

LC/MS/MS method for analysis of E₂ series prostaglandins and isoprostanes

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Abstract 15-series prostaglandins (PGE₂s) and isoprostanes (isoPGE₂s) are robust biomarkers of oxidative stress, possess potent biological activity, and may be derived through cyclooxygenase or free radical pathways. Thus, their quantification is critical in understanding many biological processes where PG, isoPG, or oxidative stress are involved. LC/MS/MS methods allow a highly selective, sensitive, simultaneous analysis for prostanoids without derivatization. However, the LC/MS/MS methods currently used do not allow for simultaneous separation of the major brain PGE₂/D₂ and isoPGE₂ without derivatization and multiple HPLC separations. The developed LC/MS/MS method allows for the major brain PGE₂/PGD₂/isoPGE₂ such as PGE₂, entPGE₂, 8-isoPGE₂, 11β-PGE₂, PGD₂, and 15(R)-PGD₂ to be separated and quantified without derivatization. The method was validated by analyzing free and esterified isoPGE₂ in mouse brains fixed with head-focused microwave irradiation before or after global ischemia. Using the developed method, we report for the first time the esterified isoPGE₂ levels in brain tissue under basal conditions and upon global ischemia and demonstrate a nonreleasable pool of esterified isoPG upon ischemia. In addition, we demonstrated that PGE₂s found esterified in the sn-2 position in phospholipids are derived from a free radical nonenzymatic pathway under basal conditions. Our method for brain PG analysis provides a high level of selectivity to detect changes in brain PG and isoPG mass under both basal and pathological conditions.—Brose, S. A., B. T. Thuen, and M. Y. Golovko. LC/MS/MS method for analysis of E₂ series prostaglandins and isoprostanes. *J. Lipid Res.* 2011. 52: 850–859.

Supplementary key words prostaglandin • isoprostane • phospholipid • brain lipids • prostanoid analysis • ischemia • microwave irradiation • high performance liquid chromatography • tandem mass spectrometry

This work was supported by the National Institutes of Health Grant 5R21NS064480-02 (M.Y.G.), North Dakota Experimental Program to Stimulate Competitive Research (ND EPSCoR) grant EPS-0814442 (M.Y.G.), University of North Dakota New Faculty Scholar Award (M.Y.G.), and the National Institutes of Health-funded Centers of Biomedical Research Excellence (COBRE) Mass Spec Core Facility Grant 5P20RR017699. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the National Institutes of Health or other granting agencies.

Manuscript received 7 December 2010 and in revised form 7 February 2011.

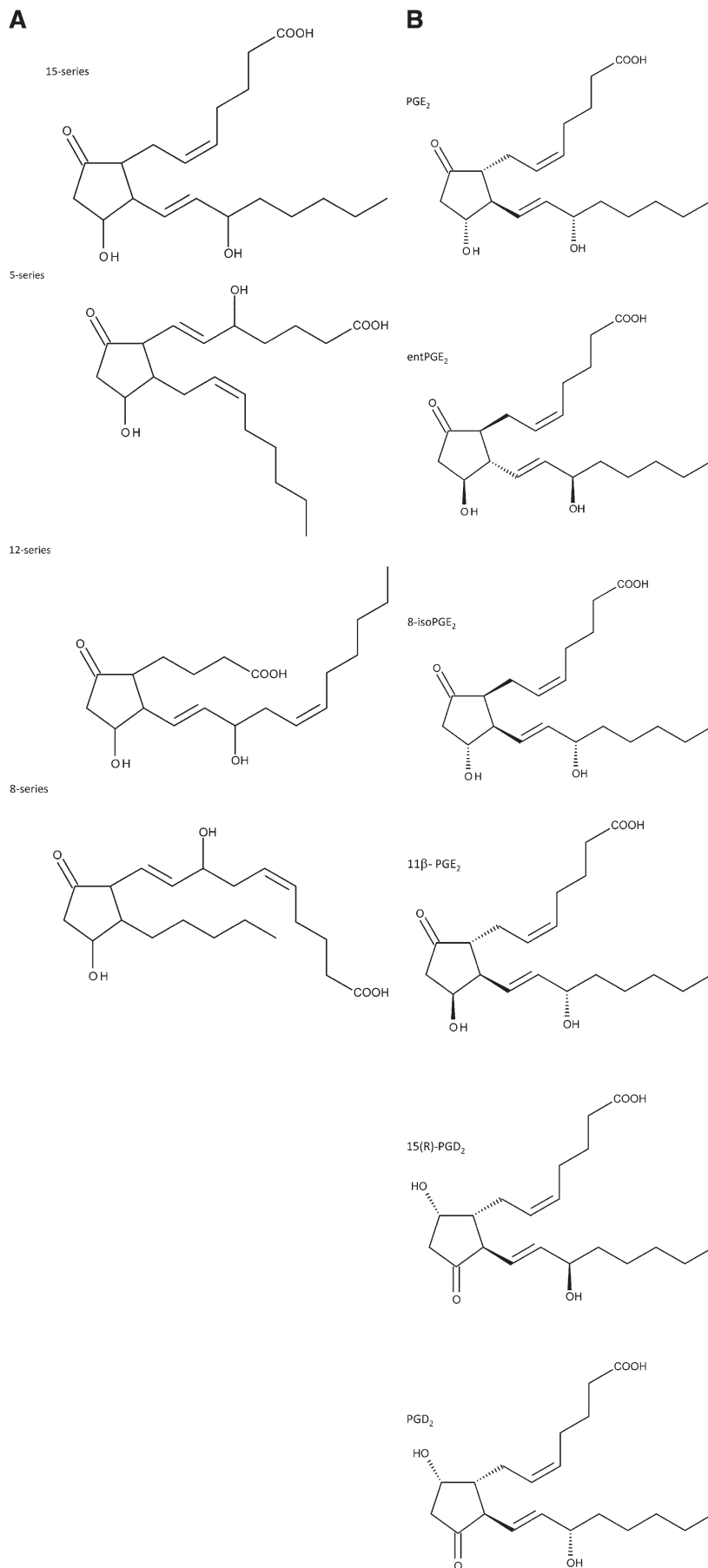
Published, JLR Papers in Press, February 10, 2011
DOI 10.1194/jlr.D013441

Prostaglandins (PGs) are important signaling molecules that are ubiquitously produced in many tissues and regulate many physiological functions under both normal and pathophysiological conditions. In the brain, they modulate synaptic plasticity through modulation of adrenergic, noradrenergic, and glutamatergic neurotransmission and regulation of membrane excitability (1–4). In addition, PGs are involved in a broad array of nervous system diseases including cancer, inflammation, neurodegenerative diseases, central nervous system injury, and neuropsychiatric conditions (5–12). The physiological importance of PGs in mediating pain, fever, and inflammation is highlighted by the use of a wide array of inhibitors of PG biosynthesis.

