Identification of prostamides, fatty acyl ethanolamines, and their biosynthetic precursors in rabbit cornea*

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Abstract Arachidonyl ethanolamine (anandamide) and prostaglandin ethanolamines (prostamides) are biologically active derivatives of arachidonic acid. Although available through different precursor phospholipids, there is considerable overlap between the biosynthetic pathways of arachidonic acid-derived eicosanoids and anandamide-derived prostamides. Prostamides exhibit physiological actions and are involved in ocular hypotension, smooth muscle contraction, and inflammatory pain. Although topical application of bimatoprost, a structural analog of prostaglandin F2α ethanolamide (PGF2α-EA), is currently a first-line treatment for ocular hypertension, the endogenous production of prostamides and their biochemical precursors in corneal tissue has not yet been reported. In this study, we report the presence of anandamide, palmitoyl-, stearoyl-, ɷ-linolenoyl docosahexaenoyl-, linoleoyl-, and oleoyl-ethanolamines in rabbit cornea, and following treatment with anandamide, the formation of PGF2α-EA, PGE2-EA, PGD2-EA by corneal extracts (all analyzed by LC/ESI-MS/MS). A number of N-acyl phosphatidyl-ethanolamines, precursors of anandamide and other fatty acyl ethanolamines, were also identified in corneal lipid extracts using ESI-MS/MS. These findings suggest that the prostamide and fatty acid ethanolamine pathways are operational in the cornea and may provide valuable insight into corneal physiology and their potential influence on adjacent tissues and the aqueous humor.—Urquhart, P., J. Wang, D. F. Woodward, and A. Nicolaou. Identification of prostamides, fatty acyl ethanolamines, and their biosynthetic precursors in rabbit cornea. J. Lipid Res. 2015. 56: 1419–1433.

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The cornea functions to refract light and protect the intraocular structures of the eye. While its outermost epithelial layer facilitates oxygen diffusion and acts to absorb UV radiation (UVR), the innermost endothelial layer contributes to corneal transparency that is essential for optimum vision and regulates ocular pressure (1). Although a healthy cornea is avascular of blood and lymph vessels, hypertension or glaucoma can cause injury through abrasion of the endothelial cell lining leading to neovascularization, which if uncontrolled causes scarring and can lead to blindness (2, 3). Prostanoids are important regulators of corneal homeostasis with prostaglandin (PG) E2 and thromboxane (TX) A2 mediating corneal endothelial cell proliferation (4–6). While PGE2 is also involved in angiogenesis and polymorphonuclear monocyte recruitment (7), PGD2 can suppress corneal tumor growth and hyperpermeability (8). Furthermore, analogs of PGs are used as ocular hypotensive agents with bimatoprost, a structural analog of PGF2α ethanolamide (PGF2α-EA), being widely used to treat glaucoma (9).

Prostaglandin ethanolamines (prostamides; PG-EAs) are sequentially biosynthesized from the endocannabinoid arachidonoyl ethanolamine (anandamide; A-EA) by cyclooxygenase (COX)-2 followed by various prostaglandin synthases (PGSs) (10–13). Anandamide is released from precursor phospholipids N-arachidonoyl phosphatidylethanolamines (NArPE) via N-acyl phosphatidylethanolamine (NAPE)-specific phospholipase (PL) D (NAPE-PLD), although recent findings have indicated the existence of other pathways mediated by either α,β-hydrolase 4 followed by cleavage of glycerophosphate to yield A-EA, or PLC and subsequent dephydroxylation of phosphoanandamide to A-EA [reviewed in (14)]. The majority of studies investigating these

Abbreviations: 15-PGDH, 15-prostaglandin hydroxydehydrogenase; A-EA, arachidonoyl ethanolamine; AL-EA, α-linolenoyl ethanolamine; COX, cyclooxygenase; DH-EA, docosahexaenoyl ethanolamine; EP-EA, eicosapentaenoyl ethanolamine; FAAH, fatty acid amide hydrolase; FA-EA, fatty acyl ethanolamine; L-EA, linoleoyl ethanolamine; MRM, multiple reaction monitoring; N-AAA, N-acyl ethanolamine-hydrolyzing acid amidase; NAPE, N-acyl phosphatidylethanolamine; NArPE, N-arachidonoyl phosphatidylethanolamine; O-EA, oleoyl ethanolamine; PC, phosphatidylcholine; P-EA, palmitoyl ethanolamine; PG, prostaglandin; PG-EA, prostaglandin ethanolamine; PGS, prostaglandin synthase; PL, phospholipase; PLD, phospholipase D; ST-EA stearoyl ethanolamine; TX, thromboxane.

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pathways have been carried out in mice and rat tissues, and, interestingly, their prevalence appears to be time and cell specific (15). Finally, A-EA may also be catabolized to arachidonic acid and ethanolamine by fatty acid amide hydrolase (FAAH; EC 3.5.1.99) or N-acylethanolamine-hydrolyzing acid amidase (N-AAA) (16, 17) (Fig. 1).

