HHS Public Access

Author manuscript

Methods Mol Biol. Author manuscript; available in PMC 2017 February 02.

Published in final edited form as:

Methods Mol Biol. 2016 ; 1412: 205–215. doi:10.1007/978-1-4939-3539-0_21.

Assay of Endocannabinoid Oxidation by Cyclooxygenase-2

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Summary

The endocannabinoids, 2-arachidonoylglycerol (2-AG) and arachidonylethanolamide (AEA) are endogenous ligands for the cannabinoid receptors (CB1 and CB2) and are implicated in a wide array of physiological processes. These neutral arachidonic acid (AA) derivatives have been identified as efficient substrates for the second isoform of the cyclooxygenase enzyme (COX-2). A diverse family of prostaglandin glycerol esters (PG-Gs) and prostaglandin ethanolamides (PG-EAs) is generated by the action of COX-2 (and downstream prostaglandin synthases) on 2-AG and AEA. As the biological importance of the endocannabinoid system becomes more apparent, there is a tremendous need for robust, sensitive and efficient analytical methodology for the endocannabinoids and their metabolites. In this chapter we describe methodology suitable for carrying out oxygenation of endocannabinoids by COX-2, and analysis of products of endocannabinoid oxygenation by COX-2 and of endocannabinoids themselves from *in-vitro* and cell assays.

Keywords

Cyclooxygenase-2; endocannabinoids; PG-Gs; PG-EAs; *in-vitro* assay; cell assay; LC-MS/MS

1. Introduction

The endocannabinoids 2-archidonoylglycerol (2-AG) and arachidonylethanolamide (AEA) are neutral arachidonic acid (AA) derivatives that exert analgesic and anti-inflammatory

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effects via the activation of cannabinoid receptors, CB1 and CB2 (1,2). Much like arachidonic acid (AA), 2-AG and AEA are oxygenated by the second isoform of the cyclooxygenase enzyme, COX-2. The oxygenation of 2-AG and AEA by COX-2 produces prostaglandin H_2-glycerol ester (PGH_2-G) and prostaglandin H_2-ethanolamide (PGH_2-EA), respectively (3,4). Each PGH_2 derivative undergoes further metabolism via prostaglandin synthases to a range of PG-glycerol esters (PG-Gs) and PG-ethanolamides (PG-EAs) that exhibit biological activities such as activation of calcium mobilization in tumor cells and macrophages, modulation of inhibitory synaptic transmission, induction of neurotoxicity by enhancement of excitatory glutamatergic synaptic transmission, and induction of hyperalgesia and anti-inflammatory responses (5–10) (Fig. 1). Additionally, when the macrophage cell line (RAW264.7) is treated with LPS and ionomycin, PG-Gs are produced which stimulate Ca^{2+} mobilization in the RAW264.7 cells (11,7), suggesting that PG-Gs may exert independent biological activities.

Given the physiological importance of the endocannabinoids and the potential biological relevance of their COX-2-derived oxygenated products, dependable methods for investigating these interactions are indispensable. In this chapter, we provide step-wise instructions for (1) establishing reactions of 2-AG and/or AEA with COX-2 both in vitro and in the RAW264.7 macrophage cell line, and (2) LC-MS/MS methodology that enables quantitative analysis of the endocannabinoids and their oxygenated metabolites.

We have utilized two LC-MS/MS methods; one for the analysis of COX-2 substrates (2-AG and AEA) and a second for the analysis of COX-2 oxygenation products (PG-Gs and PG-EAs). A silver cation (Ag^+) coordination, liquid-chromatography, electrospray-ionization, and tandem mass spectrometry (LC-ESI-MS-MS) method for analyzing 2-AG and AEA has been developed by our lab (12). In this method, the silver cation coordinates with the 4 double bonds of the arachidonate backbone of 2-AG and AEA, which is rich in π electrons. This coordination of silver to 2-AG and AEA forms an $[M+Ag]^+$ complex that is amenable to electrospray ionization and tandem mass spectrometric techniques. Figure 2 shows the chromatograms of 2-AG, AEA and their respective internal standards coordinated with silver. In Figure 2A, two peaks are seen corresponding to 1-AG and 2-AG, which are two isomers of arachidonoylglycerol. While 2-AG is the biologically relevant isomer of arachidonoylglycerol, 2-AG readily undergoes acyl migration under biological settings to form 1-AG (13).

