methods

LC/MS/MS method for analysis of E_2 series prostaglandins and isoprostanes

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Abstract 15-series prostaglandins (PGE_2s) and isoprostanes ($isoPGE_2$s) 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 PGE_2/D_2 and $isoPGE_2$ without derivatization and multiple HPLC separations. The developed LC/MS/MS method allows for the major brain $PGE_2/PGD_2/isoPGE_2$ such as PGE_2, $entPGE_2$, 8-$isoPGE_2$, 11β-PGE_2, PGD_2, and 15(R)-PGD_2 to be separated and quantified without derivatization. The method was validated by analyzing free and esterified $isoPGE_2$ 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 $isoPGE_2$ 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 PGE_2s 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 E_2 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

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 PGE_2 belonging to the 15-series (18) (**Fig. 1A**). Because $isoPGE_2/D_2$ have four chiral centers, there are 16 possible chiral isoforms within each group. Within the 15-series $isoPGE_2$, the most thermodynamically stable are PGE_2, PGE_2 enantiomer (entPGE2), 8-$isoPGE_2$, and 11β-PGE_2 that are found in abundance in tissues (13, 19–21) (Fig. 1B). Al-

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Abbreviations: 20:4n-6, arachidonic acid; BHT, butylated hydroxytoluene; COX, cyclooxygenase; $entPGE_2$, 9-oxo-11β,15R-dihydroxy-(8β,12α)-prosta-5Z,13E-dien-1-oic acid; isoPG, isoprostane; PG, prostaglandin; PGD_2, 9α,15*S*-dihydroxy-11-oxo-prosta-5*Z*,13*E*-dien-1-oic acid; 15(R)-PGD_2, 9α,15R-dihydroxy-11-oxo-prosta-5*Z*,13*E*-dien-1-oic acid; PGE_2, 9-oxo-11α,15*S*-dihydroxyprosta-5*Z*,13*E*-dien-1-oic acid; 11β-PGE_2, 9-oxo-11β,15S-dihydroxyprosta-5Z,13E-dien-1-oic acid; 8-$isoPGE_2$, 9-oxo-11α,15S-dihydroxy-(8β)-prosta-5Z,13E-dien-1-oic acid; PGE_2-d_4, 9-oxo-11α,15*S*-dihydroxyprosta-5*Z*,13*E*-dien-1-oic-3,3,4,4-$^2H_4$ acid; PGE_2-d_9, 9-oxo-11α,15*S*-dihydroxyprosta-5*Z*,13*E*-dien-1-oic-17,17,18,18,19,19,20,20,20 -$^2H_4$ acid; PL, phospholipid; PLA_2, phospholipase A_2; $sPLA_2$, secreted phospholipase A_2.

