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The Role of Pro-resolving Lipid Mediators in Ocular Diseases

Jessica Wei¹ and Karsten Gronert^{1,2,3}

¹Vision Science Graduate Program, University of California Berkeley, Berkeley, CA 94720

²School of Optometry, University of California Berkeley, Berkeley, CA 94720

³Infectious Disease and Immunity Graduate Program, University of California Berkeley, Berkeley, CA 94720

1. Introduction

The tissue graft experiments by Peter Medawar launched the basic principles of ocular immune privilege in the 1940s, with the eye being one of the special organs able to tolerate foreign antigens without rejecting the donor tissue. The eye's unique ability to manage foreign antigen exposure without mounting an inflammatory response likely evolved from the ocular surface's constant contact with the environment, the recruitment of immune cells via the dense retinal vasculature and the evolutionary pressure to preserve vision as an essential primary sense. To provide visual function, the cornea, aqueous humor, vitreous humor and the fovea are avascular in order to undergo phototransduction and convert photons into electric signals for transmission to the visual cortex in the brain. The necessity of protecting the delicate visual axis is underscored by the fact that approximately 50% of the cerebral cortex function is dedicated to visual analysis and perception [1]. Hence, a case can be made that innate, adaptive and reparative immune responses in the visual axis are among the most highly evolved. Sight threatening inflammation could be triggered by routine exposure to microbes, pathogens, irritants, neutrophils (PMN) of nocturnal tears [2] at the ocular surface, or through aberrant immune responses resulting from injury or diseases in the neural retina. However, the transparent visual axis has evolved sophisticated mechanisms to tightly control inflammation, raise the threshold for triggering immune responses, and maintain active immune tolerance. Experiments from the past few decades have established immune privilege as active surveillance where immunosuppression and regulatory cells are actively deployed to avert full-fledged innate or adaptive immune responses [3, 4]. In addition, the eye effectively minimizes immune responses through physical barriers at two sites, the corneal epithelium and the blood retinal barrier. In the eye, specialized pro-resolving mediators (SPM) lipoxin A₄ (LXA₄) and neuroprotectin D1 (NPD1/PD1) were first identified as endogenous lipid mediators in the immune privileged cornea [5]. Ongoing studies have identified SPM as essential endogenous factors for

Corresponding author: Karsten Gronert, 594 Minor Hall, School of Optometry, University of California Berkeley, Berkeley, CA 94720. kgronert@berkeley.edu.

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maintaining immune homeostasis in the visual axis, uphold an elevated basal anti-inflammatory tone, amplify wound healing, control leukocyte functional responses, downregulate inflammatory responses and drive nerve regeneration and neuroprotection [6, 7, 8, 9].

Photoreceptors have the highest DHA content and retention in comparison to all cells in the body [10], providing an abundant substrate for 5- and 15- lipoxygenase (5-, 15-LOX) to potentially generate most of the ω -3 SPM resolvins, protectins and maresins in the retina. Several lines of evidence have established protection against macular degeneration and retinopathy with DHA-enriched diets [11, 12], which underscore the importance of endogenous levels of DHA-derived SPM in the eye. SPM circuits have provided new targets to combat dysregulated ocular innate and adaptive immune responses as current treatment options are limited and aimed at broad immune suppression. Treatment with resolvin E1 (RvE1), RvE1 analogs, LXA₄, NPD1/PD1 and resolvin D1 (RvD1) in murine and *in vitro* models of ocular diseases have established the efficacy of SPM as potential treatment options to limit inflammatory pathogenesis in the cornea and conjunctiva, prevent adaptive immune responses and protect against retinopathy due to injury or oxidative stress.

5- and 15-LOX are the rate-limiting enzymes responsible for generating SPM, especially the eicosanoid LXA₄, which was the first identified SPM [13] and is generated in many tissues of the eye. A striking feature of the cornea is the high expression of 15-LOX in human and mouse corneal epithelial cells. 15-LOX-LXA₄-ALX/FPR2 circuit has been identified as an important resident circuit that controls wound healing and immune responses at the ocular surface. Two 15-LOX enzymes (*ALOX15*, *ALOX15B*) have been identified in the human cornea [14, 15, 16] and the mouse homolog 12/15-LOX (*Alox15*) is expressed in the corneal epithelium, retinal pigment epithelium (RPE) and lens [17, 18]. Knockdown of 15-LOX (*ALOX15*) in the human RPE increased susceptibility to oxidative stress induced apoptosis [19]. More importantly, 12/15-LOX knockout mice exhibit a phenotype of impaired LXA₄ formation and induction of cytoprotective heme oxygenase-1 (HO-1), which correlated with exacerbated inflammation and delayed wound healing [20, 21]. 5- and 15-LOX dependent formation of LXA₄ and/or NPD1 has been established in the cornea, retina and ocular draining lymph nodes and 15-LOX has a key role in preventing RPE cell death during oxidative stress and pathological angiogenesis in the avascular cornea [8, 18].