Our laboratory has also published a method for the simultaneous analysis of several PG-Gs and PG-EAs (14). A method describing the analysis of $PGF_{2\alpha}$-EA also exists in the literature (15). The methodology developed in our laboratory (14) involves complexing the neutral PG-Gs and PG-EAs with either ammonium (NH_4^+) or a proton (H^+). The resultant $[M+NH_4]^+$ or $[M+H]^+$ complexes yield multiple intense fragments upon collisionally-induced dissociation (CID), several of which may be employed in selected-reaction monitoring (SRM). Chromatograms of different species of PG-Gs and PG-EAs along with the respective internal standards are shown in Figure 3.

2. Materials

2.1 General

1. 2-AG, AEA, deuterated 2-AG and deuterated AEA. Additionally, the penta-deuterated analogue of PGE_2-G and tetra-deuterated PGE_2-EA were synthesized as described previously (3) (*see* Note 1).
2. 96-well plate or auto-sampler vials.

2.2 In vitro Assays

1. 5 mM hematin stock solution in dimethyl sulfoxide (DMSO). Store at room temperature.
2. Reaction buffer: 100 mM Tris--HCL (pH 8.0) with 500 μM phenol. Store at room temperature.
3. Purified COX-2 enzyme (16).
4. Substrates or inhibitors of interest.
5. Quench solution: Internal standards dissolved in ethyl acetate with 0.5% acetic acid. Place on ice before use in assay. The concentration of the internal standards in the quench solution should be such that the amount of internal standard delivered to each sample upon quenching is within 10-fold of the amount of the analyte that the internal standard will be used to quantitate.

2.3 Cell Assay

1. RAW264.7 macrophages.
2. Dulbecco's Modified Eagle Medium (DMEM) media supplemented with 10% fetal bovine serum.
3. 100 ng/ml working solution of Kdo_2-lipid A (KLA) in Ca^{2+}/Mg^{2+} free sterile Dulbecco's phosphate buffered saline (DPBS buffer). Make the working solution of KLA in serum-free medium.
4. 2 mM ionomycin stock solution in DMSO. Our lab uses 2–5 μM of ionomycin as the final concentration.
5. Inhibitors of interest (*see* Note 2).
6. Extraction solution; similar to "Quench solution" in section 2.2.5.

2.4 LC-MS/MS

1. Silver complexation mobile phase components;

[1]Many vendors sell deuterated internal standards listed in Section 2.1.3. It is in the researchers' best interest to establish the isotopic purity of purchased internal standards before use with unknown samples. Our lab has found that some isotopically labeled compounds have a range of stable isotope incorporation and that some isotopically labeled compounds will give a signal in the SRM channel for the native compound of interest.
[2]Stock solutions of inhibitors are made in DMSO.

A. 150 μM silver acetate in HPLC grade water plus 0.1% formic acid.

B. 150 μM silver acetate in HPLC grade methanol plus 0.1 % formic acid.

2. Ammonium complexation mobile phase components;

A. 5 mM ammonium acetate in HPLC grade water, pH adjusted to 3.2–3.4 with formic acid.

B. 6% component A in HPLC grade acetonitrile with 0.1 % formic acid.

3. HPLC column: C18, 5 × 0.2 cm, either 3 or 5 um particle size.

4. LC-MS system: an HPLC system with a binary pump and autosampler in-line with a triple quadrupole mass spectrometer and appropriate data acquisition software (*see* Note 3).

5. Standard mixture of analytes in methanol at a concentration of 2 μM--store at −20°C.

6. Internal standard recovery solution (*see* Note 4).

3.0 Methods

3.1 *In-vitro* Assay

1. Prepare a 40X substrate stock solution whose concentration is 40 times the concentration of substrate in the reaction vessel. Prepare in DMSO and make enough to provide 5 μL for each 200 μL reaction.