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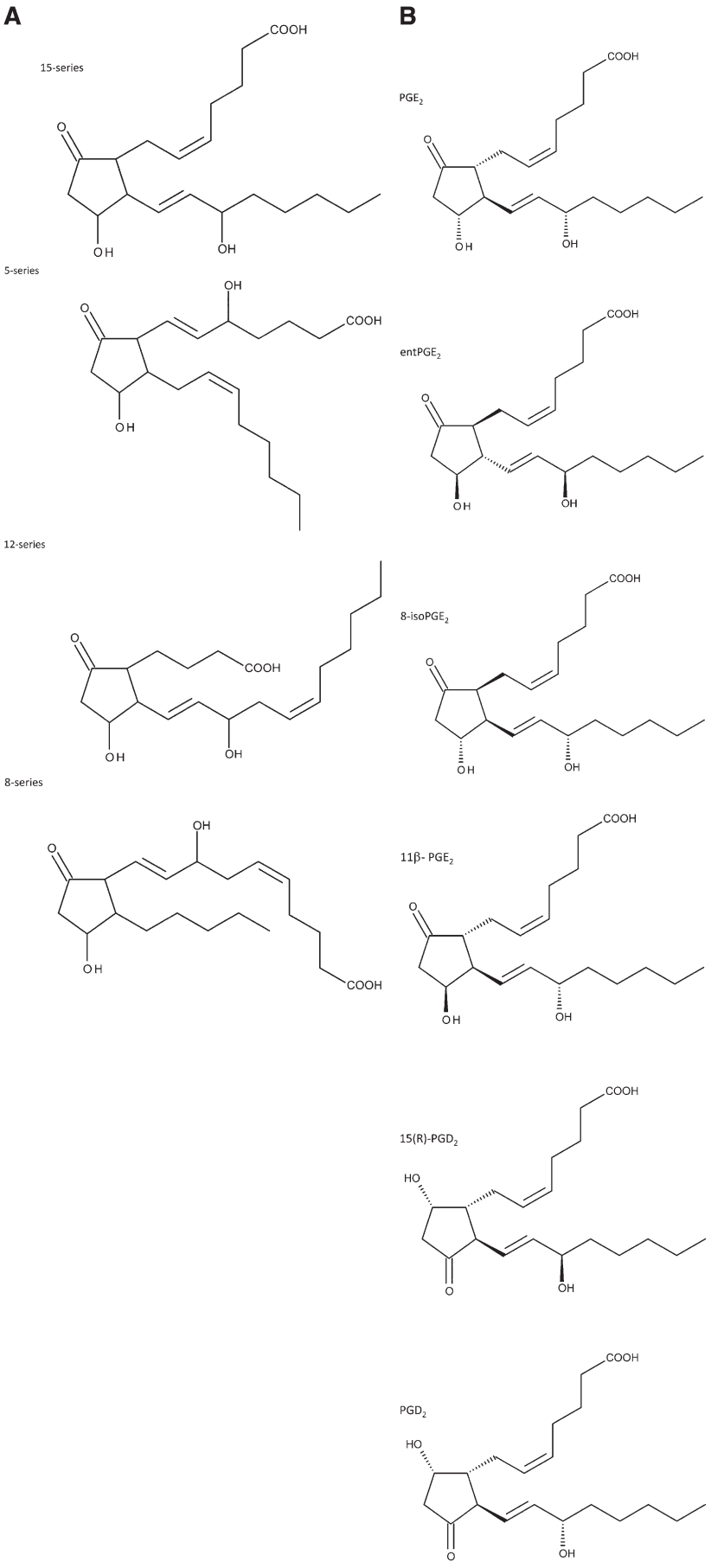

though isoPGs are stable robust biomarkers of oxidative stress (22, 23), 15-series isoPG might also possess potent biological activity (15, 21). Esterified PGE_2 and $isoPGE_2$ might be released from PL by platelet activating factor acetylhydrolase II (24) that is activated at early stages of tissue 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 PGE_2 and 15-series $isoPGE_2$ 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 $isoPGE_2$ 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 PGE_2/D_2 and $isoPGE_2$ such as PGE_2, $entPGE_2$, PGD_2, 8-$isoPGE_2$, and 11β-PGE_2 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 PGE_2/D_2 series molecules such as PGD_2, 15(R)-PGD_2, PGE_2, $entPGE_2$, 8-$isoPGE_2$, and 11β-PGE_2 to be separated and quantified without derivatization and validated the method by analyzing free and esterified $isoPGE_2$ in brains fixed with head-focused microwave irradiation before or after global ischemia. Using the developed method, we report for the first time esterified $isoPGE_2$ 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 PGE_2s 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,15*S*-dihydroxyprosta-5*Z*,13*E*-dien-1-oic acid (PGE_2), 9-oxo-11β,15R-dihydroxy-(8β,12α)-prosta-5Z,13E-dien-1-oic acid ($entPGE_2$), 9-oxo-11β,15*S*-dihydroxyprosta-5*Z*,13*E*-dien-1-oic acid (11β-PGE_2), 9-oxo-11α,15S-dihydroxy-(8β)-prosta-5Z,13E-dien-1-oic acid (8-*iso*PGE_2), 9-oxo-11α,15*S*-dihydroxyprosta-5*Z*,13*E*-dien-1-oic-3,3,4,4-$^2H_4$ acid (PGE_2-d4), 9-oxo-11α,15*S*-dihydroxyprosta-5*Z*,13*E*-dien-1-oic-17,17,18,18,19,19,20,20,20 -$^2H_4$ acid (PGE_2-d_9), 9α,15*S*-dihydroxy-11-oxo-prosta-5*Z*,13*E*-dien-1-oic acid (PGD_2), 9α,15R-dihydroxy-11-oxo-prosta-5*Z*,13*E*-dien-1-oic acid (15(R)-PGD_2), 9α,15*S*-dihydroxy-11-oxo-prosta-5*Z*,13*E*-dien-1-oic-3,3,4,4-$^2H_4$ acid (PGD_2-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 PGE_2-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 PGE_2 and $entPGE_2$, 12 μl of 35% acetonitrile was used to redissolve samples.