SPM exert their actions through G-protein coupled receptors (GPCR) that are widely expressed in the eye. ALX (also called FPR2), the GPCR for LXA₄ and its DHA-homolog RvD1, is expressed in the corneal epithelium, RPE, PMN, macrophages, T cells [22, 23, 24] and conjunctiva [25]. In humans, GPR32 has been identified as a second receptor for LXA₄ and RvD1 [26] and its expression has been established in conjunctival goblet cells [27] and human retinal endothelial cells [28].

Emerging evidence has identified endogenous SPM circuits in the ocular surface, retina and draining lymph nodes of the eye. These intrinsic protective circuits have key roles in immune regulation, injury responses, infection, inflammatory neovascularization, neuropathy and autoimmunity. The concept of SPM as promising therapeutic targets has been validated in pre-clinical animal models of ocular diseases and early clinical trials with

RvE1 analogs in Dry Eye Disease (DED), which have established the efficacy and broad protective action of local and systemic treatment with SPM [29, 30, 31]. This review aims to provide an overview of the endogenous role of SPM in ocular trauma and diseases (Figure 1).

2. Specialized Pro-resolving Mediators in Ocular Diseases

2.1 Corneal Injury and Wound Healing

The cornea is predominantly composed of the corneal epithelium, the stroma and a monolayer of corneal endothelium lining the inner surface. The corneal epithelium maintains tight junctions that prevent microbes, viruses and irritants from penetrating the cornea. The essential role of SPM circuits in the cornea is supported by the high expression of 12/15-LOX circuit in the corneal epithelium [14–16], and both human and mouse corneal epithelial cells can generate LXA₄. Mouse models of acute corneal injury have demonstrated that loss of epithelial cells or genetic deletion of 12/15-LOX abrogated LXA₄ formation in the cornea and led to impaired epithelial wound healing, while topical treatment with LXA₄, NPD1 as well as 17-HDHA, a metabolic precursor for several members of the DHA-resolvin family, accelerated re-epithelialization in acute corneal abrasions [5, 8].

Contrary to LXA₄'s established role of inhibiting PMN recruitment in various disease models, both topically administered and endogenously formed LXA₄ and NPD1 increase PMN numbers after epithelial injury in the cornea [5]. The requirement of PMN for normal corneal wound healing and nerve regeneration in acute and self-resolving epithelial injury responses is well established [32, 33]. Multiple lines of evidence also suggest that these wound healing PMN as well as nocturnal tear PMN are a non-inflammatory phenotype [2] that is distinct from stereotypical vascular inflammatory PMN. Unlike LXA₄'s endogenous role in healthy and self-resolving corneal inflammatory-reparative responses, topically added or endogenous LXA₄ in chronic or infectious injuries inhibit recruitment of inflammatory PMN, formation of inflammatory cytokines and chemokines, platelet activating factor and pathological angiogenesis [20, 34, 35, 36]. The protective actions of the 15-LOX-LXA₄ circuit in the cornea are in part mediated by the cytoprotective hemeoxygenase-1 (HO-1) system. In a positive feedback mechanism, HO-1 expression and 15-LOX activity depend on one another to maintain an elevated anti-inflammatory tone that raises the threshold for triggering full inflammatory responses in the immune privileged cornea (20, 21). In view of endogenous formation of several SPM pathway markers and expression of their receptors (ALX, ChemR23), it is likely that in addition to NPD1 and LXA₄, other SPM are formed in the ocular surface and have roles in executing healthy inflammatory and reparative responses in the cornea. A notion that is supported by a recent lipidomic analysis that document the presence of SPM in human tears, namely RvD1, RvD2, RvD5, NPD1, LXA₄ and aspirin-triggered LXA₄ [37].