PGs are produced in two distinguished pathways: enzymatically through a cyclooxygenase (COX)1/2 dependent mechanism, and nonenzymatically through an isoprostane (isoPG) mechanism (13). In the isoPG pathway, PGs and isoPGs are formed in situ via the nonenzymatic oxidation of esterified arachidonic acid (20:4n-6) and remain in the esterified form on phospholipids (PLs) (13–17). Thus, PLs contain both PG and PG-like isoPG in the esterified form. There are four positional isoforms of isoPG that belong to 5-, 12-, 8-, and 15-series with COX1/2 derived PGE₂ belonging to the 15-series (18) (Fig. 1A). Because isoPGE₂/D₂ have four chiral centers, there are 16 possible chiral isoforms within each group. Within the 15-series isoPGE₂, the most thermodynamically stable are PGE₂, PGE₂ enantiomer (entPGE₂), 8-isoPGE₂, and 11β-PGE₂ that are found in abundance in tissues (13, 19–21) (Fig. 1B). Al-

Abbreviations: 20:4n-6, arachidonic acid; BHT, butylated hydroxytoluene; COX, cyclooxygenase; entPGE₂, 9-oxo-11β,15R-dihydroxy-(8β,12α)-prosta-5Z,13E-dien-1-oic acid; isoPG, isoprostane; PG, prostaglandin; PGD₂, 9α,15S-dihydroxy-11-oxo-prosta-5Z,13E-dien-1-oic acid; 15(R)-PGD₂, 9α,15R-dihydroxy-11-oxo-prosta-5Z,13E-dien-1-oic acid; PGE₂, 9-oxo-11α,15S-dihydroxyprosta-5Z,13E-dien-1-oic acid; 11β-PGE₂, 9-oxo-11β,15S-dihydroxyprosta-5Z,13E-dien-1-oic acid; 8-isoPGE₂, 9-oxo-11α,15S-dihydroxy-(8β)-prosta-5Z,13E-dien-1-oic acid; PGE₂-d₉, 9-oxo-11α,15S-dihydroxyprosta-5Z,13E-dien-1-oic-3,3,4,4-²H₄ acid; PGE₂-d₉, 9-oxo-11α,15S-dihydroxyprosta-5Z,13E-dien-1-oic-17,17,18,18,19,19,20,20,20-²H₄ acid; PL, phospholipid; PLA₂, phospholipase A₂; sPLA₂, secreted phospholipase A₂.

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though isoPGs are stable robust biomarkers of oxidative stress (22, 23), 15-series isoPG might also possess potent biological activity (15, 21). Esterified PGE₂ and isoPGE₂ might be released from PL by platelet activating factor acetylhydrolase II (24) that is activated at early stages of tissue stimulation (25). Because the initial rapid increase in PG mass triggers downstream phases of tissue response upon stimulation (26–28), the released PG and isoPG from PL at early stages of tissue stimulation may also have a critical role during the later phases of tissue response. Because of the important role for PGE₂ and 15-series isoPGE₂ in normal and pathophysiological processes, as well as biomarkers of oxidative stress, their quantification is critical in understanding many biological processes where PG, isoPG, or oxidative stress are involved.

A number of methods have been used for brain and other tissue-esterified isoPGE₂ quantification. Liquid chromatography with tandem mass spectrometer detection (LC/MS/MS) methods are considered to be the most specific and less laborious for isoPG measurement as compared with enzyme immunoassay and radioimmunoassay methods, high performance liquid chromatography (HPLC) with ultraviolet or fluorescence detection, and gas chromatography with MS or flame ionization detection because of a better isomer separation and no derivatization or multi-step purification requirements (29, 30). However, the LC/MS/MS methods currently used do not allow for simultaneous separation of the major brain PGE₂/D₂ and isoPGE₂ such as PGE₂, entPGE₂, PGD₂, 8-isoPGE₂, and 11β-PGE₂ without derivatization and multiple HPLC separations (13, 20, 29, 31–36).

In the present study, we developed an LC/MS/MS method that allows for the major brain PGE₂/D₂ series molecules such as PGD₂, 15(R)-PGD₂, PGE₂, entPGE₂, 8-isoPGE₂, and 11β-PGE₂ to be separated and quantified without derivatization and validated the method by analyzing free and esterified isoPGE₂ in brains fixed with head-focused microwave irradiation before or after global ischemia. Using the developed method, we report for the first time esterified isoPGE₂ levels in brain tissue under basal conditions and upon global ischemia and demonstrate a nonreleasable pool of isoPG esterified onto brain PL. In addition, we demonstrate that PGE₂s found esterified in the sn-2 position in PL are derived from free radical nonenzymatic pathway under basal conditions.

MATERIALS AND METHODS

Chemicals

9-oxo-11α,15S-dihydroxyprosta-5Z,13E-dien-1-oic acid (PGE₂), 9-oxo-11β,15R-dihydroxy-(8β,12α)-prosta-5Z,13E-dien-1-oic acid (entPGE₂), 9-oxo-11β,15S-dihydroxyprosta-5Z,13E-dien-1-oic acid (11β-PGE₂), 9-oxo-11α,15S-dihydroxy-(8β)-prosta-5Z,13E-dien-1-oic acid (8-isoPGE₂), 9-oxo-11α,15S-dihydroxyprosta-5Z,13E-dien-1-

oic-3,3,4,4²H₄ acid (PGE₂-d4), 9-oxo-11α,15S-dihydroxyprosta-5Z,13E-dien-1-oic-17,17,18,18,19,19,20,20,20²H₄ acid (PGE₂-d₆), 9α,15S-dihydroxy-11-oxo-prosta-5Z,13E-dien-1-oic acid (PGD₂), 9α,15R-dihydroxy-11-oxo-prosta-5Z,13E-dien-1-oic acid (15(R)-PGD₂), 9α,15S-dihydroxy-11-oxo-prosta-5Z,13E-dien-1-oic-3,3,4,4²H₄ acid (PGD₂-d4), and arachidonic acid (20:4n-6) were purchased from Cayman Chemical Co. (Ann Arbor, MI). Other chemicals of analytical or higher quality were from Merck KGaA (Darmstadt, Germany).

