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Quantification of Prostaglandin E_2 Concentration in Interstitial Fluid from the Hypothalamic Region of Free-moving Mice

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[Abstract] Prostaglandin E_2 (PGE_2) is a well-established chemical mediator for the generation of the fever at the hypothalamus of the brain. PGE_2 mediates fever generation via PGE receptor 3 (*i.e.*, EP3) on neurons in the preoptic area. The role of PGE_2 has been analyzed by measuring PGE_2 concentration in cerebrospinal fluid (C_{csf}); however, local PGE_2 concentration at the hypothalamus may not necessarily be consistent with C_{csf}. In this protocol, we introduce our method to measure directly the alteration in PGE_2 concentration in interstitial fluid in the hypothalamus (C_{isf}) of awake (free-moving) mice using a microdialysis technique. Male mice (c57BL/6J) were anesthetized and fixed in the stereotaxic instrument, and a microdialysis probe was inserted into the hypothalamus through a guide cannula. On the fifth postoperative day, C_{isf} was monitored in free-moving mice that were intraperitoneally (*i.p.*) injected with lipopolysaccharide (LPS). PGE_2 and other eicosanoids recovered in Krebs-Ringer phosphate buffer and defused through a microdialysis probe were extracted into ethyl acetate/formic acid and then quantified with LC-MS/MS. Our method is useful to understand the role of key regulators of prostaglandin concentration such as those of transporters, which have been unappreciated in inflammation-based brain diseases.

Keywords: Prostaglandin E_2 (PGE_2), Microdialysis, Hypothalamus, Cerebrospinal fluid, Interstitial fluid, Mice, Free-moving, Transporters

[Background] PGE_2 is intracellularly synthesized by cyclooxygenase pathways and released into extracellular spaces, where it binds to PGE receptors (*i.e.*, EP1-4) to exhibit its activity. Prostanoids such as PGE_2 are present in an anionic form under physiological conditions, and membrane transport has been suggested to be important for the local distribution and physiological action of PGE_2 (Schuster, 2012; Nakanishi and Tamai, 2018). To date, several carrier proteins that are involved in PGE_2 membrane transport have been identified and characterized; however, their roles in PGE_2 distribution are not fully understood, particularly in the brain. Among these, organic anion transporting polypeptide 2A1 (OATP2A1) encoded by *SLCO2A1* is a membrane transporter responsible for cellular uptake of prostanoids including PGE_2, PGD_2, and $PGF_2\alpha$, which are important for inflammatory reactions and homeostasis (Kanai *et al.*, 1995). The roles of OATP2A1 in PGE_2 disposition have been analyzed in *Slco2a1*-deficient (*Slco2a1*$^{-/-}$) mice (Chang *et al.*, 2010; Nakanishi *et al.*, 2015). More recently, we

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reported that OATP2A1 located in intracellular organelles was involved in exocytotic PGE_2 secretion from certain types of cells including macrophages (Shimada *et al.*, 2015). Some previous articles described a potential role of OATP2A1 in the febrile response (Ivanov *et al.*, 2003; Ivanov and Romanovsky, 2004). Indeed, OATP2A1 is expressed at the apical membranes of the choroid plexus (Tachikawa *et al.*, 2012) and subarachnoidal blood vessels in rodents (Hosotani *et al.*, 2015) and in primary cultured cerebral endothelial cells prepared from rats (Kis *et al.*, 2006). Furthermore, in patients with Alzheimer's disease, immunoreactivity for anti-OATP2A1 antibody has been reported in some glial cells including microglia and astrocytes (Choi *et al.*, 2008). Therefore, our laboratory performed microdialysis to measure alteration in C_{isf} (concentration in interstitial fluid) of PGE_2 at the hypothalamus of the brain to understand the physiological significance of OATP2A1 in LPS-induced fever generation. Our recent study determined C_{isf} of PGE_2 at the hypothalamus in LPS-treated *Slco2a1*$^{+/+}$ and *Slco2a1*$^{-/-}$ mice and clearly demonstrated that it was neither identical to C_{csf} nor its concentration in hypothalamic tissues (C_{hyp}) and was maintained by OATP2A1 independently (Nakamura *et al.*, 2018). As far as we know, there is no report showing C_{isf} of PGE_2 measured in free-moving small mammals like mice, although it has been analyzed by a microdialysis technique in rats (Gerozissis *et al.*, 1995), rabbits (Kao, T. Y. *et al.*, 2007; Kao, C. H. *et al.*, 2007), and guinea pigs (Sehic *et al.*, 1996). In the case of small animals such as mice, C_{isf} of PGE_2 is expected to be lower than the detection limit of enzyme immunoassays, although an immunoassay is the most convenient and prevalent way to determine prostanoid concentration (Pradelles *et al.*, 1985). However, protanoids can be easily detected using LC-MS/MS when extracted from biological fluids and concentrated using a solid phase column because accurate and sensitive quantification methods for prostanoids are established with LC-MS/MS (Schmidt *et al.*, 2005; Cao *et al.*, 2008). Fine microdialysis techniques to measure alteration in prostanoid concentration in the extracellular spaces of the brain in mouse models have been used widely in the field of neuroscience to promote an understanding of the mechanisms underlying neuroinflammation. Therefore, in this article, we present our protocols for analysis of PGE_2 in microdialysis samples collected from the hypothalamus of mice, where PGE_2 functions as a chemical mediator for fever generation.