Fig. 1. Positional and selected chiral isoforms of $isoPGE_2$ and PGD_2. A: Positional isoforms for $isoPGE_2$. For simplicity, stereochemical orientation is not indicated in the figure. B: Selected $isoPGE_2$ and $isoPGD_2$ stereoisoforms used in the study.

HPLC of isoPGE$_2$ and PGE$_2$

The separation of isoPGE$_2$ and PGE$_2$ 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 $CaCl_2$, 8 mM Triton X-100, 60% glycerol, and 2 mg/ml BSA) containing 100 pg of PGE$_2$d$_4$ with or without secretory (s)PLA$_2$ (~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 sPLA$_2$. After 1 h of incubation at ambient temperature, PGs were extracted and quantified as described above. The difference between prostanoid levels with and without sPLA$_2$ treatment represents prostanoids esterified onto the sn-2 position in PL.

Chiral HPLC of PGE$_2$ enantiomers

The separation of PGE$_2$ and entPGE$_2$ 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. PGE$_2$, entPGE$_2$, and coeluted internal standard PGE$_2$d$_4$ 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 entPGE$_2$ LC/MS/MS analysis is presented in Fig. 6.

Electrospray ionization mass spectrometry

For PG quantification, a quadrapole mass spectrometer (API3000, AB Sciex, Foster City, CA) equipped with TurboIonSpray 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.

PGE$_2$ and isoPGE$_2$ were quantified using PGE$_2$-d4 as the internal standard. For PGE$_2$ and isoPGE$_2$ monitoring in the MRM