Prostamides exhibit a range of activities in various systems. PGE$_2$-EA is involved in inflammatory pain and dorsal horn nociceptive neuron excitability, while PGE$_2$-EA increases blood flow and reduces mean arterial pressure in the renal medulla, exhibits strong neuroprotective properties in cerebellar neurons, and along with PGD$_2$-EA, induces apoptosis in an in vitro model of colorectal carcinoma (18–21). Prostamides do not show potent interaction with cannabinoid receptors, and studies using isolated feline iris cells have suggested the presence of prostamide-sensitive receptors different from the ones responding to PGs (22–24). The prostamide precursor A-EA has also been shown to exhibit neuroprotective and analgesic roles in inflammation and pain models (25, 26), while topical administration reduces intraocular pressure (27). However, its mode of action is mediated through the CB$_1$ and CB$_2$ cannabinoid and vanilloid subtype-1 (TRPV1) receptors that are not activated by prostamides (24, 28).

Although A-EA has been found in the cornea as a minor lipid (30), neither its metabolism through COX-2 to form prostamides nor the prevalence of its biochemical precursor NAPE have been investigated. In this study, we explored the endogenous production of PGE$_2$-EA, PGE$_2$-EA, and PGD$_2$-EA by the cornea and show its capability to form these prostamides when A-EA is added externally. We also present data detailing the levels of A-EA and its congeners, as well as NAPE and other fatty acyl NAPE species in rabbit corneal tissue. Given the pharmacological potency of prostamides in ocular health, detailed information on their profile and biochemical precursors in cornea could provide valuable insight into ocular physiology and potential therapeutics.

**MATERIALS AND METHODS**

**Materials**

PGE$_2$, PGF$_2$-$\alpha$, PGF$_2$-$\gamma$, PGD$_2$, 15-deoxy-$\Delta^{12,14}$PGJ$_2$, PGJ$_2$, 15-deoxy-$\Delta^{12}$PGJ$_2$, PGE$_{\gamma}$, PGD$_{\gamma}$, PGE$_{\alpha}$, PGD$_{\alpha}$, 13,14 dihydro 15-keto PGE$_2$, 13,14 dihydro 15-keto PGF$_{2\alpha}$, TXB$_2$, 6-keto PGF$_{1\alpha}$, PGB$_{2\alpha}$-$d_4$, A-EA, A-EA-$d_8$, palmitoyl ethanolamine (P-EA), docosahexaenoyl ethanolamine (DH-EA), a-linolenoyl ethanolamine (AL-EA), oleoyl ethanolamine (O-EA), stearoyl ethanolamine (ST-EA), linoleoyl ethanolamine (L-EA), PGE$_2$-EA, PGF$_{2\alpha}$-EA, PGD$_{2\alpha}$-EA, and FAAH inhibitor PF3845 (51) were purchased from Cayman Chemical Co. (Ann Arbor, MI). N-arachidonoyl dipalmitoyl phosphatidyl-ethanolamine was purchased from Enzo Life Sciences (Exeter, UK). Security guard cartridges C18 (5 μm, 4 × 2.0 mm), C18-E solid phase extraction cartridges (SPE; 500 mg sorbent), amber glass vials (1.5 ml), insert glass vials (0.15 ml), Teflon septa and lids were from Phenomenex (Macclesfield, UK). Male white New Zealand rabbit corneas were provided by Sera Laboratories International Ltd. (Haywards Heath, UK). Chloroform, methanol, ethanol, acetonitrile, hexane (all HPLC grade), and methyl formate (97% spectroscopy grade) were from Fisher Scientific (Loughborough, UK). HPLC-grade glacial acetic acid, Trizma Base, indomethacin, and protease inhibitor cocktail [4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (104 mM), aprotinin (80 μM), bestatin (4 mM), E-64 (1.4 mM), leupeptin (2 mM), pepstatin A (1.5 mM)] were purchased from Sigma-Aldridge (Dorset, UK). EDTA was sourced from BDH (Poole, UK). Ultrapure water was tapped by a MilliQ Gradient system (Millipore, Volketswil, Switzerland).

**Tissue homogenization and extraction of prostamides and fatty acid ethanolamines**

Rabbit corneas (~75–100 mg each) were individually homogenized, using a glass Dounce tissue grinder (1 ml) (Fisher Scientific) with a tightly fitting pestle, in 1 ml ice-cold Tris-hydrochloride buffer with a tightly fitting pestle, in 1 ml ice-cold Tris-hydrochloride...
buffer (100 mM, pH 8 adjusted with 1 M HCl) containing EDTA (1 mM), FAAH inhibitor PF3845 (100 nM), and a protease inhibitor cocktail (1:100 dilution). During homogenization the tissue grinder and homogenate were kept on ice. When endogenous production of prostamides was monitored, corneal tissue homogenates were pooled. Subsequent ex vivo investigations of prostamide formation were carried out by incubating corneal tissue homogenates (eight corneas) were pooled. The resulting suspensions were kept on ice for 30 min with occasional vortexing. Each sample was vortexed and centrifuged at 4,000 rpm for 8 min to separate the organic and aqueous phases. The organic layer (bottom) from each sample was then removed into a clean wide-neck vial. The pooled supernatant was evaporated under a fine stream of nitrogen, and the remaining residue was reconstituted in 50–100 μl of ethanol and stored at −20°C, for no more than 1 week, awaiting LC/ESI-MS/MS analysis.