2. Prepare a 40X inhibitor stock solution whose concentration is 40 times the concentration of inhibitor in the reaction vessel. Prepare in DMSO and make enough to provide 5 μL for each 200 μL reaction.

3. Prepare a COX-2 solution in 100 mM Tris--HCL with 500 μM phenol, pH.8.0 buffer (the usual concentration of COX-2 is 50–100 nM). Add 3 equivalents of hematin solution to this enzyme solution 10–15 mins prior to the experiment. Keep this enzyme solution on ice.

4. Prepare the quench solution and keep on ice.

5. For inhibition assays, aliquot 190 μL of the COX-2 solution into a 1.5 mL microfuge tube and incubate in heat block set at 37 °C for 3 mins. For assays with weak-reversible inhibitors, add 5 μL of 40X inhibitor stock solution to the enzyme solution and incubate at 37°C for an additional 3 mins. For assays with slow-tight binding inhibitors, add 5 μL of 40X inhibitor stock solution to 190 μL of the enzyme solution and incubate at 37°C for an additional 15 mins. After the

[3]Our lab has employed both a Thermo Quantum triple quadrupole (with Xcalibur software) and a SCIEX 3200 QTrap (with Analyst software) instruments. Any reasonably modern triple quadrupole or ion trap mass spectrometer should give reasonable results for the methods discussed here.

[4]The recovery internal standard is a standard where the amount of internal standard in each sample is dissolved in the reconstitution volume used for each sample. This sample is important because it (a) establishes whether the instrument is working acceptably and (b) gives the experimenter the recovery level of his/her analytes, which is a useful parameter when assessing the experimental results.

incubation period, add 5 μL of stock substrate solution and wait 30 sec (*see* Note 5).

6. For assays with only COX-2 and substrate, aliquot 195 μL of the COX-2 solution into a 1.5 mL microfuge tube and incubate in a heat block set at 37 °C for 3 min. Then add 5 μL of 40X substrate stock solution and wait 30 sec (*see* Note 5).

7. After the 30 sec reaction period, quench the reaction by adding 200 μL of the quench solution. Vortex vigorously and keep on ice.

8. Collect the top layer from the quenched reaction and add it to a small glass tube. Dry the solution under a stream of N_2.

9. Re-suspend the dried solution with 200 μL of 1:1 methanol:water (HPLC grade water) solution and vortex vigorously.

10. Aliquot ~200 μL of this solution to either auto-sampler vials or a 96-well plate.

11. Load the vials or plates into the autosampler of the LC-MS system; prepare a queue with an appropriate method and start the program as described in section 3.3.

3.2 Cells Assay

1. Plate 3×10^6 cells in 8mL DMEM (10% FBS).

2. After 24 h, co-treat with Kdo_2-lipid A (KLA) and inhibitor solution for 6hrs (for COX-2 activation) (*see* Note 6).

3. Collect the media and transfer it to a Falcon tube (15 mL).

4. Extract PG-G or PG-EA species from the media by adding the extraction solution (2.3.6 -- using 2 times the media volume) to the Falcon tube containing the media. Vortex vigorously and keep on ice.

5. Transfer the top layer to a clean vessel and dry it down under N_2

6. Re-suspend the dried solution with 200 μL of 1:1 methanol:water solution and vortex vigorously

7. Aliquot ~200 μL of this solution to either auto-sampler vials or a 96-well plate.

8. Load the vials or plates into the autosampler of the LC-MS system, prepare a queue with an appropriate method and start the program as described in section 3.3.

3.3 LC-MS analysis

This section will describe two LC-MS/MS method; one for the analysis of COX-2 substrates (2-AG and AEA) and a second for the analysis of COX-2 oxygenation products (PG-Gs and

[5]It is important to limit the percent DMSO to ≤ 5 % to prevent protein precipitation.
[6]Slow-tight binding inhibitors are added along with KLA for 6 hours. In contrast, weak-reversible inhibitors plus 5 μM of ionomycin are added after 6 hours of treatment with KLA. The addition of ionomycin releases 2-AG. Cells are incubated for an additional 45 mins before extracting PGs.