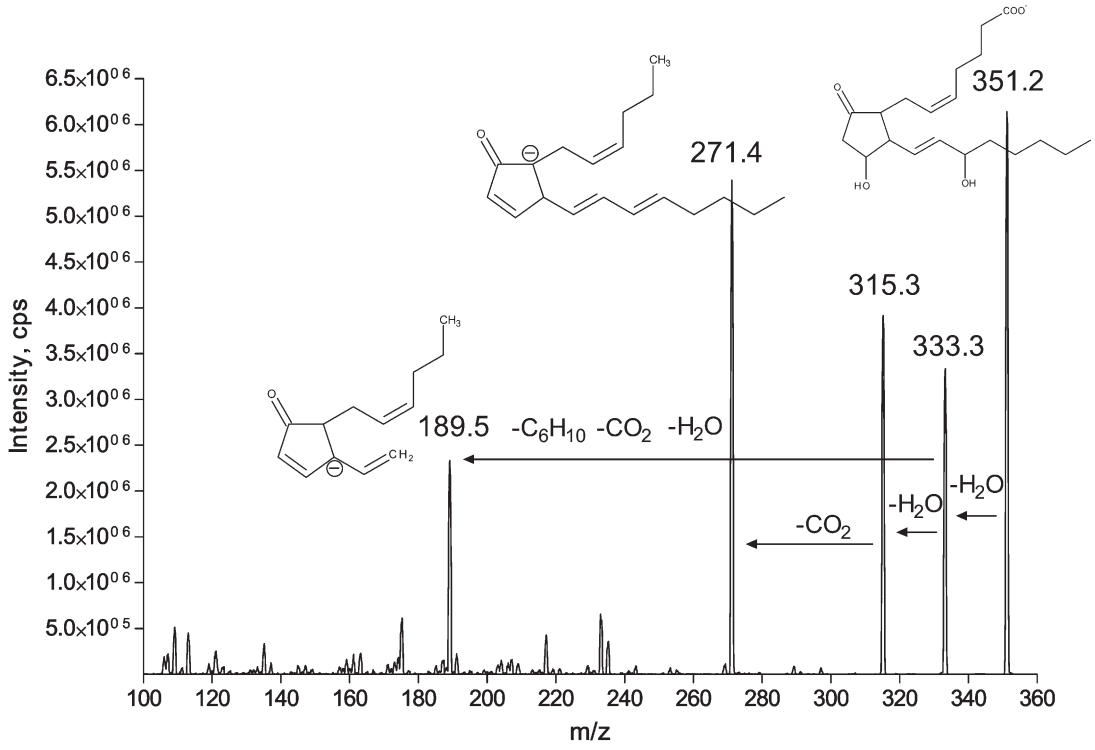

Fig. 2. MS/MS spectrum for PGE$_2$. Negative ion MS/MS spectrum of PGE$_2$ standard was generated by direct infusion into API5500Q electrospray ion source.

mode, 351.2/189.5 mass transition was used, and for PGE_2d_4, 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 PGE_2, PGD_2, and 15-series $isoPGE_2$ 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 PGE_2/PGD_2-like molecules (13, 20, 29, 31–36). However, to the best of our knowledge, none of the reported conditions allowed for PGE_2, PGD_2, and major $isoPGE_2$ separation in a single run without derivatization.

Selection of MS/MS mass transition

To resolve PGE_2/PGD_2-like molecules, we first optimized MS/MS parameters to achieve higher selectivity for 15-series $isoPGE_2$. Most of the product ions produced from PGE_2 are generated by water and carboxyl group loss (product ions 333.3, 315.3, and 271.4) that are not specific toward 15-series $isoPGE_2$ (**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, PGE_2 standards labeled with deuterium at 17, 17, 18, 18, 19, 19, 20, 20, 20 (PGE_2d_9) or at 3, 3, 4, 4 (PGE_2d_4) positions were used to confirm the pathway for an ion with m/z 189 generation from PGE_2. Upon PGE_2d_9 collision, the ion with m/z = 189.5 was predominant over the 198.5 ion, whereas PGE_2d_4 produced mainly 193.5 rather than 189.5 product ions, thereby confirming the loss of side chain from PGE_2 ω-end (data not shown). Because 5-, 12-, and 8-series $isoPGE_2$s 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 PGE_2 and PGD_2 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-$isoPGE_2$ that were found in both 20:4n-6 standard and

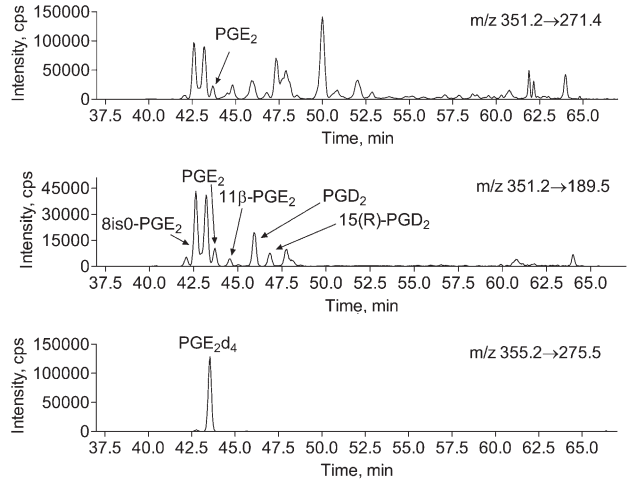

Fig. 3. Higher specificity of 351/189 over 351/271 mass transitions for LC/MS/MS analysis of arachidonic acid peroxidation products. Three hundred micrograms of arachidonic acid standard was extracted and analyzed after 6 months of storage in ethanol at −80°C as described in Materials and Methods. Ten nanograms of PGE_2d_4 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 PGE_2d_4.

brain extracts (Figs. 3 and 8). The 315, 271, and 189 product ion ratio was similar to that of PGE_2 and $isoPGE_2$ (data not shown), indicating the possibility that these peaks are $isoPGE_2$. Unfortunately, we were not able to identify them because none of the commercially available isoPG standards coeluted with these peaks.

