

# An improved LC-MS/MS procedure for brain prostanoid analysis using brain fixation with head-focused microwave irradiation and liquid-liquid extraction

Mikhail Y. Golovko\* and Eric J. Murphy<sup>1,\*†</sup>

Department of Pharmacology, Physiology, and Therapeutics,\* and Department of Chemistry,<sup>†</sup> University of North Dakota, Grand Forks, ND 58202-9037

**Abstract** High-performance liquid chromatography with tandem mass spectrometry detection (LC-MS/MS) allows a highly selective, sensitive, simultaneous analysis for prostanoids (PG) without derivatization. However, high chemical background noise reduces LC-MS/MS selectivity and sensitivity for brain PG analysis. Four common methods using different solvent systems for PG extraction were tested. Although these methods had the same recovery of PG, the modified acetone extraction followed by liquid/liquid purification had the greatest sensitivity. This method combined with hexane/2-propanol extraction permits the simultaneous analysis of other lipid molecules and PG in the same extract. We also determined that PG mass in brain powder stored at  $-80^{\circ}\text{C}$  was reduced 2- to 4- fold in 4 weeks; however, PG were stable for long periods ( $>3$  months) in hexane/2-propanol extracts. PG mass was increased significantly when mice were euthanized by decapitation and the brains rapidly flash-frozen rather than euthanized using head-focused microwave irradiation. This reduction is not the result of PG trapping or destruction in microwave-irradiated brains, demonstrating its importance in limiting mass artifacts during brain PG analysis. Our improved procedure for brain PG analysis provides a reliable, rapid means to detect changes in brain PG mass under both basal and pathological conditions and demonstrates the importance of sample preparation in this process.—Golovko, M. Y., and E. J. Murphy. An improved LC-MS/MS procedure for brain prostanoid analysis using brain fixation with head-focused microwave irradiation and liquid-liquid extraction. *J. Lipid Res.* 2008. 49: 893–902.

**Supplementary key words** prostaglandin • isoprostane • high-performance liquid chromatography-tandem mass spectrometry

Prostanoids (PG) are the major lipid-signaling molecules that are enzymically synthesized from arachidonic

acid and related 20 carbon polyunsaturated fatty acids. They are ubiquitously produced in the nervous system and modulate many brain functions under basal and pathological conditions. PG are involved in a broad array of diseases, including cancer, inflammation conditions, and central nervous system injury (1–8). Because of the broad involvement of PG in normal and pathophysiological processes in the nervous system, their quantification under these conditions is critical to understanding their role in brain function.

Several methods for measuring PG mass in brain tissue have been used, including enzyme immunoassay and radioimmunoassay methods, HPLC with ultraviolet light, fluorescence, or mass spectrometric detection, and gas chromatography with mass spectrometric or flame ionization detection (8–11). Compared with other methods, high-performance liquid chromatography with tandem mass spectrometry detection (LC-MS/MS) has several advantages because of its high selectivity, its high sensitivity, and its potential for simultaneous quantification for several PG and isoprostanes without any derivatization with relatively low cost of analysis (9, 11). However, the ap-

Abbreviations: BHT, butylated hydroxytoluene; LC-MS/MS, high-performance liquid chromatography with tandem mass spectrometry detection; PG, prostanoid; PGD<sub>2</sub>, 9 $\alpha$ ,15*S*-dihydroxy-11-oxo-prosta-5*Z*,13*E*-dien-1-oic acid; PGD<sub>2</sub>-d4, 9 $\alpha$ ,15*S*-dihydroxy-11-oxo-prosta-5*Z*,13*E*-dien-1-oic-3,3,4,4-<sup>2</sup>H<sub>4</sub> acid; PGE<sub>2</sub>, 9-oxo-11 $\alpha$ ,15*S*-dihydroxyprosta-5*Z*,13*E*-dien-1-oic acid; 11 $\beta$ -PGE<sub>2</sub>, 9-oxo-11 $\beta$ ,15*S*-dihydroxyprosta-5*Z*,13*E*-dien-1-oic acid; 8-isoPGE<sub>2</sub>, 9-oxo-11 $\alpha$ ,15*R*-dihydroxyprosta-5*Z*,13*E*-dien-1-oic acid; PGE<sub>2</sub>-d4, 9-oxo-11 $\alpha$ ,15*S*-dihydroxyprosta-5*Z*,13*E*-dien-1-oic-3,3,4,4-<sup>2</sup>H<sub>4</sub> acid; PGF<sub>2 $\alpha$</sub> , 9 $\alpha$ ,11 $\alpha$ ,15*S*-trihydroxyprosta-5*Z*,13*E*-dien-1-oic acid; 11 $\beta$ -PGF<sub>2 $\alpha$</sub> , 9 $\alpha$ ,11 $\beta$ ,15*S*-trihydroxyprosta-5*Z*,13*E*-dien-1-oic acid; 8-isoPGF<sub>2 $\alpha$</sub> , 9 $\alpha$ ,11 $\alpha$ ,15*S*-trihydroxy-(8 $\beta$ )-prosta-5*Z*,13*E*-dien-1-oic acid; PGF<sub>2 $\alpha$</sub> -d4, 9 $\alpha$ ,11 $\alpha$ ,15*S*-trihydroxyprosta-5*Z*,13*E*-dien-1-oic-3,3,4,4-<sup>2</sup>H<sub>4</sub> acid; 6-oxo-PGF<sub>1 $\alpha$</sub> , 6-oxo-9 $\alpha$ ,11 $\alpha$ ,15*S*-trihydroxyprosta-13*E*-en-1-oic acid; 6-oxo-PGF<sub>1 $\alpha$</sub> -d4, 6-oxo-9 $\alpha$ ,11 $\alpha$ ,15*S*-trihydroxyprosta-13*E*-en-1-oic-3,3,4,4-<sup>2</sup>H<sub>4</sub> acid; TXB<sub>2</sub>, 9 $\alpha$ ,11,15*S*-trihydroxythromba-5*Z*,13*E*-dien-1-oic acid; TXB<sub>2</sub>-d4, 9 $\alpha$ ,11,15*S*-trihydroxythromba-5*Z*,13*E*-dien-1-oic-3,3,4,4-<sup>2</sup>H<sub>4</sub> acid.

<sup>1</sup>To whom correspondence should be addressed.  
e-mail: emurphy@medicine.nodak.edu

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plication of LC-MS/MS requires special care for brain PG extraction and sample preparation before analysis, because of the complicated brain biological matrix that produces high chemical background noise, thereby reducing the selectivity and sensitivity of detection. Several different methods are used for brain PG extraction, including methanol extraction followed by solid-phase extraction (9, 12, 13), hexane/2-propanol extraction (14), ether extraction (15), and acetone/chloroform extraction (16). Although methanol extraction followed by solid-phase extraction (9) and ether extraction (15) have been used in different LC-MS/MS procedures, it is important to note that the simultaneous evaluation of these different extraction methods for use in brain PG analysis using LC-MS/MS has not been done.