Animals

This study was conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (NIH publication 80-23) and under an animal protocol approved by the IACUC at the University of North Dakota (Protocol #0903-1 and #0806-1). Male CD1 strain mice (30–35 g) were maintained on standard laboratory chow diet and water ad libitum. The ages of the mice used in this study were between 8-11 months.

Brain prostanoid extraction

To analyze PG under basal conditions, male mice were anesthetized with isoflurane (1-3%) and euthanized by head-focused microwave irradiation (3 kW, 1.33 s; Cober Electronics, Inc., Norwalk, CT) to heat denature enzymes in situ. To model global ischemia, mouse brains were subjected to head-focused microwave irradiation 5 min after decapitation (29, 37, 38). The whole brain was frozen in liquid nitrogen and pulverized under liquid nitrogen temperatures to a fine homogeneous powder.

Prostanoids were extracted with acetone using liquid/liquid extraction as previously described (29). This method demonstrates an ~90% of extraction efficiency and demonstrates the greatest sensitivity during MS analysis as the result of reduction of basal noise (29). Briefly, pulverized tissue was homogenized in 3 ml of acetone/saline (2:1) containing 100 pg of PGE₂-d4 as an internal standard and 0.005% of butylated hydroxytoluene (BHT) to prevent fatty acid oxidation using a Tenbroeck tissue grinder (Kontes Glass Co., Vineland, NJ). After 10 min of centrifugation (2000 g) at 4°C, the supernatant was washed using 3 × 2.0 ml of hexane, acidified with formic acid to pH = 3.5 (30 μl of 2M formic acid), and extracted with 2 ml of chloroform. Chloroform extract containing PG was transferred to silanized with Sigmacote® (Sigma Chemical Co., St. Louis, MO) tube, flushed with nitrogen, and cooled at –80°C for at least 15 min. This cooling allowed the separation of any residual upper phase, which was then removed and discarded before analysis.

Sample preparation for LC/MS/MS

Samples were prepared for LC/MS/MS analysis as previously described (29). After the residual upper phase was discarded, 200 μl of methanol was added to the extract and dried down under a stream of nitrogen. The dried extract was transferred to 300 μl silanized microvial inserts (National Scientific, Rockwoods, TN) using 2 × 0.1 ml of chloroform containing 10% methanol. The solvent in microvial inserts was dried down under a stream of nitrogen and redissolved in 30 μl of acetonitrile:water (1:2) for isoPG separation. For chiral chromatography of PGE₂ and entPGE₂, 12 μl of 35% acetonitrile was used to redissolve samples.

Fig. 1. Positional and selected chiral isoforms of isoPGE₂ and PGD₂. A: Positional isoforms for isoPGE₂. For simplicity, stereochemical orientation is not indicated in the figure. B: Selected isoPGE₂ and isoPGD₂ stereoisomers used in the study.

HPLC of isoPGE₂ and PGE₂

The separation of isoPGE₂ and PGE₂ was carried out using a Luna C-18(2) column (3 μm, 100 Å pore diameter, 150 × 2.0 mm, Phenomenex, Torrance, CA) with a stainless steel frit filter (0.5 μm) and security guard cartridge system (C-18) (Phenomenex). The HPLC system consisted of an Agilent 1100 series LC pump equipped with a wellplate autosampler (Agilent Technologies, Santa Clara, CA). The autosampler was set at 4°C. A 25 μl out of 30 μl sample was injected onto a chromatographic column.

The solvent system was composed of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The flow rate was 0.2 ml/min. Solvent B was increased from 20% to 42.5% over 50 min, at 50 min was increased further to 90% over 10.5 min to wash the column, and at 65.5 min, it was returned to 20% over 1 min for column equilibration. Equilibration time between runs was 14 min. An example of brain PG LC/MS/MS analysis is presented in Fig. 8.

Esterified PG determination

Brain samples (~35.5 mg) were incubated in 200 μl of buffer (80 mM Hepes, pH 7.4, containing 300 mM sodium chloride, 20 mM CaCl₂, 8 mM Triton X-100, 60% glycerol, and 2 mg/ml BSA) containing 100 pg of PGE₂d₄ with or without secretory (s)PLA₂ (~0.9 μmole/min of total activity). This enzyme was tested for contamination with isoPG and no contamination was detected in the quantities of enzyme used in the experiments. To validate completeness of PG hydrolysis from PL under these conditions, a time course between 1 to 240 min was built. Esterified PGs were completely released during 1 h of incubation (data not shown). In addition, there was no nonenzymatic hydrolysis during tissue incubation in the buffer without sPLA₂. After 1 h of incubation at ambient temperature, PGs were extracted and quantified as described above. The difference between prostanoid levels with and

without sPLA₂ treatment represents prostanoids esterified onto the sn-2 position in PL.

Chiral HPLC of PGE₂ enantiomers

The separation of PGE₂ and entPGE₂ was carried out on two chiral Lux Amylose2 columns connected in tandem (3 μm, 100 Å pore diameter, 150 × 2.0 mm; Phenomenex). Tissue PG extracts were separated on a C18(2) Luna column as described above. PGE₂, entPGE₂, and coeluted internal standard PGE₂d₄ fractions from HPLC run (~150 μl) were collected, dried down in rotary vacuum concentrator, redissolved in 12 μl of 35% acetonitrile in water, and 11 μl were loaded onto the column. PGs were eluted at 50 μl/min with isocratic 35% acetonitrile in 0.1% aqueous formic acid solvent system. An example of brain entPGE₂ LC/MS/MS analysis is presented in Fig. 6.

Electrospray ionization mass spectrometry

For PG quantification, a quadrupole mass spectrometer (API3000, AB Sciex, Foster City, CA) equipped with TurboIon-Spray ionization source was used. Analyst software version 1.5.1 (Applied Biosystem) was used for instrument control, data acquisition, and data analysis. The mass spectrometer was optimized in the multiple reaction-monitoring mode. The source was operated in negative ion electrospray mode at 350°C, electrospray voltage was -4250 V, nebulizer gas was zero grade air at 8 L/min, and curtain gas was ultrapure nitrogen at 11 L/min. Declustering potential, focusing potential, and entrance potential were optimized individually for each analyte. The quadrupole mass spectrometer was operated at unit resolution. For high resolution exact mass analysis of PG product ions, API5500Q (AB Sciex) in product ion scan mode operated in electrospray negative ionization mode was used.