Materials and reagents

1. 1.5-ml microtube (BIO-BIK, catalog number: CF-0150)
2. 0.6-ml microtube (Thermo Fisher Scientific, catalog number: 502-GRD-Q)
3. 2.0-ml microtube (Watson, catalog number: 132-620C)
4. 10-ml glass conical tube (EMKS-16.5, Nichiden-Rika Glass, catalog number: 113111)
5. Capillary glass tube (0.69 mm inner diameter, 1.19 mm outer diameter, 90 mm length, Drummond Scientific Company, catalog number: 3-000-210-G)
6. 0.2-μm filter (Kanto Chemical, catalog number: 96923-00)
7. Glass slide
8. BD Lo-Dose U-100 insulin syringe (29 gauge of needle, Nippon Becton Dickinson, catalog

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number: 326666)

9. Cotton wool (Osaki Medical, catalog number: 100006)
10. Glass micropipette
    a. Attach a capillary glass tube to the glass micropipette puller, and heat it with the instrument.
    b. Pull a capillary glass tube according to the product protocol, and prepare the glass micropipette.
    c. Brake the pipette at approximately 1 mm from the tip with tweezers before use.
11. Male c57BL/6J mice (10-25 weeks old)
12. Pentobarbital sodium salt (Nacalai Tesque, catalog number: 26427-14)
13. Paraffin (Leica, catalog number: 39601095)
14. Liquid nitrogen
15. Instant adhesive (Aron Alpha A, Daiichi-Sankyo)
16. LPS O111:B4 (LPS, Sigma-Aldrich, catalog number: L4130)
17. Formic acid (Wako Pure Chemical Industries, catalog number: 063-05895)
18. Acetonitrile (Wako Pure Chemical Industries, catalog number: 015-08633)
19. Ethyl acetate (Wako Pure Chemical Industries, catalog number: 058-00361)
20. Ethanol (Wako Pure Chemical Industries, catalog number: 054-00461)
21. Xylene (Wako Pure Chemical Industries, catalog number: 244-00081)
22. Mayer's hematoxylin solution (Muto Pure Chemicals, catalog number: 30001)
23. 1% eosin Y solution (Muto Pure Chemicals, catalog number: 32001)
24. Paraformaldehyde (PFA, Wako Pure Chemical Industries, catalog number: 162-16065)
25. Dental cement (Fuji I, GC, catalog number: 219AFB2X00208000)
26. Woodworking glue (Konishi, catalog number: 10122)
27. Dibutylhydroxytoluene (BHT, Wako Pure Chemical Industries, catalog number: 047-29451)
28. PGE_2 (Cayman Chemicals, catalog number: 14010)
29. d_4-PGE_2 (Cayman Chemicals, catalog number: 314010)
30. Glycerol (Nacalai Tesque, catalog number: 17045-65)
31. KCl (Wako Pure Chemical Industries, catalog number: 163-03545)
32. $MgSO_4$ (Nacalai Tesque, catalog number: 210-32)
33. NaH_2PO_4 (Wako Pure Chemical Industries, catalog number: 197-09705)
34. $Na_2HPO_4 \cdot 12H_2O$ (Kanto Chemical, catalog number: 37240-00)
35. $CaCl_2$ (Kanto Chemical, catalog number: 07057-00)
36. NaCl (Wako Pure Chemical Industries, catalog number: 191-01665)
37. HCl (5 N, Wako Pure Chemical Industries, catalog number: 081-05435)
38. NaOH solution (5 N, Wako Pure Chemical Industries, catalog number: 196-05375)
39. Pentobarbital solution (see Recipes)
40. 10% glycerol solution (see Recipes)
41. Krebs-Ringer phosphate buffer (KRPB, see Recipes)
42. 0.5-M phosphate buffer (PB, see Recipes)