Another important factor that must be considered during brain PG analysis is the method used to euthanize the animal and the subsequent handling of the brain sample. Brain PG mass in rodents euthanized by head-focused microwave irradiation is 10- to 40-fold lower than in animals euthanized by decapitation (17, 18). Although it is assumed that this reduction in PG mass is the result of heat inactivation of the enzymes involved in postmortem PG formation (17–19), this reduction could also be the result of the trapping or destruction of PG in microwaved brain. Several lines of evidence support the assumption that PG are not trapped or destroyed during microwave treatment. Brain PG mass found in indomethacin-treated animals euthanized by decapitation does not differ from PG mass in brains from animals euthanized by microwave irradiation (18). Also, after intracerebral ventricular injection of radiolabeled PG before microwave irradiation, most of the recovered radioactivity was in the form of PG; however, the recovery of the radiolabeled PG from brains subjected to microwave irradiation was not examined in this study (19). Importantly, these studies do not provide direct evidence that PG are not trapped or destroyed in microwaved brains.

In the present study, we evaluated and modified existing methods for brain PG extraction and sample preparation for LC-MS/MS analysis. The modified method improved the limits of tissue PG detection by 4- to 20-fold in an individual PG-dependent manner and allowed the analysis of PG in <10 mg of brain tissue with an extraction recovery that ranged from 85% to 95%. We also evaluated PG stability during storage and analysis and provide direct evidence that PG are not trapped or destroyed in microwaved brains.

## MATERIALS AND METHODS

Several alternative procedures and conditions for PG analysis were tested and are presented in Results and Discussion. Described below are the final recommended methods.

### Chemicals

9-Oxo-11 $\alpha$ ,15*S*-dihydroxyprosta-5*Z*,13*E*-dien-1-oic acid (PGE<sub>2</sub>), 9-oxo-11 $\beta$ ,15*S*-dihydroxyprosta-5*Z*,13*E*-dien-1-oic acid (11 $\beta$ -

PGE<sub>2</sub>), 9-oxo-11 $\alpha$ ,15*R*-dihydroxyprosta-5*Z*,13*E*-dien-1-oic acid (8-isoPGE<sub>2</sub>), 9-oxo-11 $\alpha$ ,15*S*-dihydroxyprosta-5*Z*,13*E*-dien-1-oic-3,3,4,4-<sup>2</sup>H<sub>4</sub> acid (PGE<sub>2</sub>-d4), 9 $\alpha$ ,15*S*-dihydroxy-11-oxo-prosta-5*Z*,13*E*-dien-1-oic acid (PGD<sub>2</sub>), 9 $\alpha$ ,15*S*-dihydroxy-11-oxo-prosta-5*Z*,13*E*-dien-1-oic-3,3,4,4-<sup>2</sup>H<sub>4</sub> acid (PGD<sub>2</sub>-d4), 9 $\alpha$ ,11 $\alpha$ ,15*S*-trihydroxyprosta-5*Z*,13*E*-dien-1-oic acid (PGF<sub>2 $\alpha$</sub> ), 9 $\alpha$ ,11 $\beta$ ,15*S*-trihydroxyprosta-5*Z*,13*E*-dien-1-oic acid (11 $\beta$ -PGF<sub>2 $\alpha$</sub> ), 9 $\alpha$ ,11 $\alpha$ ,15*S*-trihydroxy-(8 $\beta$ )-prosta-5*Z*,13*E*-dien-1-oic acid (8-isoPGF<sub>2 $\alpha$</sub> ), 9 $\alpha$ ,11 $\alpha$ ,15*S*-trihydroxyprosta-5*Z*,13*E*-dien-1-oic-3,3,4,4-<sup>2</sup>H<sub>4</sub> acid (PGF<sub>2 $\alpha$</sub> -d4), 6-oxo-9 $\alpha$ ,11 $\alpha$ ,15*S*-trihydroxy-prosta-13*E*-en-1-oic acid (6-oxo-PGF<sub>1 $\alpha$</sub> ), 6-oxo-9 $\alpha$ ,11 $\alpha$ ,15*S*-trihydroxy-prosta-13*E*-en-1-oic-3,3,4,4-<sup>2</sup>H<sub>4</sub> acid (6-oxo-PGF<sub>1 $\alpha$</sub> -d4), 9 $\alpha$ ,11,15*S*-trihydroxy-thromba-5*Z*,13*E*-dien-1-oic acid (TXB<sub>2</sub>), and 9 $\alpha$ ,11,15*S*-trihydroxy-thromba-5*Z*,13*E*-dien-1-oic-3,3,4,4-<sup>2</sup>H<sub>4</sub> acid (TXB<sub>2</sub>-d4) 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 (Publication 80-23) and under an animal protocol approved by the Institutional Animal Care and Use Committee at the University of North Dakota (Protocol 0409-9). Male 129/SvEv strain mice (25–30 g) were maintained on standard laboratory chow diet and water ad libitum. The ages of the mice used in this study were between 9 and 11 months.

### Brain PG extraction

Fasted male mice were anesthetized with halothane (1–3%) and euthanized using head-focused microwave irradiation (2.8 kW, 1.35 s; Cober Electronics, Inc., Norwalk, CT) to heat-denature enzymes in situ. The whole brain was removed, frozen in liquid nitrogen, and pulverized under liquid nitrogen temperatures to a fine, homogeneous powder.

The extraction protocol was a modification of a previously published procedure (16) that was adapted for tissue extraction. Pulverized tissue (10–20 mg) was homogenized in 3 ml of acetone-saline (2:1) containing PGE<sub>2</sub>-d4 and 6-oxo-PGF<sub>1 $\alpha$</sub> -d4 (100 pg in 10  $\mu$ l of acetonitrile) as internal standards and 0.005% butylated hydroxytoluene (BHT) to prevent PG oxidation using a Tenbroeck tissue grinder (Kontes Glass Co., Vineland, NJ). The homogenate was transferred to a screw-top tube, vortexed for 4 min, and subjected to 10 min of centrifugation (2,000 g) at 4°C. The supernatant was transferred to another screw-top tube and mixed with 2.0 ml of hexane by vortexing for 0.5 min. Then, the mixture was subjected to 10 min of centrifugation (2,000 g) at 4°C. The upper phase containing hexane with extracted lipids was discarded, the lower phase was acidified with formic acid to pH 3.5 (30  $\mu$ l of 2 M formic acid), and 2 ml of chloroform containing 0.005% BHT was added. The mixture was vortexed for 0.5 min and again subjected to 10 min of centrifugation (2,000 g) at 4°C to aid in the separation of the two phases. The lower phase containing chloroform was transferred to a screw-top tube silanized with Sigmacote® (Sigma Chemical Co., St. Louis, MO), flushed with nitrogen, and cooled at –20°C for at least 2 h. This cooling allows the separation of any residual upper phase, which is then removed and discarded before analysis.