**LC/ESI-MS/MS analysis of prostamides**

Analysis and characterization of PG-EA produced by corneal tissue was performed on an electrospray (ESI) tandem quadrupole Xevo TQS mass spectrometer (Waters, Ettre, Hertfordshire, UK) coupled to an Acquity UHPLC® system. The system was controlled by MassLynx™ v4.1 software. TargetLynx was used to construct calibration lines and calculate the concentration of analytes of interest. Optimized ESI-MS/MS conditions were achieved through use of Intellistart within MassLynx software. TargetLynx was used to construct calibration lines and calculate the concentration of analytes of interest. Optimized ESI-MS/MS conditions were achieved through use of Intellistart within MassLynx software. Individual standards (100 pg/μl) were introduced into the spectrometer by direct infusion via the Xevo TQS integrated syringe pump (flow rate 10 μl/min) with and without UPLC solvent flow (rate 0.2 ml/min). All analytes were monitored on the positive ionization mode. Capillary voltage was set at 2,000 V, source temperature at 100°C, desolvation temperature at 400°C, and the cone voltage at 35 V. The collision energy was optimized for each compound to obtain optimum sensitivity using argon as collision gas and was set to 0.0–10.00 min, 30% solvent B increasing linearly to 70% solvent B; 10.00–40.00 min 70% solvent B decreasing linearly to 60% solvent B; 40.00–41.00 min 60% solvent B increasing linearly to 90% solvent B; 41.00–55.00 min 90% solvent B; 55.00–56.00 90% solvent B decreasing linearly to 30% solvent B; 56.00–69.00 min 30% solvent B. A shallow gradient was put in place between 10 and 40 min (70% to 60% solvent B) to improve the resolution of FA-EA. MRM assays were set up using the following transitions: P-EA, m/z 300 > 62; AL-EA, m/z 322 > 62; L-EA, m/z 324 > 62; O-EA, m/z 326 > 62; ST-EA, m/z 328 > 62; EP-EA, m/z 346 > 62; A-EA, m/z 348 > 62; DH-EA, m/z 372 > 62; A-EA-d8, m/z 356 > 63. Results are expressed as picograms metabolite per milligrams wet tissue, using calibration lines constructed with commercially available standards.

**Extraction and LC/ESI-MS/MS analysis of prostanoids**

Prostanoids were extracted and analyzed as previously described (34, 35). Briefly, individual corneas were homogenized in 500 μl of ice-cold 15% methanol (v/v) using PGB2-d4 (40 μl of a 1 ng/μl methanol solution) as internal standard. The homogenates were acidified to pH 5.0 with 1 M hydrochloric acid, semipurified using SPE and eluted with methyl formate. The solvent was then evaporated under nitrogen, and the lipid residue reconstituted in 100 μl ethanol and stored at −20°C. LC/ESI-MS/MS analysis of prostanoids was based on MRM assays using the following transitions: 15-deoxy-D12/13 PGJ2, m/z 315 > 271; PGJ2, m/z 333 > 271; D6 PGJ2, m/z 333 > 271; PGJ2, m/z 349 > 269; PGD2, m/z 349 > 269; PGD2, m/z 351 > 271; PGD2, m/z 351 > 317; 13,14-dihydro-15-keto PGE2, m/z 351 > 333; 13,14-dihydro-15-keto PGD2, m/z 353 > 113; 15-PGD2, m/z 353 > 193; PGE1, m/z 353 > 317; PGD2, m/z 353 > 317; 6-keto PG1b, m/z 369 > 163; TXB2, m/z 369 > 169; PGD2-d4, m/z 373 > 174. Results are expressed as picograms metabolite per milligrams wet tissue, using calibration lines constructed with commercially available prostanoid standards.

**Extraction and ESI-MS/MS analysis of NAPE species**

Two corneas were homogenized individually, using a glass Dounce tissue grinder (1 ml) in ice-cold chloroform-methanol (2:1, v/v) (0.5 ml aliquots to a volume of 3 ml per cornea). The sample was then kept on ice for 90 min with occasional vortexing. Water (0.5 ml) was added to each sample and the vials vortexed before being centrifuged at 5,000 rpm for 8 min to separate the organic and aqueous phases. The organic layer (bottom) from...
ESI-MS spectra were recorded over the range \( m/z 800-1,250 \). Ions with \( m/z [M-H]^- \) corresponding to NArPE and NAPE were further analyzed by ESI-MS/MS to confirm their identity and obtain information on the sn-1 and sn-2 acyl chains.

**RESULTS**

**LC/ESI-MS/MS analysis of prostamides in rabbit cornea**

The ESI-MS, MS/MS spectra and fragmentation patterns of prostamides PGF_{2\alpha}-EA, PGE_{2\alpha}-EA, and PGD_{2\alpha}-EA were studied using commercially available standards (Fig. 2). All prostamide standards were found to form stable sodiated each sample was then removed and pooled into a clean wide-neck vial, and the solvent evaporated under a fine stream of nitrogen. The lipid residue was reconstituted in 100 \( \mu l \) chloroform-methanol (1:4, v/v) and stored at \(-20^\circ C\) awaiting ESI-MS/MS analysis (36).