PGE₂ and isoPGE₂ were quantified using PGE₂d₄ as the internal standard. For PGE₂ and isoPGE₂ monitoring in the MRM

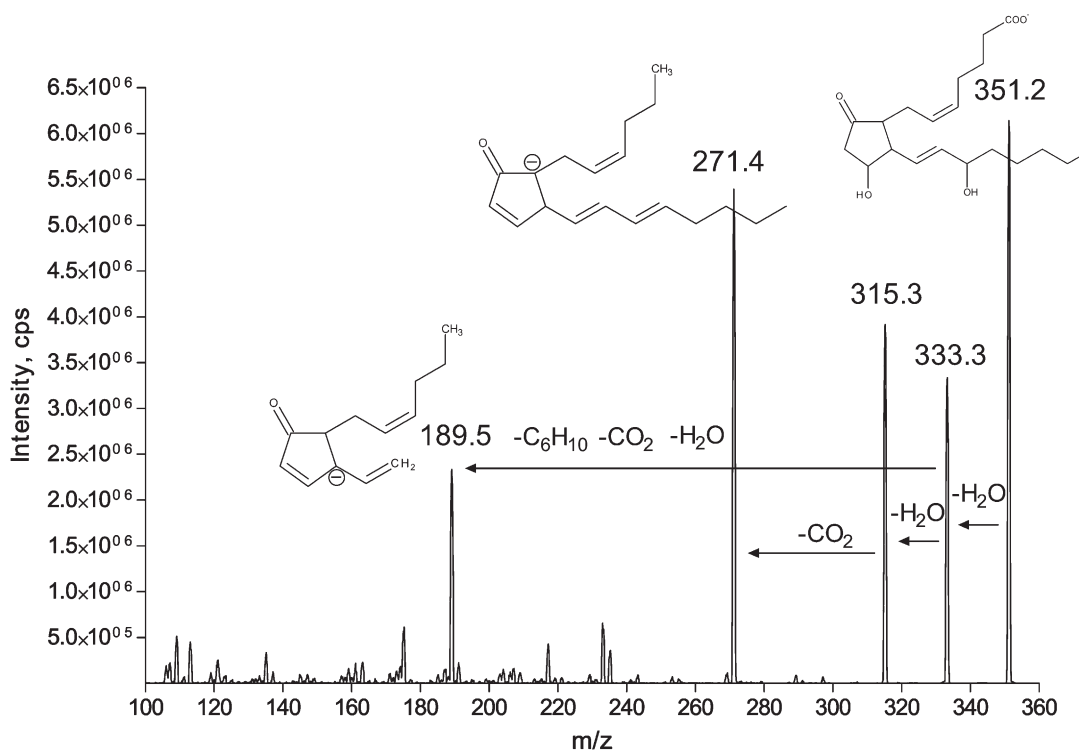


Fig. 2. MS/MS spectrum for PGE₂. Negative ion MS/MS spectrum of PGE₂ standard was generated by direct infusion into API5500Q electrospray ion source.

mode, 351.2/189.5 mass transition was used, and for PGE₂d₄, 355.2/275.5 was used.

Statistical analysis

All statistical comparisons were calculated using a two-way unpaired Student's *t*-test using GraphPad Prism 5 (Graphpad, San Diego, CA). Statistical significance was defined as <0.05. All values were expressed as mean ± SD.

RESULTS AND DISCUSSION

LC/MS/MS methods are considered to be the most specific and less laborious for PG and isoPG quantification as compared with other methods of analysis (29, 30). However, because PGE₂, PGD₂, and 15-series isoPGE₂ have the same molecular weight and similar product ion spectrums, it is important to separate them in time before MS/MS detection. A number of HPLC conditions have been used to resolve PGE₂/PGD₂-like molecules (13, 20, 29, 31–36). However, to the best of our knowledge, none of the reported conditions allowed for PGE₂, PGD₂, and major isoPGE₂ separation in a single run without derivatization.

Selection of MS/MS mass transition

To resolve PGE₂/PGD₂-like molecules, we first optimized MS/MS parameters to achieve higher selectivity for 15-series isoPGE₂. Most of the product ions produced from PGE₂ are generated by water and carboxyl group loss (product ions 333.3, 315.3, and 271.4) that are not specific toward 15-series isoPGE₂ (Fig. 2). However, a product ion with *m/z* = 189.5 is generated by the side chain loss (Fig. 2) that allows a more selective quantification for 15-series isoPG, thereby eliminating up to 48 possible isoforms from the analysis. The exact *m/z* of this product ion was determined using a high resolution mass spectrometer with linear ion trap (API5500Q) and appears to be *m/z* = 189.1279. This is consistent with the elemental composition of the presented structure (+1.2 mDa mass difference). The presented structure of the ion with *m/z* = 189 is also consistent with published data (39). In addition, PGE₂ standards labeled with deuterium at 17, 17, 18, 18, 19, 19, 20, 20, 20 (PGE₂d₉) or at 3, 3, 4, 4 (PGE₂d₄) positions were used to confirm the pathway for an ion with *m/z* 189 generation from PGE₂. Upon PGE₂d₉ collision, the ion with *m/z* = 189.5 was predominant over the 198.5 ion, whereas PGE₂d₄ produced mainly 193.5 rather than 189.5 product ions, thereby confirming the loss of side chain from PGE₂ ω-end (data not shown). Because 5-, 12-, and 8-series isoPGE₂s are not commercially available, we demonstrated the selectivity of the 189 versus 271 ion using 20:4n-6 peroxidation products. We analyzed 300 μg of 20:4n-6 standard using 351/271 and 351/189 mass transitions (Fig. 3). The 351/189 extracted ion chromatogram had less peaks indicating higher specificity of this mass transition as compared with 351/271. Although 351/271 mass transition was previously used for PGE₂ and PGD₂ quantification (29, 32, 34–36), the presented results indicate that 351/189 mass transition gains higher selectivity for the analysis. There were two unidentified peaks eluted before and after 8-isoPGE₂ that were found in both 20:4n-6 standard and

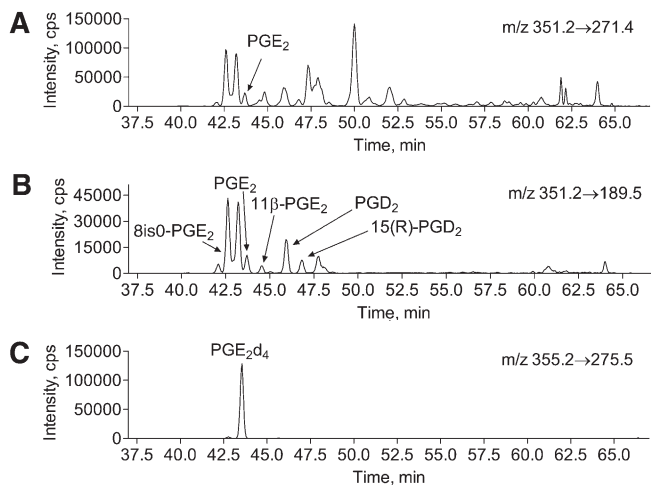


Fig. 3. Higher specificity of 351/189 over 351/271 mass transitions for LC/MS/MS analysis of arachidonic acid peroxidation products. Three hundred micrograms of arachidonic acid standard was extracted and analyzed after 6 months of storage in ethanol at -80°C as described in Materials and Methods. Ten nanograms of PGE₂d₄ was used as an internal standard. A: 351/271 extracted mass chromatogram. B: 351/189 extracted mass chromatogram. C: 355/275 extracted mass chromatogram for the internal standard PGE₂d₄.