### Sample preparation for LC-MS/MS

After the residual upper phase was discarded, 200  $\mu$ l of methanol was added to the extract and it was dried down under a stream of nitrogen. The dried extract was transferred to 100  $\mu$ l silanized microvial inserts (National Scientific, Rockwoods, TN; catalog No. C4010-S630) using 2  $\times$  0.1 ml of chloroform

containing 10% methanol and 0.005% BHT. The solvent in microvial inserts was dried down under a stream of nitrogen. The transfer procedure was repeated twice. Ten microliters of acetonitrile was added to the insert with dried extract, vortexed for 30 s, and mixed with 20  $\mu$ l of water.

### Reverse-phase HPLC electrospray ionization mass spectrometry

The separation was carried out using a Luna C-18(2) column (3  $\mu$ m, 100 Å pore diameter, 150  $\times$  2.0 mm; Phenomenex, Torrance, CA) with a stainless-steel frit filter (0.5  $\mu$ m) 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. Twenty-five microliters out of a 30  $\mu$ l sample was injected onto a chromatographic column.

The solvent program for elution was modified from a previously described method (20). This modification was made to increase the sensitivity of detection by increasing peak sharpness and resolving PG from other chemical compounds coextracted from brain tissue. 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, and the initial solvent conditions started with 10% solvent B. At 2 min, the percentage of B was increased to 65% over 8 min; at 15 min, the percentage of B was increased to 90% over 5 min; and at 35 min, it was reduced to 10% over 2 min. Equilibration time between runs was 13 min.

MS analysis was performed using a quadrupole mass spectrometer (API3000; Applied Biosystems, Foster City, CA) equipped with a TurbolonSpray ionization source. Analyst software version 1.4.2 (Applied Biosystems) 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 450°C, electrospray voltage was -4,250 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 as presented in **Table 1**. Focusing potential was -200 V, and entrance potential was -10 V for all analytes. The quadrupole mass spectrometer was operated at unit resolution.

PGE<sub>2</sub>, PGD<sub>2</sub>, PGF<sub>2 $\alpha$</sub> , and TXB<sub>2</sub> were quantified using PGE<sub>2</sub>-d4 as the internal standard, whereas 6-oxo-PGF<sub>1 $\alpha$</sub>  was quantified using 6-oxo-PGF<sub>1 $\alpha$</sub> -d4 as the internal standard. Initially, we used PGD<sub>2</sub>-d4, PGF<sub>2 $\alpha$</sub> -d4, and TXB<sub>2</sub>-d4 to normalize PGD<sub>2</sub>, PGF<sub>2 $\alpha$</sub> , and TXB<sub>2</sub>, respectively; however, this approach did not improve variability or recovery results compared with quantification using only PGE<sub>2</sub>-d4. An example of brain PG LC-ESI-MS/MS analysis is presented in **Fig. 1**.

### Statistical analysis

All statistical comparisons were calculated using a two-way, unpaired Student's *t*-test or a one-way ANOVA and a Tukey-Kramer posthoc test when appropriate, using Instat II (Graphpad, San Diego, CA). Statistical significance was defined as *P* < 0.05. All values are expressed as means  $\pm$  SD.

## RESULTS AND DISCUSSION

### PG extraction

The brain has a complicated biological matrix that produces high chemical background noise, so that the use of LC-MS/MS to measure brain PG mass requires special care with regard to sample preparation before analysis. Although several methods for brain PG extraction and purification are currently used (9, 12, 14–16, 21), these methods have not been evaluated for use by LC-MS/MS analysis.

Here, we have evaluated the background chemical noise and limits of sensitivity for four different methods currently used for PG extraction and analysis. The efficiency of the extraction procedures was estimated by the recovery of the deuterium-labeled PG added to the samples before extraction and ranged from 85% to 95% for each of the methods tested; there were no significant differences between methods.

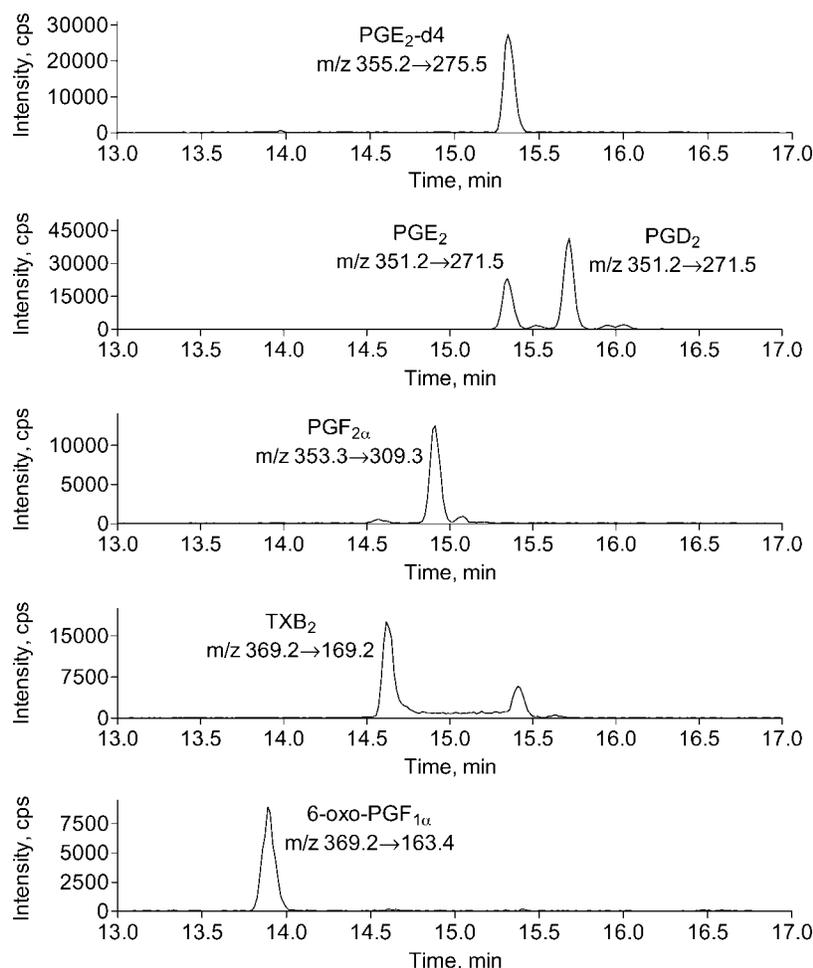
### Brain extraction with methanol followed by solid-phase extraction on C<sub>18</sub> columns