In order to optimize the ESI-MS and ESI-MS/MS conditions for NAPE analysis, commercially available N-arachidonoyl dipalmitoyl phosphatidyl ethanolamine was used. Using direct infusion (flow rate 10 \( \mu l/min \)), the optimum collision energy was found to be 40 eV, using argon as collision gas. The analyte was monitored on negative ionization mode and was found to fragment in a similar way to previously published data (36). The corneal extract was diluted 1:10 (v/v) with chloroform-methanol-water-acetic acid (2: 6.95:1:0.05, v/v/v/v) and analyzed through direct infusion.

Fig. 2. Analysis of prostamide standards PGF_{2\alpha}-EA, PGE_{2\alpha}-EA, and PGD_{2\alpha}-EA. The ESI-MS spectrum of PGF_{2\alpha}-EA standard (A) shows that the sodium adduct \([M+Na]^+ m/z 420 and [M+H-H_2O]^+ m/z 380 are readily formed; while [M+H]^+ m/z 398 is found at extremely low levels and was not easy to fragment (B), product ion scan of \([M+H-H_2O]^+ m/z 380 (C) indicates that the dehydration products \( m/z 362 and 344, and specific fragments \( m/z 283 and 62, can be used for the detection and quantitation of PGF_{2\alpha}-EA. The ESI-MS spectrum of PGE_{2\alpha}-EA standard shows the formation of sodiated ion \([M+Na]^+ m/z 418 (D) and low prevalence of \([M+H]^+ m/z 396, while product ion scans of \([M+H]^+ m/z 396 (E) and \([M+H-H_2O]^+ m/z 378 (F) show the dehydration products \( m/z 360 and 342, and specific fragments \( m/z 299 and 62, that can be used for the detection and quantitation of PGE_{2\alpha}-EA. The ESI-MS spectra of the PGD_{2\alpha}-EA standard show the formation of the sodiated, dehydration, and specific fragment ions identical to the ones produced by PGE_{2\alpha}-EA (G, H, and I). Sample LC/ESI-MS/MS reconstructed ion chromatograms of prostamides PGF_{2\alpha}-EA (J) and PGE_{2\alpha}-EA and PGD_{2\alpha}-EA (K) using the following MRM transitions: \( m/z 380 > 62 and 378 > 62 (all analytes at 10 pg/\mu l); chromatographic conditions are described in Materials and Methods. Commercially available standards were used.
ions \([M+Na]^-\) \(m/z\) 420 for PGF\(_{2\alpha}\)-EA, and \(m/z\) 418 for both PGE\(_2\)-EA and PGD\(_2\)-EA, possibly reflecting their storage in glass vials. Notably, the relative abundance of [M+H\(^+\)]\(^+\) species (\(m/z\) 398 for PGF\(_{2\alpha}\)-EA, and \(m/z\) 396 for both PGE\(_2\)-EA and PGD\(_2\)-EA) was found to be very low (Fig. 2A, D, G, respectively), and, for all prostamides examined here, the predominant ions corresponded to [M+H-H\(_2\)O]\(^+\) (\(m/z\) 380 for PGF\(_{2\alpha}\)-EA and \(m/z\) 378 for PGE\(_2\)-EA and PGD\(_2\)-EA). Further fragmentation of [M+H\(_2\)O]\(^+\) ions resulted in the product ions [M+H-2H\(_2\)O]\(^+\) \(m/z\) 362, [M+H-3H\(_2\)O-H\(_2\)NCH\(_2\)CH\(_2\)OH]\(^+\) \(m/z\) 283 for PGF\(_{2\alpha}\)-EA (Fig. 2C), and [M+H-2H\(_2\)O]\(^+\) \(m/z\) 360, [M+H-3H\(_2\)O]\(^+\) \(m/z\) 342, and [M+H-2H\(_2\)O-NH\(_2\)CH\(_2\)CH\(_2\)OH]\(^+\) \(m/z\) 299 for PGE\(_2\)-EA and PGD\(_2\)-EA (Fig. 2E, F). The protonated 2-amino ethanol ion [NH\(_3\)CH\(_2\)CH\(_2\)OH]\(^+\) \(m/z\) 62, characteristic of ethanolamine metabolites, was also detected following fragmentation of [M+H]\(^+\) (PGE\(_2\)-EA and PGD\(_2\)-EA; Fig. 2E, H) and [M+H-H\(_2\)O] \(^+\) (PGF\(_{2\alpha}\)-EA, PGE\(_2\)-EA, and PGD\(_2\)-EA; Fig. 2C, F, I). All these findings are in agreement with previously published data on the prostamide formation and identification in vitro and FAAH knockout mice (10, 12, 13, 37).