brain extracts (Figs. 3 and 8). The 315, 271, and 189 product ion ratio was similar to that of PGE₂ and isoPGE₂ (data not shown), indicating the possibility that these peaks are isoPGE₂. Unfortunately, we were not able to identify them because none of the commercially available isoPG standards coeluted with these peaks.

Chiral chromatography for PGE₂ and entPGE₂ separation

Next, to resolve 15-series isoPGE₂ and PGD₂, we tested a variety of columns: Luna C18(2) (Phenomenex), Luna phenyl-hexyl (Phenomenex), Lux cellulose1 (Phenomenex), Lux Cellulose2 (Phenomenex), Lux Amylose2 (Phenomenex), and Cheralpack AD-RH (Diacel Chemical) columns) and HPLC conditions (acetonitrile/water, methanol/acetonitrile, methanol/water, 2-propanol/water, 2-propanol/hexane, 2-propanol/hexane/methanol with formic acid or ammonium acetate). We first aimed to resolve PGE₂ and entPGE₂. Because PGE₂ and entPGE₂ are produced in equal quantities through free radical oxidation of 20:4n-6 (13, 19, 20), but only PGE₂ is the major product of COX1/2 dependent pathway, the determination of PGE₂/entPGE₂ ratio might be important in determining PGE₂ origin in biological samples. However, the LC/MS/MS methods used to date do not allow for separation of PGE₂ and entPGE₂ without derivatization and multiple HPLC separations (13, 19, 20). PGE₂ and entPGE₂ were not resolved on nonchiral HPLC even when the best conditions for the other isoPGE₂ separation were used (Luna C18(2) column eluted with acetonitrile/water/0.1% formic acid gradient). We also could not resolve PGE₂ and entPGE₂ on CSP Chiralpak AD-RH column that was reported to separate enantiomers of *trans*-4-hydroxy-2-nonenic acid (40). The only column that resolved PGE₂ and entPGE₂ was the Lux Amylose2 column when eluted with

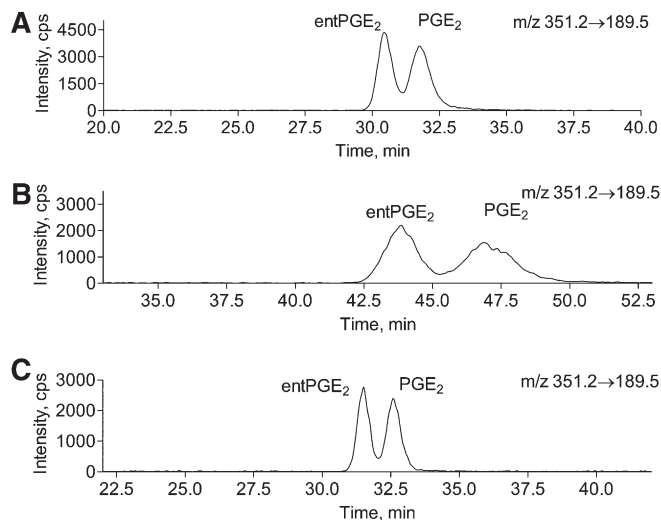


Fig. 4. The effect of acetonitrile concentration on chiral HPLC of PGE₂ and entPGE₂. PGE₂ and entPGE₂ standards (100 pg each) were loaded on one or two Lux Amylose2 columns connected in tandem and eluted using isocratic acetonitrile/water with 0.1% formic acid solvent composition at 50 μ l/min. A: 27% acetonitrile, one column. B: 25% acetonitrile, one column. C: 35% acetonitrile, two columns connected in tandem.

an acetonitrile/water/formic acid gradient (Fig. 4). Elution of the Lux Amylose2 column with methanol/water, or 2-propanol/water did not resolve these components. Both lower acetonitrile concentration (Fig. 4) and lower flow rate significantly increased separation. However, at a lower flow rate (50 μ l/min) and acetonitrile concentration (25% in water with 0.1% formic acid) when the best separation was achieved, the peaks were broadened, resulting in reduction of a signal-to-noise ratio (Fig. 4B). The use of two columns in tandem improved peak sharpness while basal resolution was maintained (Fig. 4C). However, this HPLC method did not allow for entPGE₂ and 8-isoPGE₂ separation (Fig. 5). To separate PGE₂ from 8-isoPGE₂, pre-separation on nonchiral C18(2) Luna column is required as indicated below.

PGE₂ and entPGE₂ determination in brain tissue

To demonstrate the application of the chiral HPLC method for biological samples, we quantified PGE₂ and entPGE₂ from brain tissue subjected to head-focused microwave irradiation and treated with bee venom sPLA₂ to release esterified isoprostanes (15, 20). The PGE₂ peak was collected from a HPLC run on the C18(2) Luna column and loaded onto the chiral Lux Amylose2 column. As indicated in Fig. 6, entPGE₂ and PGE₂ were generated in equal quantities ($48.4 \pm 3.5\%$ and $51.7 \pm 3.7\%$, respectively, $n = 3$), which is consistent with the presence of esterified isoPG onto PL (13, 19, 20). However, in brains subjected to ischemia followed by microwave irradiation, PGE₂ mass was significantly higher as compared with entPGE₂ (Fig. 6), which is consistent with COX1/2-dependent production of PGE₂ upon ischemia (41, 42). These data indicate that PGE₂ found esterified onto brain PL are derived from free radical oxidation pathway and demon-

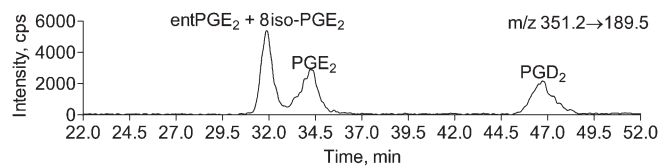


Fig. 5. Siso-PGE₂ and entPGE₂ are not resolved with chiral HPLC. PGE₂, entPGE₂, 8-isoPGE₂, and PGD₂ standards (100 pg each) were loaded on one Lux Amylose2 column and eluted with 27% acetonitrile in 0.1% formic acid aqueous solution at 50 μ l/min.

strate the appropriateness of the developed method for PGE₂ and entPGE₂ analysis.