The most common method is methanol extraction followed by solid-phase extraction (9, 12, 13). Here, we describe our modified extraction methodology based upon a previously described procedure (10). Briefly, ~20 mg of brain microwaved tissue was homogenized in 3 ml of 15% methanol in water at pH 3 containing PGE<sub>2</sub>-d4 and 6-oxo-PGF<sub>1 $\alpha$</sub> -d4 (100 pg in 10  $\mu$ l of acetonitrile) as internal standards and 0.005% BHT to prevent PG oxidation using a 7 ml Tenbroeck tissue grinder (Kontes Glass Co.). The homogenate was then transferred to a screw-top tube, vortexed for 4 min, and subjected to 10 min of centrifugation (2,000 *g*) at 4°C. The supernatant was loaded onto a prewashed C<sub>18</sub> Sep-Pak classic cartridge (Waters Corp., Milford, MA). To remove the bound PG, the cartridge was washed with 20 ml of 15% methanol followed by 20 ml

TABLE 1. Multiple reaction monitoring parameters optimized for PG of interest

PG	Q1 Mass	Q3 Mass	Declustering Potential	Collision Cell Energy	Collision Cell Exit Potential
PGE <sub>2</sub> and PGD <sub>2</sub>	351.2	271.5	-30	-28	-10
PGE <sub>2</sub> -d <sub>4</sub>	355.2	275.5	-30	-28	-10
PGF <sub>2<math>\alpha</math></sub>	353.3	193.2	-40	-34	-11
6-oxo-PGF <sub>1<math>\alpha</math></sub>	369.2	163.4	-40	-37	-29
6-oxo-PGF <sub>1<math>\alpha</math></sub> -d <sub>4</sub>	374.2	167.4	-40	-37	-29
TXB <sub>2</sub>	369.2	169.2	-40	-30	-9

PG, prostanoid; PGD<sub>2</sub>, 9 $\alpha$ ,15S-dihydroxy-11-oxo-prosta-5Z,13E-dien-1-oic acid; PGE<sub>2</sub>, 9-oxo-11 $\alpha$ ,15S-dihydroxyprosta-5Z,13E-dien-1-oic acid; PGE<sub>2</sub>-d<sub>4</sub> 9-oxo-11 $\alpha$ ,15S-dihydroxyprosta-5Z,13E-dien-1-oic-3,3,4,4-<sup>2</sup>H<sub>4</sub> acid; PGF<sub>2 $\alpha$</sub> , 9 $\alpha$ ,11 $\alpha$ ,15S-trihydroxyprosta-5Z,13E-dien-1-oic acid; 6-oxo-PGF<sub>1 $\alpha$</sub> , 6-oxo-9 $\alpha$ ,11 $\alpha$ ,15S-trihydroxy-prosta-13E-en-1-oic acid; 6-oxo-PGF<sub>1 $\alpha$</sub> -d<sub>4</sub>, 6-oxo-9 $\alpha$ ,11 $\alpha$ ,15S-trihydroxy-prosta-13E-en-1-oic-3,3,4,4-<sup>2</sup>H<sub>4</sub> acid; TXB<sub>2</sub>, 9 $\alpha$ ,11,15S-trihydroxythromba-5Z,13E-dien-1-oic acid.



**Fig. 1.** Example of brain prostanoid (PG) analysis using high-performance liquid chromatography with tandem mass spectrometry detection (LC-ESI-MS/MS). A sample of brain tissue (300 mg) from mice euthanized using head-focused microwave irradiation was analyzed using a procedure described in Materials and Methods. The concentrations of 9-oxo-11 $\alpha$ ,15*S*-dihydroxyprosta-5*Z*,13*E*-dien-1-oic acid (PGE<sub>2</sub>), 9 $\alpha$ ,15*S*-dihydroxy-11-oxo-prosta-5*Z*,13*E*-dien-1-oic acid (PGD<sub>2</sub>), 9 $\alpha$ ,11,15*S*-trihydroxythromba-5*Z*,13*E*-dien-1-oic acid (TXB<sub>2</sub>), 9 $\alpha$ ,11 $\alpha$ ,15*S*-trihydroxyprosta-5*Z*,13*E*-dien-1-oic acid (PGF<sub>2 $\alpha$</sub> ), and 6-oxo-9 $\alpha$ ,11 $\alpha$ ,15*S*-trihydroxy-prosta-13*E*-en-1-oic acid (6-oxo-PGF<sub>1 $\alpha$</sub> ) were 0.23, 0.65, 0.08, 0.29, and 0.08 ng/g wet weight, respectively. The concentration of internal standard 9-oxo-11 $\alpha$ ,15*S*-dihydroxyprosta-5*Z*,13*E*-dien-1-oic-3,3,4,4-<sup>2</sup>H<sub>4</sub> acid (PGE<sub>2</sub>-d4) was 100 pg/sample. cps, counts per second.

of water, dried with syringe air, and eluted with 10 ml of methyl formate (spectral grade; Acros Organics, Pittsburgh, PA). The methyl formate was then removed under a stream of nitrogen, and PG were prepared for LC-MS/MS analysis as described in Materials and Methods. Although this method has been used for tissue extraction before PG LC-MS analysis (9), we found that the chemical background noise was very high. This high background noise accounts for a 5- to 20-fold reduction in sensitivity for different PG compared with the acetone liquid/liquid extraction described in Materials and Methods (**Table 2**). Importantly, using another brand of methyl formate (MP Biochemicals, Solon, OH), or its substitution with ethyl acetate, resulted in much greater chemical noise and significantly reduced sensitivity (data not shown). Use of other brands of C<sub>18</sub> cartridges [Ultra-Clean™ (Alltech Associates, Inc., Deerfield, IL) or Strata®

(Phenomenex)] did not improve the limits of sensitivity (data not shown).