Although sodium adducts have been used to analyze PGF\(_{2\alpha}\)-EA (21), prostamide adducts proved to be very stable under our experimental conditions, and the collision energies required were found too high (>40 eV) to produce identifiable characteristic fragments. Furthermore, under our experimental setting the prostamide standards were readily dehydrated resulting in a very low abundance of the [M+H]\(^+\) ions (Fig. 2A, D, G), and it was necessary to use very high concentrations of the commercially available standard (>40 ng on the column) in order to detect these parent ions. Therefore, in order to set up an LC/ESI-MS/MS assay appropriate for detection and quantitation of prostamides found at low concentrations in biological samples, we followed the fragmentation of [M+H\(_2\)O]\(^+\) ions using four MRM transitions: PGF\(_{2\alpha}\)-EA, \(m/z\) 380 > 362, 380 > 344, 380 > 283, and 380 > 62; PGE\(_2\)-EA and PGD\(_2\)-EA, \(m/z\) 378 > 360, 378 > 342, 378 > 299, and 378 > 62. Chromatographic separation of PGF\(_{2\alpha}\)-EA, PGE\(_2\)-EA, and PGD\(_2\)-EA was achieved using a reverse phase C18 column with an acidified acetonitrile-based gradient system (Fig. 2f, K). Analysis of rabbit corneal tissue extracts using this assay did not offer conclusive evidence for the presence of PGF\(_{2\alpha}\)-EA, PGE\(_2\)-EA, and PGD\(_2\)-EA (Fig. 3).

Further experiments were designed to explore the capability of rabbit cornea to form prostamides. For this, tissue homogenates were incubated with exogenously added A-EA (10 and 50 \(\mu\)M) (Fig. 4). The LC-MS/MS reconstructed ion chromatograms presented in Fig. 4A-D show increased production of PGF\(_{2\alpha}\)-EA in all transitions recorded and following incubation with 50 \(\mu\)M A-EA. The ESI-MS/MS spectrum of the compound eluted at 2.14 min (retention time of the PGF\(_{2\alpha}\)-EA standard) confirms the presence of PGF\(_{2\alpha}\)-EA in the A-EA-treated corneal extract (Fig. 4E). Furthermore, when the tissue homogenate was incubated with A-EA (50 \(\mu\)M) in the presence of the COX inhibitor indomethacin, PGF\(_{2\alpha}\)-EA formation was inhibited by \(\sim\)70\% (Fig. 5A, B), while the presence of the FAAH inhibitor PF3845 showed a clear increase in PGF\(_{2\alpha}\)-EA production (\(\sim\)65\%) (Fig. 6A, B). For clarity, Figs. 5 and 6 show two of the four recorded transitions (i.e., \(m/z\) 380 > 62 and 380 > 283), the ones that gave the best signal when examining the A-EA-supplemented corneal extract and confirm the presence of 2-amino ethanol (Fig. 4).

The LC/ESI-MS/MS reconstructed ion chromatograms presented in Fig. 4F-I suggest that the A-EA-supplemented corneal extracts produce PGE\(_2\)-EA and PGD\(_2\)-EA, albeit at very low levels. The presence of these compounds is further supported by the product ion spectra corresponding to peaks eluted at 2.24 and 2.79 min, the retention times of PGE\(_2\)-EA and PGD\(_2\)-EA authentic standards, respectively (Fig. 4J, K). Inhibition of COX by indomethacin showed reduction of the putative PGE\(_2\)-EA and PGD\(_2\)-EA peaks (\(\sim\)30–70\%; peaks eluting at 2.23 and 2.79 min, respectively; Fig. 5C, D), while inhibition of FAAH appeared to increase the corresponding signals (\(\sim\)30–60\%) (Fig. 6C, D), further supporting the identification.

Treatment with indomethacin reduced the relative production of peaks eluting at retention times earlier than that of PGF\(_{2\alpha}\)-EA indicating the possible presence of 6-keto PGF\(_{1\alpha}\)-EA, the stable metabolite of prostacyclin ethanolamine (PGL\(_2\)), in the corneal tissue (11). A single ion recording (SIR) for [M+H]\(^+\) \(m/z\) 414 revealed two broad peaks at retention times 0.56 and 1.27 min, respectively (supplementary Fig. 1). Furthermore, an MRM experiment based on the dehydrated ions [M+H\(_2\)O]\(^+\) \(m/z\) 396, [M+H-2H\(_2\)O]\(^+\) \(m/z\) 378, and [M+H-3H\(_2\)O]\(^+\) \(m/z\) 360, as well as the protonated 2-amino ethanol ion [NH\(_3\)CH\(_2\)CH\(_2\)OH]\(^+\) \(m/z\) 62 (\(m/z\) 414 > 396, 414 > 378, 414 > 360, and 414 > 62), suggested the presence of these 6-keto PGF\(_{1\alpha}\)-EA-related ions at retention times 0.61 min and 1.32 min, while indomethacin appeared to reduce the formation of only one of the observed peaks (retention time 1.22 min; supplementary Fig. 1D–H). These findings indicate the putative formation of 6-keto PGF\(_{1\alpha}\)-EA by corneal tissue, although a synthetic standard would be needed to further explore and confirm this finding.