PG-EAs). Both methods employ reverse-phase chromatography and mass spectrometric detection where the mass spectrometer is equipped with an electrospray source, operated in positive ion mode and is configured for selected reaction monitoring (SRM). Obviously, not all analytes described here need be included in one's assay--the choice of analytes depends on the experimental parameters and the interest of the investigator. These methods are based on methods described in the literature for endocannabinoids (12) and prostanoids (14).

1. Prime the LC system with appropriate mobile phase (*see* **Section 2.4, 1 or 2**) and establish mobile phase flow through the chosen column at the initial conditions.
2. Create (or modify an existing) instrument method containing the desired SRM transitions and chromatographic gradient profile, as specified in tables 1–4 (*see* Note 7)
3. Place samples in sample tray and create a run sequence. It is best to bracket all unknowns with standards and then randomize the order in which the unknowns are analyzed.
4. Inject the standard solution, verify that all analytes are observed
5. Inject the Internal Standard Recovery solution, verify that the retention times are very similar to Step 4, that no analyte peak is observed in the appropriate SRM transition, and that all internal standards are observed (*see* Note 4).
6. Inject a blank and ensure that no peaks appear in any transition.
7. Start the sequence.
8. After the samples have been successfully injected, prepare a processing method and process the resultant raw files.
9. Export data to Excel or other spreadsheet and calculate analyte amounts (*see* Note 8).

The representative chromatograms of COX-2 substrates, 2-AG and AEA, and their oxygenated products are shown in figures 2 and 3. For SRM analysis of 2-AG and 2-AG-d_8, the transitions of m/z 485 → 411 and the m/z 493 → 419 are used (Fig. 2A and 2B) (see note 12). For AEA, the m/z 454 → 436 transition is employed for SRM analysis, while the m/z 462 → 444 transition is used for detection of AEA-d_8 (Fig. 2B and 2C). For PG-Gs and PG-G-d_5 PGs m/z 444 → 391 and m/z 449 → 396 transitions are used for SRM analysis, respectively (Fig. 3A and 3B). A transition of m/z 446 → 393 is used for PG-$F_{2\alpha}$-G (Fig. 3C). For SRM analysis of PG-EA and PG-EA-d_4 m/z 396 → 360 and m/z 400 → 364 transitions are used (Fig. 3D and 3E).

Acknowledgments

We are grateful to Carol Rouzer for editorial assistance. This work was supported by a research grant from the national institute of health (GM 15431).

[7]Flow rate of 0.3–0.4 mL/min is recommended for both the LC-MS-MS methods.
[8]Stable isotope dilution is used for quantification of analytes in the assays described above. With stable isotope dilution, the amount of analyte in each sample = the response ratio (analyte peak area divided by the internal standard peak area) of the analyte multiplied by the amount of internal standard added to the sample.