#### Brain extraction with diethyl ether

Diethyl ether is another solvent used for PG extraction (15). Briefly, ~20 mg of brain microwaved tissue was homogenized in 3 ml of saline at pH 3 containing PGE<sub>2</sub>-d4 and 6-oxo-PGF<sub>1 $\alpha$</sub> -d4 (100 pg in 10  $\mu$ l of acetonitrile) as internal standards and 0.005% BHT using a 7 ml Tenbroeck tissue grinder. The homogenate was extracted twice with 4 ml of diethyl ether, and the organic layer was then dried under a stream of nitrogen. PG were prepared for LC-MS/MS analysis as described in Materials and Methods. This method for extraction resulted in very high level of chemical noise, so we purified the extracts on C<sub>18</sub> cartridges as described above (10). The level of sensitivity for TXB<sub>2</sub> was improved; however, it reduced sensitivity for

TABLE 2. Effects of different methods of brain PG extraction on high-performance liquid chromatography with tandem mass spectrometry detection limits

Extraction Method	PGE <sub>2</sub> and PGD <sub>2</sub>	TXB <sub>2</sub>	PGF <sub>2α</sub>	6-Oxo-PGF <sub>1α</sub>
Methanol followed by solid-phase extraction	3.9 ± 1.0 (0.5 ± 0.1)	17.3 ± 6.2 (2.0 ± 0.7)	7.2 ± 2.6 (0.9 ± 0.3)	14.7 ± 3.8 (1.7 ± 0.5)
Diethyl ether followed by solid-phase extraction	5.6 ± 2.5 (0.7 ± 0.3)	5.5 ± 1.9 (0.7 ± 0.2)	20.0 ± 8.0 (2.4 ± 0.9)	33.3 ± 8.3 (3.9 ± 1.0)
Hexane/2-propanol followed by solid-phase extraction	5.0 ± 1.7 (0.6 ± 0.2)	14.5 ± 3.6 (1.7 ± 0.4)	15.4 ± 5.4 (1.8 ± 0.6)	13.3 ± 4.0 (1.6 ± 0.5)
Hexane/2-propanol followed by acetone	0.5 ± 0.1 (0.1 ± 0.0)	0.7 ± 0.2 (0.1 ± 0.0)	1.2 ± 0.1 (0.1 ± 0.0)	2.1 ± 0.5 (0.3 ± 0.1)
Acetone	0.6 ± 0.1 (0.1 ± 0.0)	0.9 ± 0.2 (0.1 ± 0.0)	1.5 ± 0.5 (0.2 ± 0.1)	3.3 ± 0.7 (0.4 ± 0.1)

Limits of detection were determined as lower analyte mass for which the signal-to-noise ratio was >3. First values indicate limits of detection in picograms on-column, and second values (in parentheses) indicates mass in nanograms per gram wet weight for 20 mg brain samples. Values are expressed as means ± SD (n = 3).

all other analyzed PG compared with methanol extraction followed by solid-phase extraction on C<sub>18</sub> columns (Table 2). Overall, the chemical background noise was much higher for all PG, accounting for a 6- to 13-fold reduction in sensitivity for PG compared with acetone extraction with liquid/liquid purification.

### Brain extraction with hexane/2-propanol

We also tested hexane/2-propanol extraction (3:2, v/v), which allows for simultaneous extraction of brain phospholipids, neutral lipids, and PG (14). Briefly, ~20 mg of brain microwaved tissue was homogenized in 1 ml of hexane/2-propanol (3:2, v/v) containing PGE<sub>2</sub>-d<sub>4</sub> and 6-oxo-PGF<sub>1α</sub>-d<sub>4</sub> (100 pg in 10 μl of acetonitrile) as internal standards using a 2 ml Tenbroeck tissue grinder. The homogenizer was rinsed three times with 1 ml of hexane/2-propanol. The homogenate was subjected to 10 min of centrifugation (2,000 g) at 4°C, and the supernatant was removed. The supernatant was then dried under a stream of nitrogen, redissolved in 15% methanol at pH 3, and purified on C<sub>18</sub> cartridges as described above (10). PG were prepared for LC-MS/MS analysis as described in Materials and Methods. The chemical background noise was similar to values for methanol extraction followed by solid-phase extraction on C<sub>18</sub> columns. Also, the sensitivity was 4- to 16-fold lower compared with the acetone liquid/liquid extraction described in Materials and Methods (Table 2).

Because purification of hexane/2-propanol lipid extracts on C<sub>18</sub> cartridges did not increase the levels of sensitivity of LC-MS/MS analysis compared with other methods, we used acetone to reextract PG from hexane/2-propanol lipid extracts. To purify hexane/2-propanol lipid extracts with acetone, aliquots of extracts were transferred into silanized tubes, solvent was removed under a stream of nitrogen and redissolved in 2 ml of acetone containing 0.005% BHT, and then 1 ml of saline was added. This mixture was mixed by vortexing for 4 min. We then followed the procedures for PG analysis as described in Materials and Methods. Although the purification of lipid extract with acetone followed by liquid/liquid extraction did not significantly improve the levels of sensitivity compared with direct extraction with acetone (Table 2), this approach allows the simultaneous analysis of PG and other lipid molecules in the same sample.

### Extraction recovery with acetone

We verified the recovery of PG extraction with acetone compared with extraction with hexane/2-propanol, because hexane/2-propanol affords a high recovery of PG from tissue (14). Because we found that hexane/2-propanol extraction has reduced sensitivity as a result of high background, we induced brain PG formation by injecting mice with lipopolysaccharide (1 mg/kg ip) (22) at 3 h before head-focused microwave irradiation. We extracted the same brain samples using either acetone or hexane/2-propanol (3:2, v/v). There were no differences in PG mass found after hexane/2-propanol extraction compared with acetone extraction (Fig. 2), indicating that acetone yields a high extraction ratio of PG from the brain tissue. Because acetone extraction produced significantly less background chemical noise compared with hexane/2-propanol extraction, the standard deviations for the individual PG are smaller in samples analyzed from the acetone extract compared with the hexane/2-propanol extract.

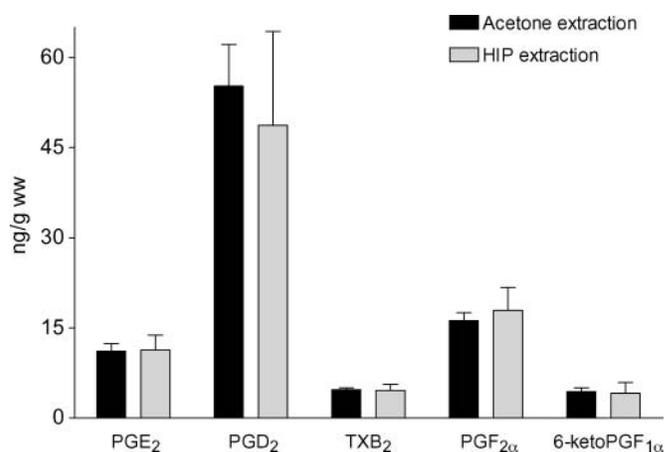
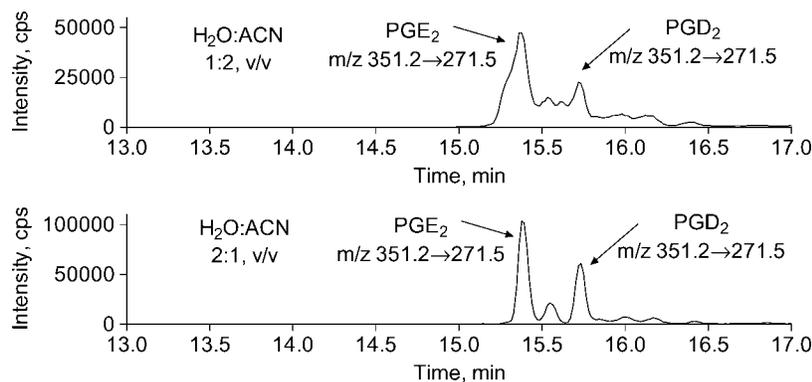