**LC/ESI-MS/MS analysis of prostanoids in rabbit cornea**

Although A-EA is the substrate for enzymatic conversion by COX-2 to PGL\(_2\)-EA (13), it is the expression and activity of the individual PGSs that ultimately determines the tissue profile of prostamides. We have, therefore, assessed the profile of prostanoids in rabbit cornea as means of appreciating the range and relative activity of PGSs in this tissue. Prostacyclin (PG\(_I\_2\), measured as 6-keto PGF\(_{1\alpha}\)-EA; Table 1) appeared to be the predominant prostanoid produced at 270.9 \(\pm\) 109.8 pg/mg tissue, while PGF\(_{2\alpha}\), PGE\(_2\), and PGD\(_2\) were produced at lower levels (40.5 \(\pm\) 15.0, 151.9 \(\pm\) 103.5, and 187.1 \(\pm\) 73.4 pg/mg tissue, respectively) (Table 2). Interestingly, PGE\(_3\), PGD\(_3\), and PGF\(_3\), derived from COX metabolism of dihomo \(\gamma\)-linolenic acid (20:3) and EPA (20:5) respectively, were also detected albeit at very low levels (0.9–5.3 pg/mg tissue). Furthermore, the cyclopentanone PGs PG\(_J\_2\), \(\Delta^9\)PG\(_{12}\), and 15 deoxy \(\Delta^9\)PG\(_{12}\) were also detected at 3–47 pg/mg tissue, showing that PGD\(_2\) may also act as precursor to anti-inflammatory species in the cornea.
Finally, the presence of 13,14 dihydro-15-keto metabolites of PGE\(_{2}\) and PGF\(_{2\alpha}\) shows the expression of 15-prostaglandin dehydrogenase (15-PGDH) and PG keto reductases in rabbit cornea, suggesting that the tissue actively controls the levels of PGs through their metabolism and deactivation (38). Overall, these data clearly show the presence of an active arachidonic acid cascade through COX, while the PG profile suggests the prevalence of PGIS, PGES, and PGDS isomers in rabbit cornea, suggesting that the tissue has the capability of forming the correspondent prostanoid species.

**LC/ESI-MS/MS analysis of FA-EAs in rabbit cornea**

Tissue levels of prostanoids depend on the availability of A-EA; thus, a low corneal A-EA level could explain the lack of detectable levels of PGF\(_{2\alpha}\)-EA, PGE\(_{2}\)-EA, and PGD\(_{2}\)-EA in baseline corneal extracts. This was confirmed by LC/ESI-MS/MS analysis of the corneal extract and showed A-EA at 10.7 ± 5 pg/mg tissue. Overall, seven species of FA-EA were detected in rabbit cornea; the level of A-EA was similar to ST-EA (8.3 ± 4.9 pg/mg tissue) and LEA (13.8 ± 4.2 pg/mg tissue), but lower than P-EA (32.7 ± 12.5 pg/mg tissue) and O-EA (42.1 ± 26.8 pg/mg tissue), while AL-EA and DH-EA were detected at much lower concentrations (0.4 ± 0.1 and 0.7 ± 0.3 pg/mg tissue, respectively); data shown in Fig. 7.

**Analysis of NAPEs in rabbit cornea**

The pool of A-EA and congeners is derived from membrane stores of the respective NAPE. Commercially available N-arachidonoyl dipalmitoyl phosphatidyl ethanolamine (978 Da) was used to optimize the experimental conditions for the ESI-MS/MS analysis of NAPE species by direct infusion. Fragmentation of [M-H]- \(m/z\) 977 resulted in an abundant product ion \(m/z\) 255, which was attributed to the carboxylate anion derived from the fatty acyl in position sn-2 (i.e., palmitate), while other product ions were identified as the sn-2 N-acetyl lysophospholipid \(m/z\) 739, N-arachidonoyl ethanolamine cyclic phosphate derivative \(m/z\) 482, and N-arachidonoylthanolamine phosphate \(m/z\) 426. These data were in agreement with results published by Astarita et al. (36). A general scan in the range of \(m/z\) 850–1200 indicated more than 25 potential NAPE species present in corneal lipid extracts. Following a focused MS/MS analysis of the species found in higher abundance (i.e., exceeding \(10^5\) ion intensity), five NAPE precursor ions with \(m/z\) 1041, 1085, 1097, 1055, and 1027 were identified (Table 2). These ions are consistent with the expected masses of seen NAPE species (namely, P-EA, AL-EA, LEA, O-EA, ST-EA, A-EA, and DH-EA NAPE) precursors of the main FA-EA identified in rabbit cornea (Table 2 and Fig. 7).

**DISCUSSION**

Prostamides and their biochemical precursor A-EA exhibit a range of pharmacological and physiological functions that make them an attractive basis for therapeutic intervention. Nevertheless, there is very little information available that describes endogenous prostamide levels and their biosynthetic formation pathways(\(s\)). In particular, there is a complete absence of any comprehensive analyses of biosynthetic pathways to the prostamides upstream of A-EA. The authors believe that this is the first report of such an investigative lipidomic analysis of prostanoid formation in a tissue. Beyond prostanoids, these studies also incorporated analytic detection of other FA-EAs. The function of these species concurrently detected with A-EA may form a foundation for a more complete investigation of biolipid function in the cornea and anatomically adjacent ocular tissues.

LC/ESI-MS/MS lipidomic analysis of rabbit corneal lipid extract did not provide clear evidence for the presence of endogenous prostamides. The identification was based on four fragment ions per compound, selected for increased sensitivity and corresponding to dehydrated and structure specific ions such as the diagnostic for 2-amino alcohols ion \(m/z\) 62 (Fig. 2). Although some peaks with retention times similar to the ones of commercially available PGF\(_{2\alpha}\)-EA, PGE\(_{2}\)-EA, and PGD\(_{2}\)-EA standards were detected, the presence of weak broad signals did not support their identification (Fig. 3). Therefore, evidence of the corneal tissue capability to produce prostamides was sought using externally added A-EA. The formation of PGF\(_{2\alpha}\)-EA increased following incubation with A-EA, and this production was found to be reduced when COX was inhibited and stimulated by FAAH inhibition. PGE\(_{2}\)-EA and PGD\(_{2}\)-EA showed the same response although they were produced at lower levels than PGF\(_{2\alpha}\)-EA.