Chiral chromatography for PGE_2 and $entPGE_2$ separation

Next, to resolve 15-series $isoPGE_2$ and PGD_2, 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 PGE_2 and $entPGE_2$. Because PGE_2 and $entPGE_2$ are produced in equal quantities through free radical oxidation of 20:4n-6 (13, 19, 20), but only PGE_2 is the major product of COX1/2 dependent pathway, the determination of PGE_2/$entPGE_2$ ratio might be important in determining PGE_2 origin in biological samples. However, the LC/MS/MS methods used to date do not allow for separation of PGE_2 and $entPGE_2$ without derivatization and multiple HPLC separations (13, 19, 20). PGE_2 and $entPGE_2$ were not resolved on nonchiral HPLC even when the best conditions for the other $isoPGE_2$ separation were used (Luna C18(2) column eluted with acetonitrile/water/0.1% formic acid gradient). We also could not resolve PGE_2 and $entPGE_2$ on CSP Chiralpak AD-RH column that was reported to separate enantiomers of *trans*-4-hydroxy-2-nonenoic acid (40). The only column that resolved PGE_2 and $entPGE_2$ was the Lux Amylose2 column when eluted with

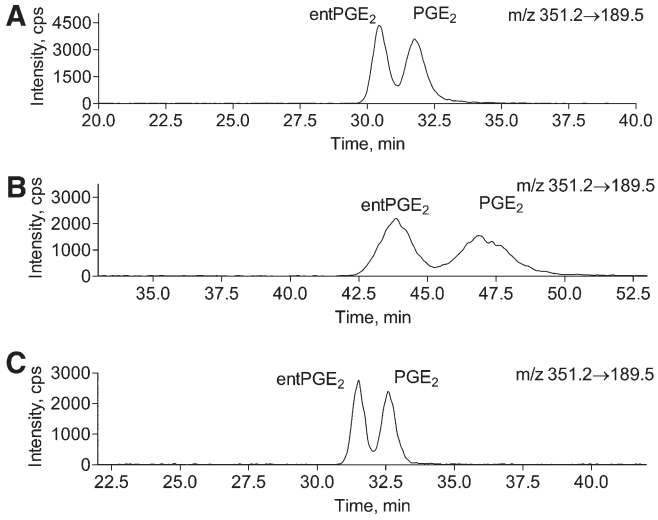

Fig. 4. The effect of acetonitrile concentration on chiral HPLC of PGE_2 and $entPGE_2$. PGE_2 and $entPGE_2$ standards (100 pg each) were loaded on one or two Lux Amylose2 columns connected in tandem and eluted using isocratic acetonitrile/water with 0.1% formic acid solvent composition at 50 μl/min. 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 μl/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 $entPGE_2$ and 8-$isoPGE_2$ separation (**Fig. 5**). To separate PGE_2 from 8-$isoPGE_2$, preseparation on nonchiral C18(2) Luna column is required as indicated below.

PGE_2 and $entPGE_2$ determination in brain tissue

To demonstrate the application of the chiral HPLC method for biological samples, we quantified PGE_2 and $entPGE_2$ from brain tissue subjected to head-focused microwave irradiation and treated with bee venom $sPLA_2$ to release esterified isoprostanes (15, 20). The PGE_2 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,** $entPGE_2$ and PGE_2 were generated in equal quantities (48.4 ± 3.5% and 51.7 ± 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, PGE_2 mass was significantly higher as compared with $entPGE_2$ (Fig. 6), which is consistent with COX1/2-dependent production of PGE_2 upon ischemia (41, 42). These data indicate that PGE_2 found esterified onto brain PL are derived from free radical oxidation pathway and demonstrate the appropriateness of the developed method for PGE_2 and $entPGE_2$ analysis.

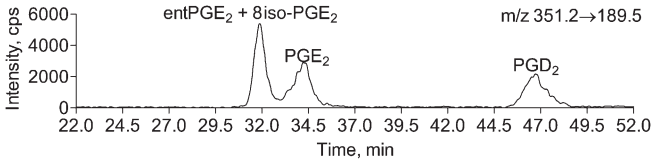

Fig. 5. 8iso-PGE_2 and $entPGE_2$ are not resolved with chiral HPLC. PGE_2, $entPGE_2$, 8-$isoPGE_2$, and PGD_2 standards (100 pg each) were loaded on one Lux Amylose2 column and eluted with 27% acetonitrile in 0.1% formic acid aqueous solution at 50 μl/min.