Fig. 2. PG mass measured after acetone and hexane/2-propanol extraction. Brain PG mass was induced by intraperitoneal injection of lipopolysaccharide (1 mg/kg) at 3 h before animals were euthanized using head-focused microwave irradiation. The same brain samples were extracted with either hexane/2-propanol or acetone and analyzed as described in Materials and Methods. Values are expressed as means ± SD (n = 3). HIP, hexane/2-propanol (3:2, v/v); ww, wet weight.



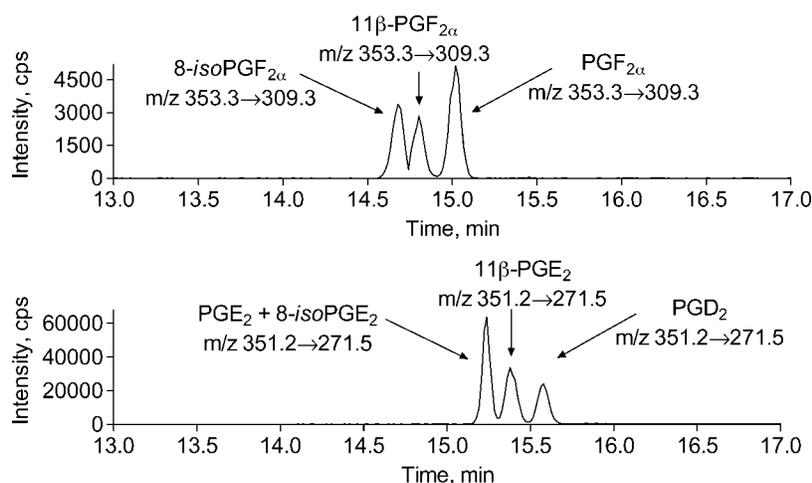
**Fig. 3.** Effect of loading solvent composition of the separation of brain PG by HPLC. A sample of brain tissue (10 mg) from mice euthanized by decapitation was extracted and analyzed using a procedure described in Materials and Methods. The sample (25  $\mu$ l) was loaded onto an HPLC column using either water-acetonitrile (1:2) (upper panel) or water-acetonitrile (2:1) (lower panel). cps, counts per second.

In summary, tissue PG extraction with acetone followed by liquid/liquid extraction significantly increased the level of sensitivity in the LC-MS/MS analysis compared with other extraction methods tested in this study. This increased sensitivity was the result of a significant reduction of background chemical noise, probably attributable to better purification of PG extract from components that affect LC-MS/MS analysis. Also, dissolving the residue from a hexane/2-propanol lipid extract with acetone permits PG analysis in common lipid extracts that contain all major lipids, thereby extending the application of this method of extraction to other lipid parameters beyond PG, which is important when sample quantity is limited. Besides better sensitivity, PG extraction with acetone followed by liquid/liquid extraction is considerably less laborious when large sets of samples are analyzed and much less expensive compared with purification on  $C_{18}$  cartridges. Therefore, we consider

acetone extraction followed by liquid/liquid extraction to be the method of choice for PG analysis using LC-MS/MS.

#### Sample preparation for LC-MS/MS analysis

The solvent composition used to apply a sample onto an HPLC column may have a significant effect on analyte separation and peak sharpness, thereby affecting the limits of detection and the accuracy of analysis. Acetonitrile would be the best solvent to dissolve a sample before application on the column because it dissolves PG and is a component of the mobile phase. However, because of the small volume of the HPLC column used for PG separation and the low flow rate of solvents used in the LC separation program, a high acetonitrile concentration used to apply the sample reduces peak sharpness and increases peak leading (**Fig. 3**). We have found that the optimal acetonitrile-water ratio is 1:2 when 25  $\mu$ l of sample is applied onto an HPLC column.



**Fig. 4.** Analysis of prostaglandin and isoprostane standard mixture using LC-ESI-MS/MS. A mixture of  $PGE_2$ , 8-iso $PGE_2$ , 11 $\beta$ - $PGE_2$ ,  $PGF_{2\alpha}$ , and 11 $\beta$ - $PGF_{2\alpha}$  (100, 100, 100, 100, 50, and 50 pg, respectively, in 10  $\mu$ l of acetonitrile) was analyzed using a procedure described in Materials and Methods. cps, counts per second.

### Specificity of the LC-MS/MS

It is well known that liver, brain, and plasma phospholipids contain esterified isoprostanes, which are PGF<sub>2</sub>-like and PGD<sub>2</sub>/E<sub>2</sub>-like molecules (23–26). These isoprostanes are also found in urine and plasma in an unesterified form (27–29). It is important to determine that these molecules are formed in situ via the nonenzymic oxidation of esterified 20:4n-6 (24, 25). To demonstrate the specificity of our method for PG, we analyzed a mixture of PGE<sub>2</sub>, 11 $\beta$ -PGE<sub>2</sub>, 8-isoPGE<sub>2</sub>, PGD<sub>2</sub>, PGF<sub>2 $\alpha$</sub> , 11 $\beta$ -PGF<sub>2 $\alpha$</sub> , and 8-isoPGF<sub>2 $\alpha$</sub>  standards (Fig. 4). All of the analyzed prostaglandins and isoprostanes were chromatographically resolved, with the exception of PGE<sub>2</sub> and 8-isoPGE<sub>2</sub>. These results are consistent with resolving isoprostanes and PG on C18 or C8 columns using a gradient of acetonitrile-based solvents (9, 28).