The cause for sex-specific differences in innate and adaptive immune responses and the striking higher prevalence of autoimmune disease in females compared to males is largely unknown. A 2011 post-hoc analysis of a prospective clinical trial identified for the first time a marked sex-specific difference in the outcome of wound healing in the cornea. Namely, after correcting for confounding factors, corneal wounds in men healed two times faster than

in women [38]. Employing the mouse model of self-resolving corneal injury, it was discovered that estrogen regulates the 15-LOX-LXA₄-ALX circuit via the epithelial ER β receptor [39]. Estrogen downregulated 15-LOX and ALX expression and LXA₄ formation in the cornea that resulted in a female-specific phenotype of delayed re-epithelialization, reduced recruitment of wound healing PMN and an amplified inflammatory response; a phenotype that can be reversed by LXA₄ treatment [39]. This study identified regulation of SPM circuits by estrogen as a potential factor in the etiology or pathogenesis of sex-specific ocular surface inflammatory diseases. Recent lipidomic analysis of human tears demonstrated that women have lower SPM levels than men [37], emphasizing the clinical relevance of animal models that have identified sex-specific regulation of SPM circuits.

The cornea is the most innervated tissue in the human body, and corneal innervation serves a vital role in maintaining homeostasis and normal injury responses. In a lamellar keratectomy model that severs corneal nerves, NPD1 treatment markedly increased nerve regeneration [40, 41, 42], a bioaction that is consistent with the originally defined role of NPD1 as a neuroprotective SPM in the retina and in mouse models of stroke [6, 43].

Several SPM have been assessed as treatment options for corneal diseases in pre-clinical animal models of infection, graft survival, inflammatory angiogenesis and keratectomy. Both NPD1 and RvE1 treatment after herpes simplex virus infection decreased pro-inflammatory cytokines, virus-induced lesions and angiogenesis [44, 45], while RvE1 and LXA₄ also reduced the severity of LPS-induced stromal keratitis [20, 46]. In a mouse model of corneal transplantation RvD1 treatment prevented dendritic cell maturation by downregulating MHCII, CD40 and IL-12 expression on dendritic cells, which led to suppressed alloimmunity and enhanced graft survival [47]. Local or topical treatment with LXA₄, LXA₄ analogs, RvD1 or RvE1 markedly reduced pathological angiogenesis by inhibiting VEGF, VEGFR2, VEGFR3, TNF α and IL1 α /IL1 β [34, 48]. In a rabbit model of keratectomy, the RvE1 analog RX-10045 reduced corneal opacity after photorefractive keratectomy [49]. These *in vivo* studies demonstrated that consistent with established bioactions of SPM in other organ systems, SPM are promising drug targets for corneal diseases, infections and graft survival. However, regulation of SPM circuits and sex-specific differences in corneal health and diseases and cellular mechanisms of action still remain to be defined.

2.2 Conjunctivitis

The conjunctiva is a tissue composed of stratified epithelium, goblet cells, resident mast cell, lymphocytes and antigen presenting cells that covers the inner eyelids and anterior sclera. As a result of being highly vascularized, the conjunctiva is responsible for immune surveillance and cell trafficking of the ocular surface but has been largely ignored as the primary tissue for initiating ocular surface immune responses. Goblet cells serve as a physical fortification by producing mucin to keep the eye lubricated and to clear microbes and irritants. Conjunctivitis is a common ocular surface disease caused by viral or bacterial infection, allergies or autoimmune diseases. SPM actions and signaling mechanisms have been studied at length *in vitro* using isolated human or rat goblet cells. *In vitro* models that recapitulate goblet cell responses in DED and allergic conjunctivitis have demonstrated that RvD1 and RvE1 can block inflammatory eicosanoids or histamine activation of ERK and intracellular

Ca²⁺ mobilization in goblet cells [27, 50]. LXA₄ has unique actions on isolated conjunctival goblet cells as it can stimulate mucin secretion via activation of phospholipases, ERK and downstream protein kinases [51]. The *in vivo* expression, endogenous formation, and therapeutic actions of SPM have not been determined in the healthy or inflamed conjunctiva or animal models of conjunctivitis. However, *in vitro* data with goblet cells and mouse models of inflammation in other mucosal tissues (i.e. lung and colon) have established the protective therapeutic actions of SPM, inferring that treatment with SPM in general should attenuate the pathogenesis of conjunctivitis.