Non-chiral HPLC for $isoPGE_2$ separation

Next, because the developed chiral chromatography method did not allow for PGE_2 and 8-$isoPGE_2$ separation, we aimed to develop an HPLC method to separate major classes of 15-series of $isoPGE_2$ and PGD_2. The selection of

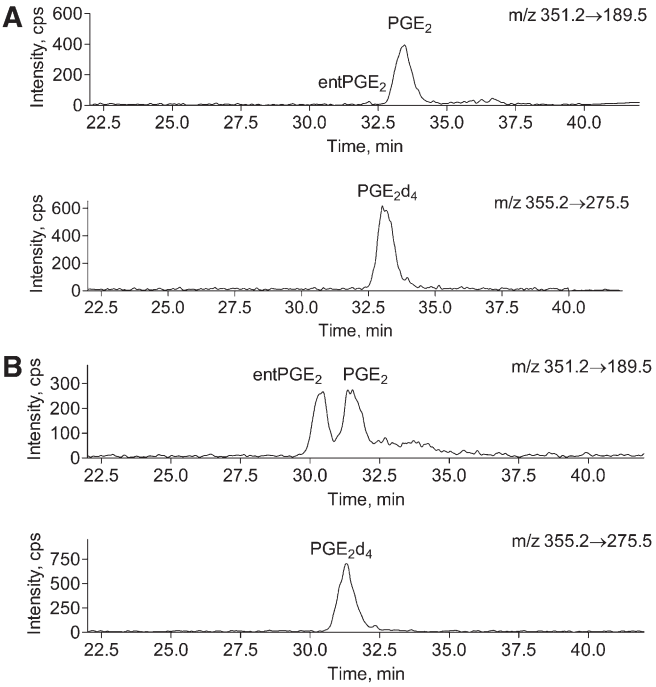

Fig. 6. Chiral HPLC of brain tissue PGE_2 and $entPGE_2$ under basal conditions and upon global ischemia. Brain PGs were extracted from fixed mouse brain tissue before or after 5 min of global ischemia. PG extracts were separated on a C18(2) Luna column as described in Materials and Methods. PGE_2, $entPGE_2$, and coeluted internal standard PGE_2d_4 fractions from an HPLC run (Fig. 8) were collected, dried down in a rotary vacuum concentrator, redissolved in 12 μl of 35% acetonitrile in water, and 11 μl were loaded onto two chiral Lux Amylose2 columns connected in tandem. PGs were eluted at 50 μl/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 $isoPGE_2$. Lower panel: extracted ion chromatogram for internal standard PGE_2d_4. B: Brain tissue (whole brain, 300 mg) fixed with head-focused microwave irradiation under basal conditions. Upper panel: extracted ion chromatogram for $isoPGE_2$. Lower panel: extracted ion chromatogram for internal standard PGE_2d_4.