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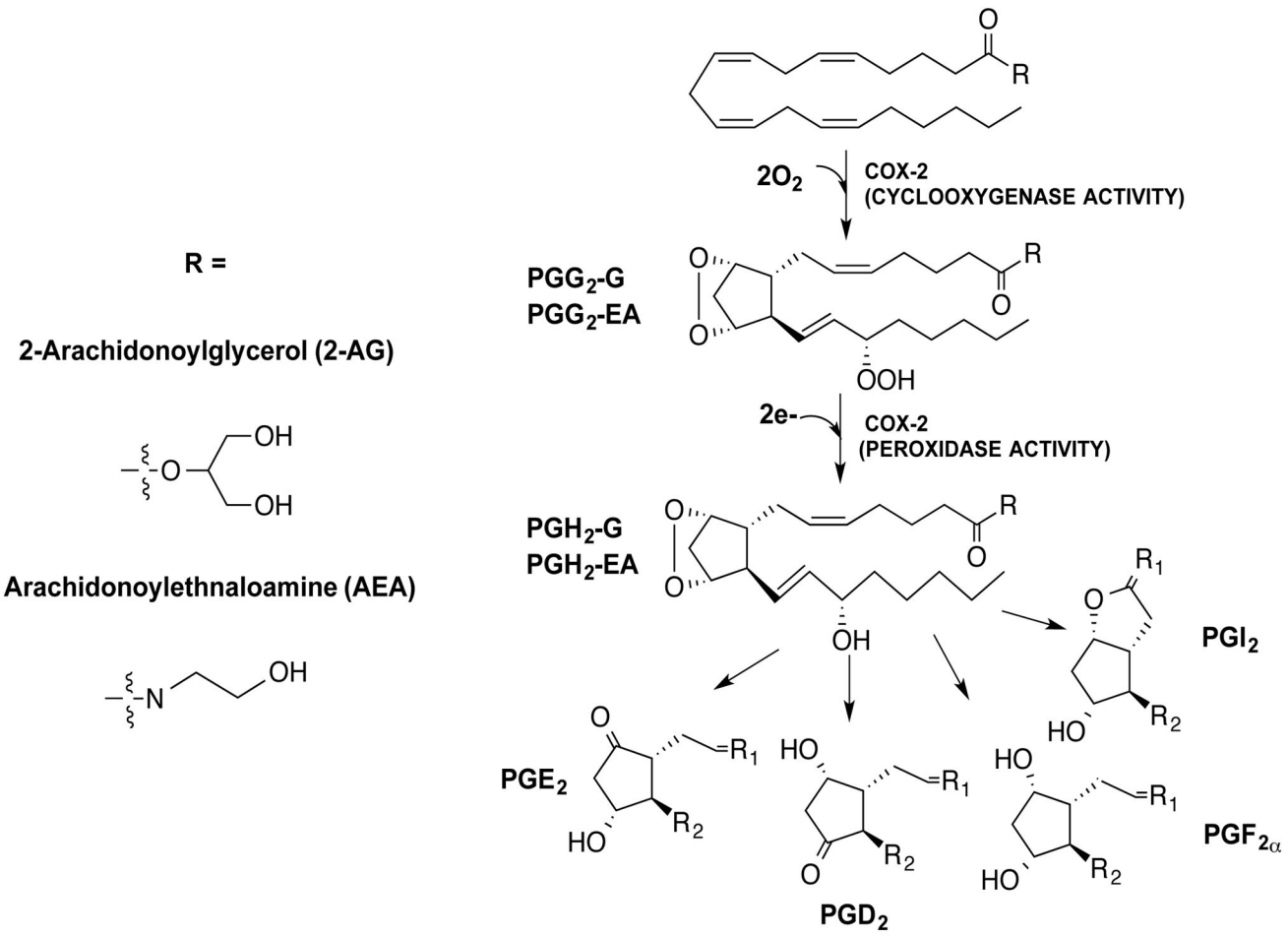

Fig. 1.

Structures of endocannabinoids, 2-arachidonoylglycerol (2-AG) and arachidonylethanolamide (AEA), and their conversion by COX-2 and various PG synthases to prostaglandin glyceryl esters (PG-Gs) and prostaglandin ethanolamides (PG-EAs), respectively.