2.3 Retinopathy

SPM's endogenous protective roles extend beyond the ocular surface to the posterior segment of the eye, especially the retina. The neural retina is composed of densely packed photoreceptors and neurons responsible for converting light to electrical signals that are transmitted to the visual cortex, where they are decoded and interpreted to provide visual perception. Unlike the self-renewal capabilities of the corneal epithelium, cells in the retina are not equipped with regenerative mechanisms thus damage to the retina could lead to irreversible vision loss. The RPE is comprised of a monolayer of highly specialized cells that are vital in maintaining the health of retina. The RPE phagocytoses the outer segments of the photoreceptors, facilitates the visual cycle, upholds the blood-retinal barrier and expresses immunosuppressive molecules. Injury to RPE or dysregulation of homeostatic function of RPE is a key factor in the etiology of retinopathy.

NPD1 is of particular interest in retinopathy due to highly enriched levels of DHA in the retina and the pathway for NPD1 formation only requires 15-LOX. A large body of work has shown that NPD1 promotes cell survival of human RPE cells upon exposure to oxidative stress and that 15-LOX is the rate-limiting enzyme for endogenous formation [52, 53, 54]. Stereospecific binding sites for NPD1 have been identified in RPE cells and PMN [55] but the receptor remains to be identified. NPD1 mediates protection against RPE apoptosis in an autocrine fashion, suggesting a critical role for the 15-LOX-NPD1 circuit in maintaining RPE homeostasis [19]. The protective and anti-apoptotic bioactivity of RPE-released NPD1 are mediated by upregulation of anti-apoptotic Bcl-2 and Bcl-xL, as well as reduction of pro-apoptotic Bax expression and caspase-3 activation [53, 54, 56]. In addition, NPD1 promotes RPE cell survival against oxidative stress by increased phosphorylation of PI3K/Akt and mTOR/p70S6K signaling pathways [57, 58].

In addition to maintaining RPE homeostasis, NPD1 also inhibits inflammatory responses in the retina by exerting direct neuroprotective actions. In laser-induced choroidal neovascularization (CNV) that recapitulates macular degeneration, NPD1 attenuated neovascularization and shifted microglia toward a non-injury inducing phenotype *in vivo* [59, 60]. NPD1 also has direct actions on neuron cell survival and apoptosis. Retinal ganglion cells are neurons that relay action potentials from photoreceptors through the optic nerve, making cells along this path indispensable for processing visual information. Treatment with NPD1 after optical nerve transection in rats markedly delayed retinal ganglion cell death [61]. *In vitro* data has demonstrated endogenous formation of NPD1 by

cone photoreceptor cell lines [62] indicating that NPD1 formation in the retina is likely not restricted to RPE cells.

Inflammation is now recognized as a prominent feature of many retinal diseases and the role of SPM in resolution of inflammation has sparked research efforts to explore their therapeutic potential in retinopathies. A leading cause of blindness is diabetic retinopathy triggered by glucose-induced inflammation of the choroid and/or the retina, characterized by dysfunction and/or abnormal growth of retinal blood vessels. Inflammatory cells are subsequently recruited and facilitate further destruction. *In vitro* experiments have established that RvE1 and RvD1 are formed during retinal endothelial-PMN interactions and inhibit PMN transmigration and pro-inflammatory cytokines production [63]. A potential role of SPM circuits in human diabetic retinopathy is underscored by marked reduction of LXA₄ in the serum and vitreous humor with a concurrent increase of IL-6 in patients with diabetic retinopathy [64]. RvD1 formation has also been demonstrated in human endothelial cells in an *in vitro* model of diabetic retinopathy. Endothelial formation of RvD1 is reduced in high glucose conditions and restored to normal by vasoactive intestinal peptide, suggesting that RvD1 has a role in maintaining retinal endothelial cell homeostasis [28]. A preclinical model of retinopathy of prematurity emphasizes the important role of enzymes in the formation of SPM circuits. Dietary DHA is protective in this model but inhibition of pathogenic angiogenesis by the dietary supplement required 5-LOX [65]. It is important to recognize that 5-LOX is the key enzyme for amplifying and initiating inflammation but is also the rate-limiting and required enzyme for formation of the majority of SPM. Potential roles of SPM circuits in controlling innate immune responses in the retina are suggested by *in vitro* experiments that demonstrated LXA₄'s ability to inhibit LPS induced IL-6 production in human RPE cells [64].