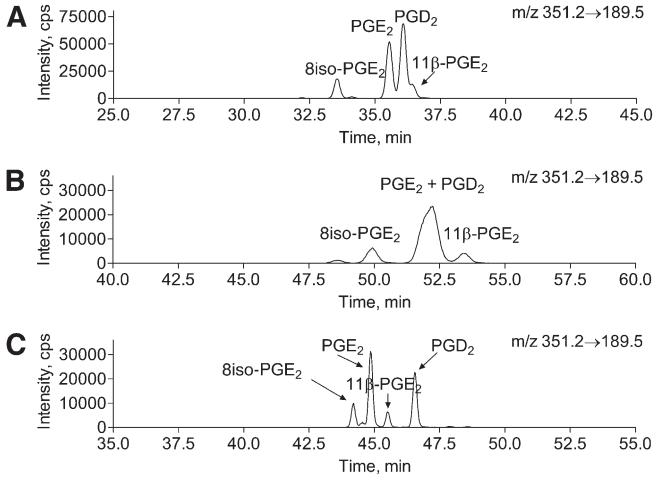

Fig. 7. Separation of a isoPGE$_2$/D$_2$ standard mixture on Luna C18(2) column using different elution solvents. One nanogram of PGE$_2$ and 0.5 ng of PGD2, 8iso-PGE$_2$, and 11β-PGE$_2$ were injected on the column. The best separation conditions are presented. A: Methanol/water gradient with 0.1% formic acid. Methanol concentration was increased from 50% to 75% in 50 min. PGE$_2$ and PGD$_2$ were not well separated and PGD$_2$ and 11β-PGE$_2$ 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. PGE$_2$ and PGD$_2$ 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 PGE$_2$/D$_2$ and isoPGE$_2$ isoforms such as PGE$_2$, PGD$_2$, 8-isoPGE$_2$, and 11β-PGE$_2$ 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-isoPGE$_2$ from PGE$_2$ (35); however, we could not separate other isoPG using this approach. The only condition that we found to separate all major isoPGE$_2$ 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, PGE$_2$ and PGD$_2$ were not separated in the presence of 2-propanol, and PGD$_2$ and 11β-PGE$_2$ 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 PGE$_2$ and PGD$_2$ 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 3:1signal-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 PGE$_2$ and PGD$_2$.

Method accuracy. To determine the method accuracy in biologically relevant PG concentration range found in brain samples, 0.100 or 1.000 ng of PGE$_2$ standard was spiked with microwaved brain samples (30.2 ± 0.7 mg, n = 6) and extracted with internal standard PGE$_2$d$_4$. A separate group of the same brain samples was analyzed without PGE$_2$ standard to correct for endogenous brain PGE$_2$ mass. The PGE$_2$ 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 ± 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 PGE$_2$d$_4$ mass (100 pg) as an internal standard, and variable concentrations of PGE$_2$ and PGD$_2$ standard mixture. For quantification, 351/189 mass transitions were used for PGE$_2$ and PGD$_2$ and 355/275 for PGE$_2$d$_4$ quantification. The instrument response was linear in the range between 1 pg to 100 ng with R^2 1.0000 and 0.9999 for PGE$_2$ and PGD$_2$, respectively. The calibration

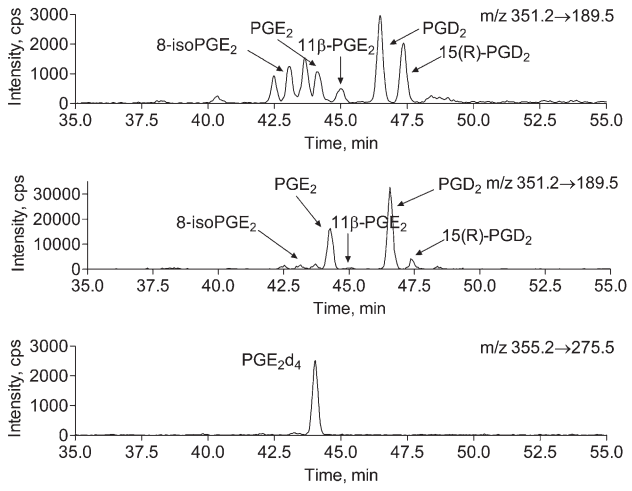

Fig. 8. HPLC of brain tissue total (free and esterified) isoPGE$_2$/D$_2$ under basal conditions and upon 5 min of global ischemia. Mouse brain total (free and esterified) PGs were extracted from fixed mouse brain tissue before or after 5 min of global ischemia. PG extracts were separated on C18(2) Luna 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 PGE$_2$d$_4$.