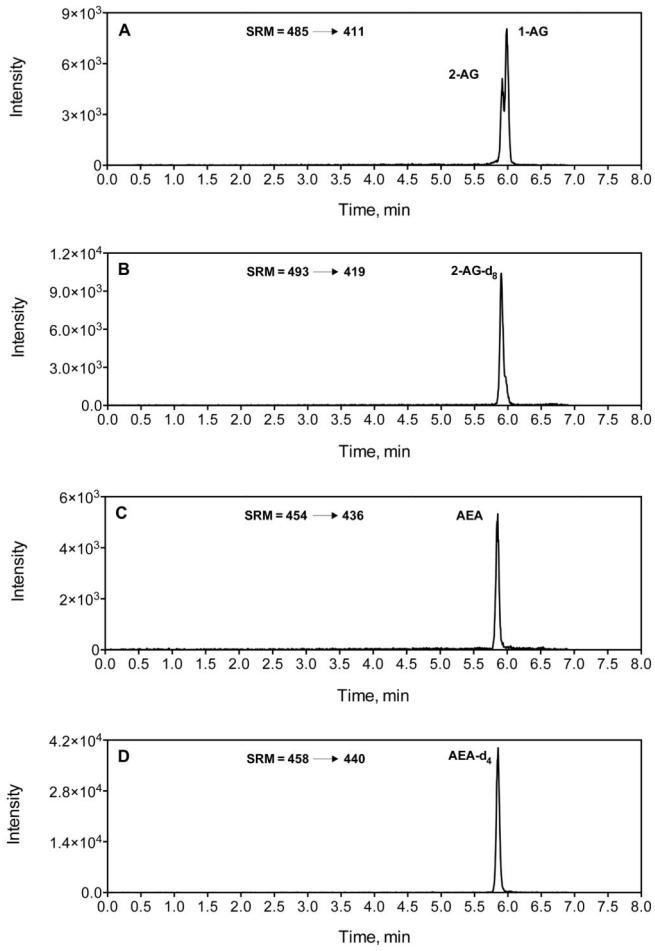

Fig. 2.

LC-MS/MS chromatograms of endocannabinoids and their deuterated internal standards, 2 and 1-AG (*A*), 2-AG-d_8 (*B*), AEA (*C*) and AEA-d_4 (*D*).

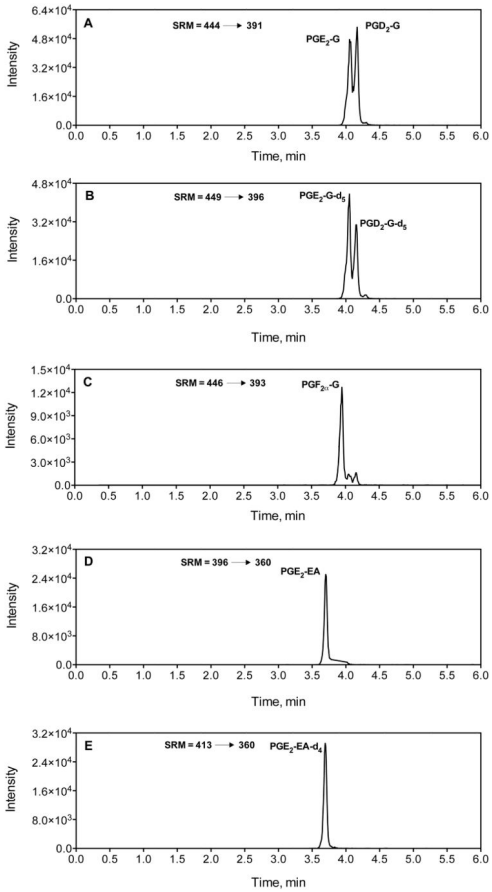

Fig. 3.

LC-MS/MS chromatograms of selected PG-Gs and PG-EAs; PGE_2-G and PGD_2-G (*A*), PGE_2-G-d_5 and PGD_2-G-d_5 *(B),* $PGF_{2\alpha}$-G *(C)*, PGE_2-EA *(D)* and PGE_2-EA-d_4 *(E).*

Table 1

SRM transitions for endocannabinoids via silver complexation analysis

Compound	M.W.	Q1 *(m/z)*	Q3 *(m/z)*	Collision Energy
2-AG	378.6	485	411	33
2-AG-d_8	386.6	493	419	33
AEA	347.5	454	436	33
AEA-d_4	351.5	458	440	33

Table 2

SRM transitions for oxygenated products of COX-2

Compound	M.W.	Q1 *(m/z)*	Q3 *(m/z)*	Collision Energy
PGE_2-G & PGD_2-G	426.6	444	391	19
$PGF_{2\alpha}$-G	428.6	446	393	19
PGE_2-G -d_5	431.6	449	396	19
PGE_2-EA	395.6	396	360	13
PGE_2-EA-d_4	399.6	400	364	13

Table 3

Gradient for silver complexation LC-MS/MS.

Time	%B
Initial	70
0.5	70
4.5	100
5.5	100
6.0	70
7.5	70

Table 4

Gradient for oxygenated product LC-MS/MS.

Time	%B
Initial	30
0.5	30
2.0	80
3.70	80
3.74	30
3.75	30