The retina has the highest metabolic rate, oxygen demand and DHA content of any tissue and thus evolved robust mechanisms to prevent photoreceptor and neuronal cell death. NPD1 was the first identified *in vivo* retinal SPM [61] and its neuroprotective actions in the retina and central nervous system after injury and oxidative stress are well established [6, 7]. Emerging evidence has identified formation and bioactions of other SPM in the retina such as RvD1 and LXA₄. However, the endogenous roles of SPM in neuroprotection, retinopathy or maintaining homeostasis still remain to be explored.

2.4 Endotoxin-Induced Anterior Uveitis

Uveitis is characterized by inflammation of the uveal layer, which includes the iris, ciliary body and the choroid. Inflammation of the middle layer of the eye can be further categorized into anterior, posterior or pan uveitis depending on the tissues affected. Anterior uveitis is mediated by PMN, macrophage and T cell infiltration, modeled by endotoxin induced uveitis (EIU) in rats and is primarily an acute and self-resolving inflammatory response. Consistent with the anti-inflammatory and pro-resolving actions of SPM, treatment with LXA₄ or LXA₄ analogs reduced the clinical inflammation score, PMN cell counts and protein levels in the aqueous humor in rats with EIU [66]. RvD1, the structural DHA homolog of LXA₄, mediates its action via the same shared receptors. Consistent with LXA₄'s action in EIU, tail vein injection of RvD1 upon LPS-induced uveitis led to a dose-

dependent reduction of MPO activity along with PMN and T cell infiltration into the eye [67], while intravitreal injection of RvD1 decreased TNF α , chemokine MIP1- α , ubiquitin proteasome [66] and caspase 3 [68]. Macrophage profiles also shifted from the M1 phenotype to high expression of M2 upon RvD1 administration [69]. Endogenous roles for LXA₄ or RvD1 in EIU have not been reported. However, the established therapeutic actions of SPM in many inflammatory diseases models and the marked inhibition of inflammation with LXA₄ and RvD1 treatment in uveitis models provide strong evidence for SPM as promising treatment options for anterior uveitis.

2.5 Autoimmune Diseases: Dry Eye Disease and Posterior Uveitis

The role of eicosanoids, especially SPM, in the etiology of ocular and other autoimmune diseases is relatively unexplored. Autoimmunity occurs when the host immune cells mistakenly recognize and/or triggers T effector cell-driven responses against self-proteins and tissues. Autoimmune DED is a debilitating disease that inflicts working-age adults with unknown triggers. This T cell-driven chronic disease targets the lacrimal gland, goblet cells in the conjunctiva and ocular surface leading to impaired tear formation, inflammation and epithelial defects. DED is multifactorial and affects approximately 4 million people in the United States, and of which 3.2 million are women [70]. The cause for this striking sex-specific prevalence in women is unknown. Current primary treatment options are limited and included the long standing use of corticosteroids, the immunosuppressant cyclosporine A and artificial tears; a new treatment approach of inhibiting lymphocyte integrin/adhesion molecule interaction (LFA-1/ICAM-1) by a small molecule (lifitegrast) received FDA approval in 2016 for treating signs and symptoms of DED. A recent study identified resident LXA₄ circuits in the ocular surface and draining lymph nodes of the eye, which demonstrated striking sex-specific differences and established LXA₄ as a key regulator of T_H1 and T_H17 effector cells and T regulatory cells (T_{reg}). In mouse models that recapitulate human T-cell driven DED, a population of regulatory PMN that highly expresses 15-LOX was identified. The regulatory PMN generate LXA₄ in the lacrimal gland, lymph nodes and ocular surface. The loss of LXA₄ formation in the lymph nodes upon resident regulatory PMN depletion during desiccating stress was female-specific, and caused amplified T_H1 and T_H17 responses and reduction of T_{reg}. The exacerbated adaptive immune response in females was reversed by LXA₄ treatment, which reduced the number of T effector cells and increased T_{reg} in the draining lymph nodes and reduced dry eye pathogenesis [24]. This provided the first evidence that LXA₄ and regulatory PMN are a resident circuit in draining lymph nodes and key factors that control T_H1, T_H17 and T_{reg}. The role the LXA₄ circuit is also under investigation in T cell-driven autoimmune uveitis. In a pre-clinical model of posterior uveitis that is induced by immunization with retinal interphotoreceptor retinoid-binding protein (IRBP), LXA₄ treatment inhibits T effector cell infiltration and prevents retinopathy [71]. The emerging roles of the LXA₄ circuit in draining lymph nodes of the eye warrant further studies to determine if sex-specific regulation of the LXA₄ circuit is a factor in the etiology and striking prevalence of autoimmune diseases in women.