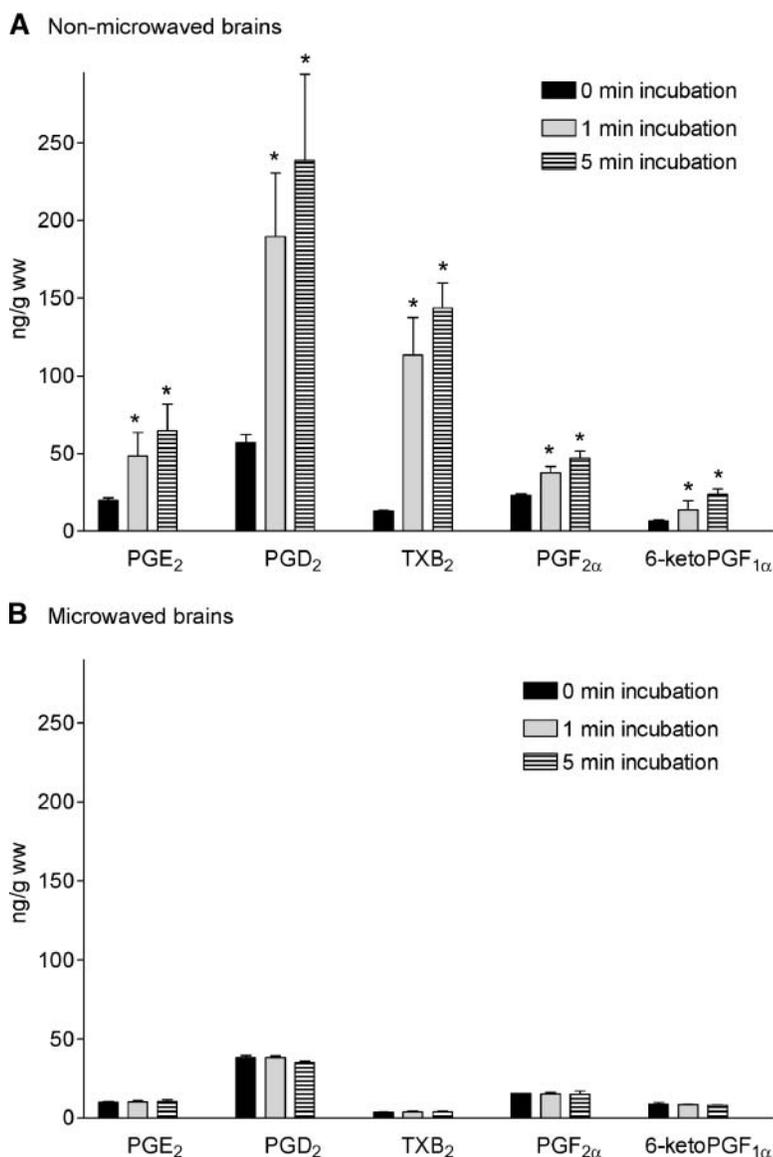
### Antioxidants prevent PG degradation during analysis

BHT is often used to prevent PG oxidation during extraction and analysis. Different concentrations of BHT

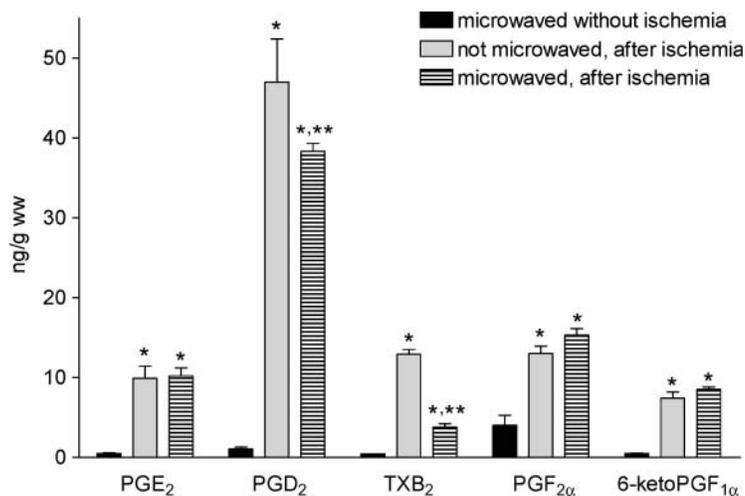
have been used in lipid analysis, ranging from 0.1% to 0.01% (3, 30), although not all investigators use antioxidants during PG analysis. To evaluate the need for antioxidants, we analyzed three identical brain samples with 0.1% BHT, 0.005% BHT, or no BHT added to acetone and chloroform used in the extraction. We found that 0.1% BHT produced a precipitate that may clog the LC system. However, using 0.005% BHT in chloroform and acetone for PG extraction and sample preparation efficiently decreased the variability of analysis and prevented a 2.8-fold reduction in 6-oxo-PGF<sub>1 $\alpha$</sub>  mass without producing a precipitate in the loading mixture.

### Use of head-focused microwave irradiation

Brain PG mass found in rodents euthanized by head-focused microwave irradiation is 10- to 40-fold lower than in animals euthanized by decapitation (17, 18). Different processes may account for this difference in results, including postmortem PG formation (17–19), induction of



**Fig. 5.** Induction of PG formation during brain tissue extraction. Two groups of animals were anesthetized with halothane (1–3%) and euthanized by decapitation, and their skulls were opened. The whole brains were removed at 1 min after animal death and either frozen in liquid nitrogen (A) or subjected to head-focused microwave irradiation (B) as described in Materials and Methods. A: Three identical nonmicrowaved brain samples were extracted either in ice-cold conditions or after sample incubation in extraction mixture for 1 or 5 min at room temperature. B: As a control for the completeness of extraction in ice-cold conditions, microwaved brains were extracted under the same conditions described for A. Values are expressed as means  $\pm$  SD ( $n = 3$ ). \*  $P < 0.05$  compared with samples extracted in ice-cold conditions. ww, wet weight.



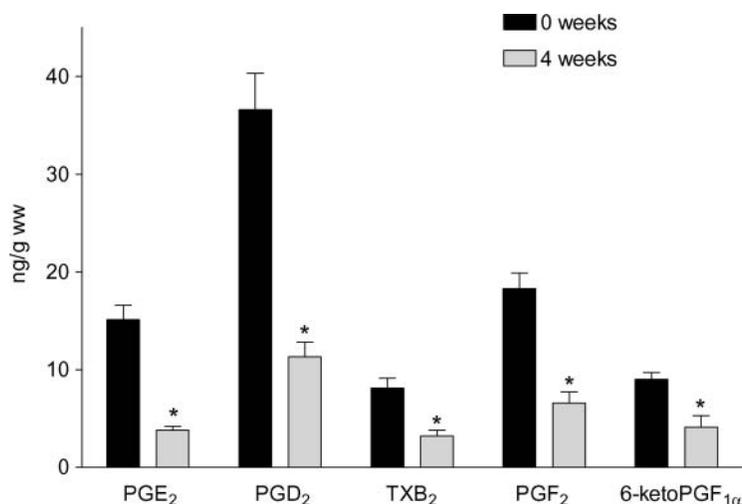
**Fig. 6.** PG are not destroyed or trapped in microwaved brain. Two groups of animals were anesthetized with halothane (1–3%) and euthanized by decapitation, and their skulls were opened. The whole brains were removed at 1 min after animal death and either frozen in liquid nitrogen or subjected to head-focused microwave irradiation as described in Materials and Methods. The temperature of microwaved brains ranged from 70°C to 85°C as measured using a thermocouple. As a control for enzyme inactivation in microwaved brains, a third group of animals was euthanized by microwave irradiation as described in Materials and Methods. Values are expressed as means  $\pm$  SD ( $n = 3$ ). \*  $P < 0.05$  compared with brains from mice euthanized by head-focused microwave irradiation without global ischemia; \*\*  $P < 0.05$  compared with brains from nonmicrowaved brains of mice exposed to global ischemia. ww, wet weight.