Non-chiral HPLC for isoPGE₂ separation

Next, because the developed chiral chromatography method did not allow for PGE₂ and 8-isoPGE₂ separation, we aimed to develop an HPLC method to separate major classes of 15-series of isoPGE₂ and PGD₂. The selection of

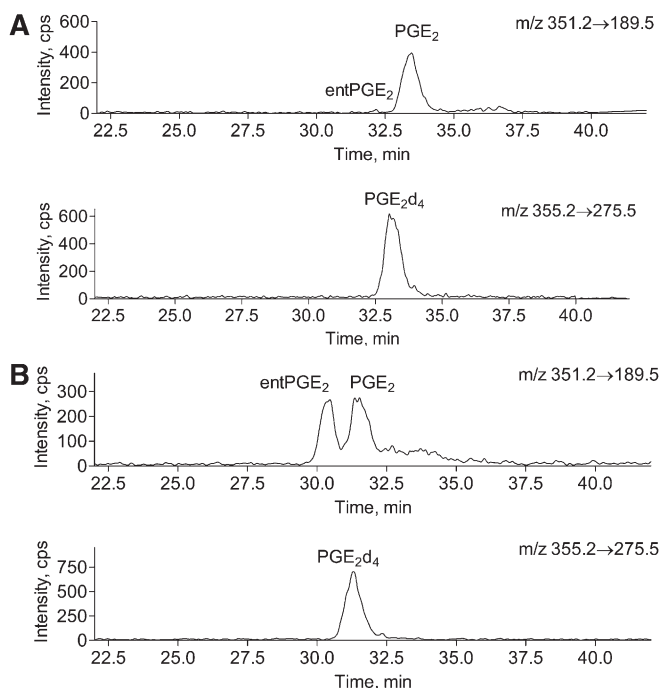


Fig. 6. Chiral HPLC of brain tissue PGE₂ and entPGE₂ under basal conditions and upon global ischemia. Brain PGs were extracted from fixed mouse brain tissue before or after 5 min of global ischemia. PG extracts were separated on a C18(2) Luna column as described in Materials and Methods. PGE₂, entPGE₂, and coeluted internal standard PGE₂d₄ fractions from an HPLC run (Fig. 8) were collected, dried down in a rotary vacuum concentrator, redissolved in 12 μ l of 35% acetonitrile in water, and 11 μ l were loaded onto two chiral Lux Amylose2 columns connected in tandem. PGs were eluted at 50 μ l/min with 35% acetonitrile in 0.1% aqueous formic acid. Please note the difference in the sample size between A and B. A: Brain tissue (20 mg) fixed with head-focused microwave irradiation after 5 min of global ischemia. Upper panel: extracted ion chromatogram for isoPGE₂. Lower panel: extracted ion chromatogram for internal standard PGE₂d₄. B: Brain tissue (whole brain, 300 mg) fixed with head-focused microwave irradiation under basal conditions. Upper panel: extracted ion chromatogram for isoPGE₂. Lower panel: extracted ion chromatogram for internal standard PGE₂d₄.

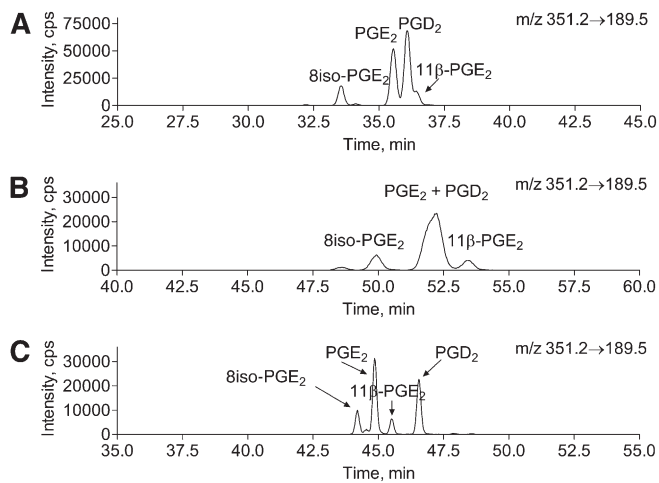


Fig. 7. Separation of a isoPGE₂/D₂ standard mixture on Luna C18(2) column using different elution solvents. One nanogram of PGE₂ and 0.5 ng of PGD₂, 8iso-PGE₂, and 11β-PGE₂ were injected on the column. The best separation conditions are presented. A: Methanol/water gradient with 0.1% formic acid. Methanol concentration was increased from 50% to 75% in 50 min. PGE₂ and PGD₂ were not well separated and PGD₂ and 11β-PGE₂ were not separated. B: 2-Propanol/water gradient with 0.1% formic acid. 2-Propanol concentration was increased from 25% to 35% in 40 min. PGE₂ and PGD₂ were not resolved. C: Acetonitrile/water with 0.1% formic acid gradient. Acetonitrile/water gradient was as described in Materials and Methods. All standards were well resolved at baseline.

the HPLC conditions was based on the reported methods for PG separation (13, 20, 29, 31–36). Although a number of LC/MS/MS methods have been developed, to the best of our knowledge, no methods have been reported to separate the major brain PGE₂/D₂ and isoPGE₂ isoforms such as PGE₂, PGD₂, 8-isoPGE₂, and 11β-PGE₂ without derivatization and multiple HPLC separations (13, 20, 29, 31–36). One of the most promising columns was phenyl-hexyl column eluted with ammonium acetate/methanol gradient that has been reported to separate 8-isoPGE₂ from PGE₂ (35); however, we could not separate other isoPG using this approach. The only condition that we found to separate all major isoPGE₂ was the 18(2) Luna column eluted with acetonitrile/water gradient with 0.1% formic acid (Figs. 7, 8). Interestingly, although addition of methanol or 2-propanol to the elution solvents increased resolving power for PG using other columns (31, 33, 40, 43–45), it decreased resolution when the C18(2) Luna column was used. For example, PGE₂ and PGD₂ were not separated in the presence of 2-propanol, and PGD₂ and 11β-PGE₂ were not separated in the presence of methanol in the elution solvents (Fig. 7).

