic nerve head found that levels of LPA and its precursor LPC were significantly lower in glaucoma compared with normal patients when measured using mass spectrometry.\textsuperscript{161} In the same study, diacylglycerol was found to be increased in glaucoma optic nerve heads compared with normal.\textsuperscript{161} LPP3 protein expression was significantly increased in glaucomatous optic nerve heads, which could explain, at least in part, the lower LPA levels observed in this study due to enhanced LPA degradation.\textsuperscript{161} Expression of ATX was not found to be significantly altered.\textsuperscript{161}

Hypoxia and oxidative stress are also thought to play a role in glaucoma pathogenesis at the optic nerve head.\textsuperscript{162,163} Retinal ganglion cells in the optic nerve head are highly susceptible to degeneration in response to hypoxia and reactive oxygen species. Hypoxia inducible factor-α (HIF-α) is overexpressed in optic nerve heads of glaucoma patients.\textsuperscript{162} Although there is a lack of evidence on the role of LPA in hypoxic injury in the optic nerve head in glaucoma, response of ovarian cancer cells to LPA signalling was found to be enhanced in hypoxic conditions.\textsuperscript{164} LPA treatment of multiple cancer cell lines under hypoxic conditions increased HIF-α expression.\textsuperscript{165} Overexpression of LPA\textsubscript{1}, in retinal ganglion cells increased susceptibility to hypoxia induced degeneration.\textsuperscript{166}

To the best of our knowledge, there is no data on the LPA axis specifically in the LC of the optic nerve head. As mentioned earlier, the LC is biochemically related to the TM, and the pro-fibrotic pathways that are dysregulated in glaucoma are similar in these two regions. Inhibition of ROCK, a prominent downstream mediator of LPA, has been shown to reduce the expression of fibrosis genes COL1A1 and TGF-B1 in response to cyclic mechanical stretch in the LC.\textsuperscript{167} Perhaps LPA is acting upstream of ROCK in the LC in glaucoma. Additionally, epigenetic regulation of ATX expression in the form of promoter hypomethylation was observed in patients with advanced biliary atresia, a fibrotic inflammatory disease of the liver.\textsuperscript{111} Promoter hypomethylation was associated with higher ATX expression, advanced stage of disease, liver dysfunction and increased tissue stiffness.\textsuperscript{111} DNA methylation has been widely implicated in regulating fibrotic diseases.\textsuperscript{168} We have previously shown that DNA methylation is an important factor regulating profibrotic genes such as TGF-β1 in glaucomatous LC cells.\textsuperscript{169} It could be possible that promoter methylation might be regulating ATX expression in LC cells, and therefore affecting downstream LPA availability and signalling. Further research is required in LC cells specifically to determine the role, if any, of the LPA axis in glaucomatous optic nerve fibrosis.

### 5 CONCLUSION

LPA and its related proteins are implicated in pathological fibrosis, including in the fibrotic changes observed in the TM in glaucoma, leading to decreased AH outflow and increased IOP. The role of the LPA axis in the optic nerve head in glaucoma is yet to be fully described. Our group have established that there is dysregulated ECM remodelling in the LC of the optic nerve head. It is within this region that retinal ganglion cell axons degenerate and produce visual field defects in glaucoma.\textsuperscript{8} To the best of our knowledge, there is no research published on LPA specifically in the LC. As the LC and the TM are biochemically similar tissues, it is plausible that LPA signalling may play a role in the ECM changes observed.

LPA signals via GPCRs, which are the receptors most frequently targeted by pharmacological agents.\textsuperscript{170} ATX represents a second promising drug target for fibrosis within the LPA axis. Of note, an inhibitor of ATX is currently in phase III trials for IPF. Whilst potentially targetable, it is important to bear in mind that ATX and LPA are also required for normal wound healing. This poses less of an issue in glaucoma as it does in fibrotic disease affecting other organs, as topical therapy can directly target the ocular structures involved, with minimal systemic absorption. Future research is required to ascertain whether LPA, its receptors or ATX are implicated in the glaucomatous changes of the optic nerve head and whether inhibiting LPA signalling can prevent fibrosis and resultant visual loss.

### CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

### ORCID

Amy O’Regan \( \text{https://orcid.org/0000-0003-0998-634X} \)

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