The therapeutic potential of amplifying endogenous SPM circuits is underscored by studies that examined the pharmacological effects of SPM treatment in experimental autoimmune DED. RvE1 increased tear production and corneal epithelium density while inhibiting

macrophage infiltration in a mouse model of immune DED [72] and an analog of RvE1 applied at the initiation of DED was able to maintain goblet cell density [73]. Based on promising data from pre-clinical animal models synthetic RvE1 analogs were tested in Phase I and Phase II clinical trials for chronic DED and initial results demonstrated significant improvements [74]. New emerging data provides compelling evidence that lipoxins [24] as well as their DHA structural homologs, RvD1 and RvD2 [75] have direct actions with T cells and regulate adaptive immune responses, which warrants further research to define the cellular mechanism of action, signaling pathways and direct T cell-targeted actions.

3. Summary

The anti-inflammatory and pro-resolving therapeutic actions of SPM are well established in many organ systems and pre-clinical inflammatory diseases models. However, there is a clear gap of knowledge regarding SPM's distinct cellular mechanisms of action, their endogenous regulation and role in disease etiology and health. The unique properties of the eye, such as highly conserved primary sense with immune-privileged tissues, neural tissues that are the direct outgrowth of the central nervous system and an exceptional high metabolic rate and DHA content, have uncovered novel bioactions and insights into the endogenous roles of SPM circuits, which extend well beyond their traditional roles of regulating acute inflammation or resolution.

Enzymes for SPM formation are highly expressed in corneal and retinal epithelial cells and tissue resident leukocytes as well as receptors for LXA₄, RvD1, NPD1 and RvE1. Studies in the eye have discovered that SPMs have novel intrinsic roles in driving corneal wound healing, preventing RPE and ganglion cell apoptosis, promoting survival of photoreceptors and controlling T effector cell responses in adaptive immune responses. More importantly, endogenous formation of LXA₄ and NPD1 in the eye is not limited to the resolution phase of inflammation (Table 1). These SPM circuits have fundamental roles in maintaining healthy homeostasis in the cornea, retina and draining lymph nodes in the absence of acute inflammation. Of particular interest is the sex-specific regulation of the intrinsic LXA₄ circuit in the ocular surface and draining lymph nodes, which has emerged as a factor in driving female-specific adaptive immune response and indicate that SPM circuits have direct actions on lymphocytes. *In vitro* studies and animal models have established the therapeutic potential of SPM on limiting inflammatory ocular diseases. Consistent with inflammatory disease models in other organ systems, SPM circuits are validated therapeutic targets for inflammatory diseases in the eye. However, many questions remain to be answered regarding the regulation of SPM circuits in health, disease and their role or dysregulation in the etiology of autoimmunity, retinopathy and neurodegeneration.

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Figure 1.

The anti-inflammatory bioactions of SPM in ocular tissues and draining lymph nodes. MØ= macrophages, Th1= T helper 1 cells, Th17= T helper 17 cells, PMN= polymorphonuclear cells, RGC= retinal ganglion cells, RPE= retinal pigmented epithelium.

Table 1

Formation of SPM in ocular tissues, tears and draining lymph node *in vivo* and *in vitro*. LXA₄= lipoxin A₄, NPD1= neuroprotectin D1, RvD1, 2, 5= resolvin D1, resolvin D2, resolvin D5. Grey columns depict *in vivo* or *in vitro* formation and references are in brackets.

<i>In Vivo + In Vitro</i> ★	LXA ₄	NPD1	RvD1, 2, 5
Corneal Epithelium	+, ★	+	[5]
Corneal Stroma	★		
Vitreous Humor	+		
Retina	+	+	[61]
Retinal Pigmented Epithelium		★	[52]
Draining Lymph Nodes	+		
Tears	+	+	[37]