Method validation

Limits of detection. We have previously reported a significant improvement of detection limits for PG analysis when acetone was used for extraction (0.6 ± 0.1 pg on column) (29). Importantly, a less selective 351/271 mass transition and HPLC separation was used for PGE₂ and PGD₂ quantification in this study. In the present study, we evaluated

whether a more selective MS/MS mass transition, as well as an improved HPLC separation, might gain better limits of detection with acetone extraction. To determine detection limits, we used PG extract from ~35 mg of brain tissue as a biological matrix for basal noise determination. A 3:1 signal-to-noise ratio was used to calculate limits of detection. Although the 189 product ion has an ~2-fold lower intensity as compared with the less selective 271 product ion (Fig. 2), the new improved method also reduced the basal noise, thus improving detection levels to 0.3 ± 0.1 pg on column for PGE₂ and PGD₂.

Method accuracy. To determine the method accuracy in biologically relevant PG concentration range found in brain samples, 0.100 or 1.000 ng of PGE₂ standard was spiked with microwaved brain samples (30.2 ± 0.7 mg, $n = 6$) and extracted with internal standard PGE₂d₄. A separate group of the same brain samples was analyzed without PGE₂ standard to correct for endogenous brain PGE₂ mass. The PGE₂ standard mass determined in the spiked samples was 0.097 ± 0.004 and 1.029 ± 0.029 ng for 0.100 and 1.000 spiked standards, respectively, indicating $3.0 \pm 2.9\%$ method accuracy in the tested mass range.

Calibration curve, linearity, and dynamic range. Dynamic range and linearity was determined using isotope dilution approach with a constant PGE₂d₄ mass (100 pg) as an internal standard, and variable concentrations of PGE₂ and PGD₂ standard mixture. For quantification, 351/189 mass transitions were used for PGE₂ and PGD₂ and 355/275 for PGE₂d₄ quantification. The instrument response was linear in the range between 1 pg to 100 ng with R^2 1.0000 and 0.9999 for PGE₂ and PGD₂, respectively. The calibration

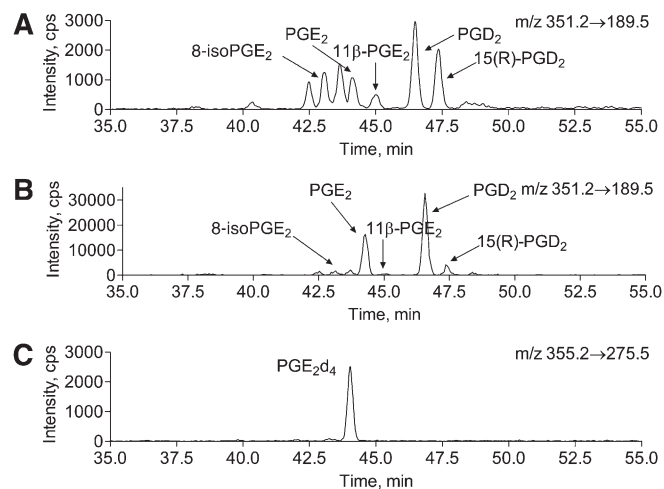


Fig. 8. HPLC of brain tissue total (free and esterified) isoPGE₂/D₂ under basal conditions and upon 5 min of global ischemia. Mouse brain total (free and esterified) PGs were extracted from fixed mouse brain tissue before or after 5 min of global ischemia. PG extracts were separated on C18(2) Luna column as described in the Materials and Methods. A: PG separation from brain tissue (20 mg) fixed with head-focused microwave irradiation under basal conditions. B: PG separation from brain tissue (10 mg) fixed with head-focused microwave irradiation after 5 min of global ischemia. C: Extracted ion chromatogram of internal standard PGE₂d₄.

TABLE 1. Intra- and inter-day precision for brain endogenous PG analysis

	Day 1				Day 2				Inter-day Difference	
	Free		Esterified		Free		Esterified		Free	Esterified
	Mean \pm SD ng/gww	RSD %	Mean \pm SD ng/gww	RSD %	Mean \pm SD ng/gww	RSD %	Mean \pm SD ng/gww	RSD %		
PGE ₂	0.07 \pm 0.01	11	1.67 \pm 0.12	7	0.08 \pm 0.01	13	1.39 \pm 0.11	8	8	-17
11 β -PGE ₂	0.02 \pm 0.01	43	0.50 \pm 0.07	14	0.03 \pm 0.01	43	0.47 \pm 0.07	15	10	-6
8-isoPGE ₂	0.05 \pm 0.02	45	1.57 \pm 0.19	12	0.05 \pm 0.13	26	1.36 \pm 0.23	17	-8	-13
PGD ₂	0.06 \pm 0.01	22	1.81 \pm 0.11	6	0.06 \pm 0.01	19	1.71 \pm 0.7	4	-12	-5
15(R)-PGD ₂	0.05 \pm 0.02	48	1.31 \pm 0.15	12	0.04 \pm 0.17	43	1.18 \pm 0.08	7	-19	-20

Two mouse brains were fixed with head-focused microwave irradiation and pulverized together under liquid nitrogen temperatures to a fine homogeneous powder. The mixed brain powder samples (\sim 35 mg) were analyzed in triplicate on the same day (Day 1) and the following day (Day 2). Samples were incubated with or without sPLA₂ as described in Materials and Methods and analyzed after extraction with acetone. PGE₂d₄ was added before incubation as an internal standard. The difference between prostanoid levels with and without sPLA₂ treatment represents PG esterified onto the sn-2 position in PL. Inter-day difference was calculated as percent difference between average values for Day 1 and Day 2. SD, standard deviation; RSD, relative standard deviation.

curve was linear within the dynamic range with the fitting curve slopes 106.4 and 110.1 for PGD₂ and 15(R)-PGD₂, respectively, and 193.9 to 198.5 for PGE₂ and isoPGE₂.

Matrix effect. To determine matrix effect, microwaved brain samples (36.3 \pm 0.7 mg) were extracted with 100 pg of PGE₂d₄ internal standard. Three PG extracts were spiked with 50 pg of PGE₂ and three other extracts were analyzed without PGE₂ standard to correct for endogenous brain PGE₂ mass. A separate set of PGE₂ standard samples (100 pg) was analyzed without having been spiked with brain extracts. PGE₂ peak area was determined after LC/MS/MS analysis. The matrix effect was determined as a percent difference between PGE₂ standard peak area with and without spiking with brain extract after correction for endogenous PGE₂ mass. The corrected integrated peak area for PGE₂ spiked with tissue extract was 9783 \pm 229, and for PGE₂ standard alone was 9496 \pm 127. Although the relative standard deviation was very low, in the range of 2%, these values were not statistically different between samples. In addition, no peaks interfering with PGE₂d₄ internal standard were detected on chromatograms (data not shown). These data indicate very little matrix effect from brain tissue samples used in the analysis and a high method precision for LC/MS/MS analysis of spiked standards.