Anandamide as well as L-EA and ST-EA were found at relatively low levels (10.7 ± 5 pg/mg, 13.8 ± 4.2 pg/mg, and 8.3 ± 4.9 pg/mg tissue, respectively) and were not the most abundant of the seven species of FA-EA identified: O-EA and P-EA were found at higher levels (42.1 ± 26.8 and 32.7 ± 12.5 pg/mg tissue, respectively) (Fig. 7). This could be attributed to the higher prevalence of their respective fatty acids at position sn-1 of the phosphatidylcholine (PC) precursor (39). DH-EA and AL-EA were minor congeners (0.4 and 0.7 pg/mg tissue, respectively), indicating very low levels of DHA and α-linolenic incorporation at the sn-1 position of corneal PC available for transacylation to the amine terminal of phosphatidylethanolamine (PE) (40). This is in contrast to the high levels of di-docosahexanoyl-PC and -PE species reported in rat and bovine retinal phospholipids (41, 42) and highlights

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**Fig. 3.** LC/ESI-MS/MS analysis of prostamides PGF\(_{2\alpha}\)-EA, PGE\(_{2}\)-EA, and PGD\(_{2}\)-EA in rabbit cornea. Sample reconstructed ion chromatograms of untreated corneal extract (baseline cornea) compared with corneal extract spiked with commercially available prostamide standards (spiked cornea) and standards (standard) at the following MRM transitions: PGF\(_{2\alpha}\)-EA (retention time 2.05 min): \(m/z\) 380 > 62 (A), \(m/z\) 380 > 283 (B), \(m/z\) 380 > 344 (C), and \(m/z\) 380 > 362 (D). PGE\(_{2}\)-EA and PGD\(_{2}\)-EA (retention times 2.15 min and 2.72 min, respectively): \(m/z\) 378 > 62 (E), \(m/z\) 378 > 299 (F), \(m/z\) 378 > 342 (G), and \(m/z\) 378 > 360 (H).
Fig. 4. Confirmation of rabbit corneal tissue capability to produce the prostamides PGF$_{2\alpha}$-EA, PGE$_2$-EA, and PGD$_2$-EA. Sample reconstructed ion chromatograms of corneal homogenate incubated (10 min at 37°C) with externally added anandamide (10 μM and 50 μM; 10 A-EA and 50 A-EA) compared with commercially available prostamide standards (standard) at the following MRM transitions: PGF$_{2\alpha}$-EA: m/z 380 > 62 (A), m/z 380 > 283 (B), m/z 380 > 344 (C), and m/z 380 > 362 (D). E: Product ions of m/z 380 corresponding to the corneal extract peak eluted at retention time (r.t.) 2.14 min (PGF$_{2\alpha}$-EA). PGE$_2$-EA and PGD$_2$-EA: m/z 380 > 62 (F); m/z 378 > 299 (G); m/z 378 > 342 (H); and m/z 378 > 360 (I). J, K: Product ions of m/z 378 corresponding to the corneal extract peak eluted at retention times (r.t.) 2.24 min (PGE$_2$-EA) and 2.79 (PGD$_2$-EA).
the tissue specific distribution of sn-1 docosahexanoyl species of PC (43).

Although A-EA is considered a minor lipid species, representing only 1–10% of total FA-EA in human membranes under basal conditions, studies carried out in brain and nervous tissue indicate A-EA levels are increased in response to injury (44, 45), and that A-EA participates as an anti-inflammatory agent of the immune response (25, 46). Few studies have examined the actions of A-EA per se in the cornea; however, it is a highly innervated tissue, and CB₁ receptors expressed on corneal sensory nerves stimulated by an agonist were found to support a role in contributing to antinociception in the anterior eye (47). Also, in a wound-healing model, both CB₁ and TRPV₁ receptor activation increased proliferation and migration in corneal epithelial cells (48, 49), thereby indirectly associating A-EA with these physiological functions. The presence of O-EA, ST-EA, L-EA, and DH-EA was previously unreported in corneal tissue (50). Reports showing a difference in the levels and distribution of 2-AG, A-EA, and P-EA in normal and glaucomatous ocular tissue, from human donors, suggest a role of these fatty acyl moieties in this disease state. The function of other FA-EA species is under investigation and effects on sleeping pattern, appetite control, and depression have been published to date (51–53). EP-EA was not detected in the cornea, which was unsurprising as eicosapentaenoic acid is a
enzymes possess no sequence homology and are optimally active under basic and acidic conditions respectively, as also reflected in their intracellular localization with FAAH found in membrane fractions and N-AAA in lysosomes.

minor species and DHA is the predominant omega-3 fatty acid found in the brain and ocular tissues (41, 54).