PG formation during extraction, or PG destruction or trapping in microwaved brains.

Because the induction of PG formation during extraction has not been tested in previous studies, we extracted identical nonmicrowaved brain samples either in ice-cold conditions or after sample incubation in extraction mixture for 1 or 5 min at room temperature. The mass of all PG analyzed was increased significantly at room temperature (Fig. 5A). As a control for the completeness of extraction in ice-cold conditions, microwaved brains with heat-inactivated enzymes were extracted under the same conditions described above. There were no differences between PG mass in microwaved tissue after extraction in ice-cold conditions compared with room temperature (Fig. 5B), indicating the same high extraction ratio in ice-cold conditions. These data indicate that induction of PG formation during extraction can occur, which is one factor contributing to the higher values and greater variability of PG mass found in nonmicrowaved brains.

Another reason for a reduction in PG mass in brains subjected to microwaved irradiation could be PG breakdown and/or trapping in irradiated tissue. Temperatures of 70°C to 100°C are reported a few seconds after expos-

ing the animal to head-focused microwave irradiation (19), similar to our observations. Because PG are known for their instability and have a short half-life, PG breakdown in heat-denatured tissue should be considered as another factor accounting for the reduced PG mass observed in microwaved brains compared with nonmicrowaved brains. In addition, because PG are bound *in vivo* by a variety of carrier proteins (31, 32), trapping of PG in heat-denatured proteins may also account for the reduction in PG mass observed after microwave irradiation. Although it is assumed that the observed reduction in PG mass in microwaved versus nonmicrowaved brain is the result of the heat inactivation of enzymes involved in postmortem PG formation (17–19), direct evidence that PG are not trapped or destroyed in microwaved brains has not been reported. What has been reported is that in indomethacin-treated rats euthanized by decapitation, brain PG mass is not different from the levels in brains from rats euthanized by microwave irradiation (18). Additional evidence is that after injection (intracerebral ventricular) of radiolabeled PG into the brain before microwave irradiation, most of the recovered radioactivity was in the form of PG (19), suggesting heat stability *in situ*. However, the recovery of



**Fig. 7.** Reduction in PG mass during tissue storage. Identical tissue samples were analyzed either the same day or 4 weeks after animals were euthanized by decapitation. Tissue powder was stored at  $-80^{\circ}\text{C}$ . Values are expressed as means  $\pm$  SD ( $n = 4$ ). \*  $P < 0.05$  compared with brains analyzed the same day as the mice were euthanized.

the radiolabeled PG from brains subjected to microwave irradiation was not examined in this study.

To address the possibility that microwave irradiation may affect the recovery of endogenous PG, we induced brain PG production by modeling global ischemia. Two groups of animals were anesthetized with halothane (1–3%) and euthanized by decapitation, and their skulls were opened. The whole brains were removed 1 min after death and either frozen in liquid nitrogen or subjected to microwave irradiation as described in Materials and Methods. The temperature of microwaved brains ranged from 70°C to 85°C as measured using a thermocouple. As a control for enzyme inactivation in microwaved brains, the third group of animals was euthanized immediately using head-focused microwave irradiation as described in Materials and Methods. The magnitude of increased PG mass found in nonmicrowaved brains was similar to the levels reported by others (17, 18) (Fig. 6). For most PG analyzed, there were no differences between the two different fixation regimens subjected to induction of PG production via ischemia (Fig. 6), indicating that the recovery of brain PG was fixation-independent.

However, PGD<sub>2</sub> and TXB<sub>2</sub> mass were increased in nonmicrowaved brains compared with microwaved brains after induction of PG formation. There are several explanations for these data. First, PGD<sub>2</sub> and TXB<sub>2</sub> are formed during extraction because their mass was affected to a greater extent than the mass of other PG during the extraction of nonmicrowaved brains (Fig. 5A). Second, PGD<sub>2</sub> and TXB<sub>2</sub> are more heat-labile. To test this assumption, we incubated a PGE<sub>2</sub>, PGD<sub>2</sub>, PGF<sub>2α</sub>, TXB<sub>2</sub>, and 6-oxo-PGF<sub>1α</sub> mixture dissolved in acetonitrile-water (1:2, v/v) at 85°C for 10 min and analyzed PG mass as described in Materials and Methods. The mass of all PG tested was decreased to the same extent (10–15%; data not shown), indicating similar heat lability of the tested PG.

Together, these data support the need to microwave brain and provide direct evidence that PG are not trapped or destroyed in microwaved brains.

#### PG stability during tissue storage

Because of PG short shelf half-lives, we tested PG stability in brain tissue and extracts during storage. PG were analyzed in the same samples either the same day or 4 weeks after animals were euthanized by decapitation. Tissue powder was stored at –80°C. Four weeks of tissue powder storage at –80°C resulted in a 2- to 4-fold decrease in PG mass (Fig. 7); however, no decrease of PG was observed in lipid extracts stored in hexane/2-propanol (3:2) for several months at –80°C under nitrogen (data not shown). These data indicate the utility of rapid tissue extraction with hexane/2-propanol (3:2), which then can be stably stored in hexane/2-propanol for PG and other lipid analysis in the future.

#### Summary

In summary, tissue PG extraction with acetone followed by liquid/liquid extraction significantly increased the level

of sensitivity of LC-MS/MS analysis compared with other extraction methods tested in this study. This increased sensitivity was the result of a significant reduction in background chemical noise. Dissolving residue from hexane/2-propanol lipid extracts with acetone allows the analysis of PG in these lipid extracts, thereby extending the application of this method of extraction. Besides better sensitivity, this method is less laborious and less expensive compared with purification on C<sub>18</sub> cartridges. We also evaluated PG stability during extraction and storage. The use of 0.005% BHT during PG extraction decreased the variability of analysis and limited 6-oxo-PGF<sub>1α</sub> oxidation. Importantly, PG were rapidly destroyed during the storage of powdered tissue; however, PG were stable in hexane/2-propanol extracts. Lastly, our data support the need to euthanize animals by head-focused microwave irradiation rather than by decapitation and provide direct evidence that PG are not trapped or destroyed in microwaved brains.

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