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

	Day 1				Day 2					
	Free		Esterified		Free		Esterified		Inter-day Difference	
	Mean ± SD ng/gww	RSD %	Mean ± SD ng/gww	RSD %	Mean ± SD ng/gww	RSD %	Mean ± SD ng/gww	RSD %	Free %	Esterified %
PGE_2	0.07 ± 0.01	11	1.67 ± 0.12	7	0.08 ± 0.01	13	1.39 ± 0.11	8	8	−17
11β-PGE_2	0.02 ± 0.01	43	0.50 ± 0.07	14	0.03 ± 0.01	43	0.47 ± 0.07	15	10	−6
8-isoPGE_2	0.05 ± 0.02	45	1.57 ± 0.19	12	0.05 ± 0.13	26	1.36 ± 0.23	17	−8	−13
PGD_2	0.06 ± 0.01	22	1.81 ± 0.11	6	0.06 ± 0.01	19	1.71 ± 0.7	4	−12	−5
15(R)-PGD_2	0.05 ± 0.02	48	1.31 ± 0.15	12	0.04 ± 0.17	43	1.18 ± 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 (~35 mg) were analyzed in triplicate on the same day (Day 1) and the following day (Day 2). Samples were incubated with or without $sPLA_2$ as described in Materials and Methods and analyzed after extraction with acetone. PGE_2d_4 was added before incubation as an internal standard. The difference between prostanoid levels with and without $sPLA_2$ 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 PGD_2 and 15(R)-PGD_2, respectively, and 193.9 to 198.5 for PGE_2 and isoPGE_2.

Matrix effect. To determine matrix effect, microwaved brain samples (36.3 ± 0.7 mg) were extracted with 100 pg of PGE_2d_4 internal standard. Three PG extracts were spiked with 50 pg of PGE_2 and three other extracts were analyzed without PGE_2 standard to correct for endogenous brain PGE_2 mass. A separate set of PGE_2 standard samples (100 pg) was analyzed without having been spiked with brain extracts. PGE_2 peak area was determined after LC/MS/MS analysis. The matrix effect was determined as a percent difference between PGE_2 standard peak area with and without spiking with brain extract after correction for endogenous PGE_2 mass. The corrected integrated peak area for PGE_2 spiked with tissue extract was 9783 ± 229, and for PGE_2 standard alone was 9496 ± 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 PGE_2d_4 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 PGE_2, 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 ± 0.6 mg) were incubated with or without $sPLA_2$ 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 ± 5% (n = 10) for esterified PG and 31 ± 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 ± 5% for esterified PG and 22 ± 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 (~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 ± 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 ± 0.01	1.55 ± 0.13	1.62 ± 0.43	14.52 ± 0.44	0.87 ± 0.37	15.39 ± 0.35
11β-PGE_2	0.09 ± 0.04	0.87 ± 0.31	0.97 ± 0.29	1.36 ± 0.15*	0.52 ± 0.33	1.88 ± 0.28*
8-isoPGE_2	0.08 ± 0.02	1.22 ± 0.4	1.30 ± 0.41	1.93 ± 0.47*	0.73 ± 0.31	2.66 ± 0.28*
PGD_2	0.06 ± 0.01	1.92 ± 0.10	1.98 ± 0.12	31.21 ± 0.53	1.02 ± 0.22	32.23 ± 0.62
15(R)-PGD_2	0.05 ± 0.02	1.25 ± 0.17	1.51 ± 0.20	2.13 ± 0.32	1.65 ± 0.85	3.78 ± 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 ~20 mg tissue were incubated with or without $sPLA_2$ and extracted with acetone as described in Materials and Methods. PGE_2d_4 was added before incubation as an internal standard. The difference between prostanoid levels with and without $sPLA_2$ treatment represents prostanoids esterified onto the sn-2 position in PL. Data are mean (ng/gww) ± SD, n = 3. * indicates statistically different values as compared with basal conditions.

microwave irradiation. The free PGE_2 and PGD_2 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 isoPGE$_2$ and isoPGE$_2$ esterified onto PL in brain tissue fixed with microwave irradiation before (basal conditions) or 1 min after ischemia (29, 37). Although free isoPGE$_2$/D$_2$ 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 $sPLA_2$ as described in Materials and Methods (15, 20). The difference between prostanoid levels with and without $sPLA_2$ 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 PGE_2 and PGD_2 were significantly increased with a more profound increase in PGD_2 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 isoPGE$_2$/D$_2$ 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 PLA_2 are activated upon ischemia (49, 50), and at least one PLA_2 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 PLA_2 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 isoPGE$_2$ and PGD_2 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 PGE$_2$s found esterified in the sn-2 position in PL are derived from a free radical nonenzymatic pathway under basal conditions. We report for the first time values for esterified isoPGE$_2$ levels in brain tissue under basal conditions and upon global ischemia and demonstrated a nonreleasable pool of isoPG esterified onto brain PL upon ischemia.

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