Inactivation of A-EA and other FA-EA occurs through hydrolysis via FAAH and N-AAA. Interestingly, these enzymes possess no sequence homology and are optimally active under basic and acidic conditions respectively, as also reflected in their intracellular localization with FAAH found in membrane fractions and N-AAA in lysosomes.

Fig. 5. The effect of COX inhibition on the formation of prostamides PGF$_{2\alpha}$-EA, PGE$_{2\alpha}$-EA, and PGD$_{2\alpha}$-EA by rabbit corneal tissue. Sample reconstructed ion chromatograms of corneal extract incubated with exogenously added anandamide (50 µM, 10 min at 37°C) in the absence (cornea + A-EA) or presence (cornea + A-EA+ PF3845) of the FAAH inhibitor PF3845 (100 nM) compared with commercially available prostamide standards (standard) at the following MRM transitions: PGF$_{2\alpha}$-EA: m/z 380 > 62 (A) and m/z 380 > 283 (B); PGE$_{2\alpha}$-EA and PGD$_{2\alpha}$-EA: m/z 378 > 62 (C) and m/z 378 > 299 (D).

Fig. 6. The effect of FAAH inhibition on the formation of prostamides PGF$_{2\alpha}$-EA, PGE$_{2\alpha}$-EA, and PGD$_{2\alpha}$-EA by rabbit corneal tissue. Sample reconstructed ion chromatograms of corneal extract incubated with exogenously added anandamide (50 µM, 10 min at 37°C) in the absence (cornea + A-EA) or presence (cornea + A-EA+ PF3845) of the FAAH inhibitor PF3845 (100 nM) compared with commercially available prostamide standards (standard) at the following MRM transitions: PGF$_{2\alpha}$-EA: m/z 380 > 62 (A) and m/z 380 > 283 (B); PGE$_{2\alpha}$-EA and PGD$_{2\alpha}$-EA: m/z 378 > 62 (C) and m/z 378 > 299 (D).
represent only a minor class of lipids, making up just 0.01% of total animal membrane phospholipids, under physiological conditions (29). Nevertheless, NAPE species are reported to exert bioactive functions independent of being the precursor of the FA-EA [reviewed in (30)]. Concentration of NAPE species can vary greatly depending on the tissue analyzed (e.g., levels described in the rat kidney are 6-fold higher than in rat brain cortex) (57, 58). In the present study, we have identified several NArPE and NAPE species present in corneal tissue (Table 2), and further studies are required to address the pathway of FA-EA generation from these parent phospholipids. Their effects on corneal tissue and cells, per se, also remain to be studied.

The data presented herein suggest that PGF$_2\alpha$-EA, PGE$_2$-EA, and PGD$_2$-EA can be produced by corneal tissue, a finding that could be attributed to the specific profile of prostanoid synthases expressed in rabbit cornea. To assess this, we analyzed corneal PGs and found that PGI$_2$ was the most prevalent metabolite, followed by PGE$_2$ and PGD$_2$ in approximately equal concentrations, and, at even lower levels, PGF$_2\alpha$. It would follow that the most prevalent PG-EA produced by the cornea would be PGI$_2$-EA, and evidence for its biosynthesis from PGH$_2$-EA has been presented by Kozak et al. (11). We have explored the possible production of PGI$_2$-EA through formation of its stable metabolite 6-keto PGF$_1\alpha$-EA, using SIR of [M+H]$^+ \text{m/z 414}$ and fragment ions predicted based on the fragmentation patterns of other prostamides (e.g., dehydration, (17)). The therapeutic potential of increasing in situ levels of A-EA has been shown in several studies through the use of FAAH inhibitors (26, 31). The use of nonsteroidal anti-inflammatory drugs (NSAIDs) is also of interest in controlling A-EA levels, with reports suggesting that lower concentrations of NSAIDs are required for the inhibition of A-EA cyclooxygenation than those required for arachidonic acid cyclooxygenation (55, 56). This would allow for a mechanism that modulates endocannabinoid levels without disrupting the effect of PGs.

While multiple pathways exist for generating free FA-EA from membrane stores (15), the precursor NAPE species represent only a minor class of lipids, making up just 0.01% of total animal membrane phospholipids, under physiological conditions (29). Nevertheless, NAPE species are reported to exert bioactive functions independent of being the precursor of the FA-EA [reviewed in (30)]. Concentration of NAPE species can vary greatly depending on the tissue analyzed (e.g., levels described in the rat kidney are 6-fold higher than in rat brain cortex) (57, 58). In the present study, we have identified several NArPE and NAPE species present in corneal tissue (Table 2), and further studies are required to address the pathway of FA-EA generation from these parent phospholipids. Their effects on corneal tissue and cells, per se, also remain to be studied.

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Prostamides and fatty acyl ethanolamines in rabbit cornea

In conclusion, the novel findings presented herein provide evidence that the pathway for the biosynthesis of PG-EA is operational in the cornea and, as such, constitutes a distinct target for modulating pain perception through use of FAAH and COX-2 inhibitors, in a way that is independent from the classical PG pathway. In addition, the congeners of A-EA were detected and quantified, which provides valuable insight into corneal physiology and those tissues that are anatomically adjacent. Thus, it is possible that corneal FA-EA and their biosynthetic precursors may influence a proximal region, such as the endothelial cells of Schlemm’s canal. These studies provide rationale for such future investigations.

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