Method precision and sample stability. To determine the method precision for endogenous brain PGE₂, we

determined intra- and inter-day variability calculated as a relative standard deviation for brain samples. Two mouse brains subjected to microwave fixation were removed, frozen in liquid nitrogen, and pulverized together under liquid nitrogen temperatures to a fine homogeneous powder. Mixed brain powder samples (35.5 \pm 0.6 mg) were incubated with or without sPLA₂ as described in Materials and Methods and analyzed after extraction with acetone for free and esterified PG quantification. When PG analysis was performed on the same day, the average intra-day variability was 11 \pm 5% (n = 10) for esterified PG and 31 \pm 14% (n = 10) for free PG (Table 1). When the same brain powder was analyzed on the following day, the inter-day variability was 10 \pm 5% for esterified PG and 22 \pm 22% for free PG (Table 1). For most of the isoPG analyzed, the concentration was decreased when brain samples were analyzed on the following day. However, this decrease was not statistically different. Importantly, we have observed statistically significant 2- to 4-fold reduction in PG mass after 4 weeks of brain powder storage at -80°C, indicating significant instability of PG in brain samples (29). Free PG had higher intra-day variability that might be the results of their low concentrations under basal conditions (\sim 1 pg/sample) that were close to the limits of detection. This variability might be decreased by the increased sample mass used for the analysis. To address this possibility, we analyzed three samples (20.3 \pm 0.2 mg, n = 3) from the same mouse brain subjected to 5 min of brain ischemia followed by head-focused

TABLE 2. Brain PG alterations upon decapitation induced ischemia

	Basal Conditions			Ischemia		
	Free	Esterified	Total	Free	Esterified	Total
PGE ₂	0.06 \pm 0.01	1.55 \pm 0.13	1.62 \pm 0.43	14.52 \pm 0.44	0.87 \pm 0.37	15.39 \pm 0.35
11 β -PGE ₂	0.09 \pm 0.04	0.87 \pm 0.31	0.97 \pm 0.29	1.36 \pm 0.15*	0.52 \pm 0.33	1.88 \pm 0.28*
8-isoPGE ₂	0.08 \pm 0.02	1.22 \pm 0.4	1.30 \pm 0.41	1.93 \pm 0.47*	0.73 \pm 0.31	2.66 \pm 0.28*
PGD ₂	0.06 \pm 0.01	1.92 \pm 0.10	1.98 \pm 0.12	31.21 \pm 0.53	1.02 \pm 0.22	32.23 \pm 0.62
15(R)-PGD ₂	0.05 \pm 0.02	1.25 \pm 0.17	1.51 \pm 0.20	2.13 \pm 0.32	1.65 \pm 0.85	3.78 \pm 0.75

Mouse brain was fixed with head-focused microwave irradiation before and after 5 min of global ischemia as described in Materials and Methods and pulverization under liquid nitrogen temperature. Samples of \sim 20 mg tissue were incubated with or without sPLA₂ and extracted with acetone as described in Materials and Methods. PGE₂d₄ was added before incubation as an internal standard. The difference between prostanoid levels with and without sPLA₂ treatment represents prostanoids esterified onto the sn-2 position in PL. Data are mean (ng/gww) \pm SD, n = 3. * indicates statistically different values as compared with basal conditions.

microwave irradiation. The free PGE₂ and PGD₂ mass was 11.03 ± 0.33 and 25.54 ± 0.97ng/gram wet weight (gww), respectively, with ~4% of variability. These data indicate a significant improvement of the method precision with increased PG mass in the samples. In addition, because isoPGs are products of free radical peroxidation that might be effected by sample handling, the sample handling might be also an important factor effecting method precision.

Brain tissue isoPG separation and quantification under basal conditions and upon ischemia

To demonstrate the application of the method for biological sample analysis, we analyzed free isoPGE₂ and isoPGE₂ esterified onto PL in brain tissue fixed with microwave irradiation before (basal conditions) or 1 min after ischemia (29, 37). Although free isoPGE₂/D₂ levels are reported upon decapitation-induced ischemia (38), to the best of our knowledge, no previous studies addressed esterified isoPG levels in fixed or ischemic brain tissue. To release esterified isoPG from the sn-2 position of PL, tissue samples were treated with bee venom sPLA₂ as described in Materials and Methods (15, 20). The difference between prostanoid levels with and without sPLA₂ treatment represents prostanoids esterified onto the sn-2 position in PL. Under basal conditions, esterified PG and isoPG were 10- to 32-fold higher as compared with free isoPG (Table 2). Upon ischemia, free PGE₂ and PGD₂ were significantly increased with a more profound increase in PGD₂ mass (Table 2), which is consistent with previous studies (29, 37, 38, 46). Total (free plus esterified) isoPGs were significantly increased ~2-fold, which is consistent with increased oxidative stress upon ischemia (47, 48) and consistent with the increase in free isoPGE₂/D₂ levels in decapitated rat brains (38). The found values for brain isoPG are in the range of the previously reported values for rat liver (15). The increase in total isoPG was mainly accounted for by the increase in free isoPG levels that were increased 15- to 43-fold. However, the esterified pool was not decreased upon ischemia (Table 2). This is intriguing because PLA₂ are activated upon ischemia (49, 50), and at least one PLA₂ platelet activating factor acetylhydrolase II has been reported to hydrolyze isoPG from PL (24). It remains to be elucidated if the stability of the esterified isoPG pool upon ischemia is the result of reesterification of free isoPG, a lack of PLA₂ activity toward esterified isoPG upon ischemia, or a balance between release and esterification processes.

In summary, the developed method allows for a complete separation and quantification of the major 15-series isoPGE₂ and PGD₂ without derivatization that works sufficiently when a standard mixture or endogenous brain tissue isoPGs are analyzed. Using the developed LC/MS/MS method, we demonstrated that PGE₂s found esterified in the sn-2 position in PL are derived from a free radical non-enzymatic pathway under basal conditions. We report for the first time values for esterified isoPGE₂ levels in brain tissue under basal conditions and upon global ischemia and demonstrated a nonreleasable pool of isoPG esterified onto brain PL upon ischemia. ■

The authors thank Dr. Johnie Brown (AB Sciex) for assistance with MS/MS spectrum generation on API5500Q mass spectrometer.

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