43. 4% PFA solution (see Recipes)

Equipment

1. Pipettes (Nichipet EXII, Nichiryo, catalog numbers: 00-NPX2-20, 00-NPX2-200, 00-NPX2-1000)
2. Scissors (Natsume Seisakusho, catalog number: B-12H)
3. Tweezers (Y-277, Hirasawa, catalog number: 9523)
4. Dental drill
   a. Power supply (Minitor, catalog number: C2012)
   b. Rotary (Minitor, catalog number: M112)
   c. Diamond bar (diameter 0.8 mm, Minitor, catalog number: AD1403)
5. -80 °C deep freezer (CLN-50U, Nihon Freezer)
6. Microsyringe pump CMA/102 (CMA Microdialysis AB, catalog number: CMA/102)
7. Microsyringe (Ito Seisakusho, catalog number: MS-GAN100)
8. Stereotaxic instrument (Narishige, catalog number: SR-5M-HT)
9. Anchor screws (Eicom, catalog number: AN-3)
10. CMA7 guide cannula (CMA Microdialysis AB, catalog number: CMA P0000137)
11. CMA7 microdialysis probe (1 mm membrane length, CMA Microdialysis AB, catalog number: CMA P000082)
12. Device for free-movement (Eicom, catalog number: TSU-25)
13. Acrylic cage (Eicom, catalog number: FC-25)
14. Ultrasonic sonicator (QSonica, catalog number: XL2000)
15. Glass micropipette puller (Narishige, catalog number: PC-10)
16. Solid phase column (Oasis®MAX 3 cc (60 mg) extraction cartridges, Waters, catalog number 186000367)
17. LCMS8050 (Shimadzu)
18. LC-30AD (Shimadzu)
19. Analytical column (Capcell Pak C18 IF2, 2.1 mm inner diameter x 100 mm, 2 μm, Shiseido, catalog number: 92887)
20. Benchtop centrifuge (CF15RXII, Hitachi)
21. Savant SpeedVac SPD2010 (Thermo Fisher Scientific, catalog number: SPD2010A-220)

Software

1. LabSolution LCMS (Shimadzu)

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Procedure

Part I: Sample collection

A. Collection of interstitial fluid from the hypothalamic area by means of a microdialysis method
   1. Administer pentobarbital solution intraperitoneally to a c57BL/6J mouse (male, at 10-25 weeks old) with a dose of 50 mg/kg body using an insulin syringe (29 gauge of needle x 12.7 mm, 0.5-ml). Tilt the mouse with its head slightly toward the ground, and insert the needle at the lower right quadrant of the abdomen and to a depth in which 1/2 of the needle length is placed in the peritoneal cavity. Use caution to avoid injection of the intestinal organs and urinary bladder.
   2. Attach a guide cannula to a manipulator of the stereotaxic instrument in the vertical plane (Figure 1).

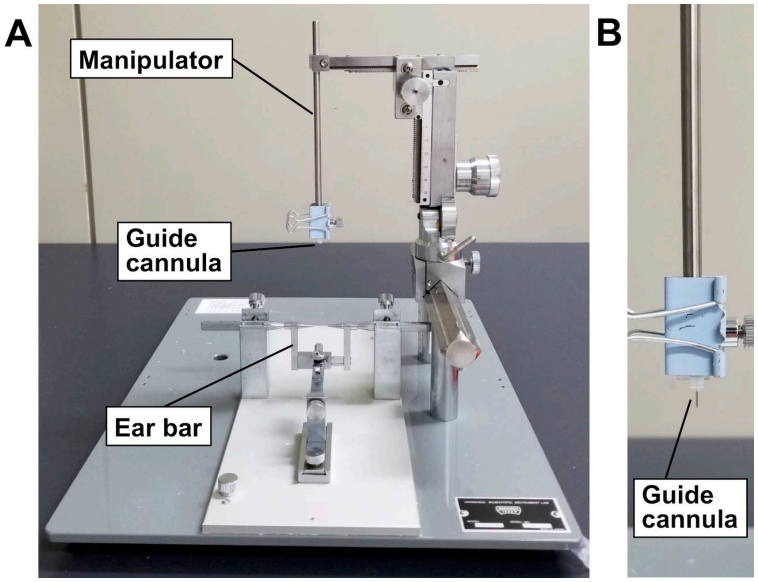

Figure 1. Attachment of a guide cannula on the stereotaxic instrument. A. Stereotaxic instrument SR-5M-HT obtained from Narishige. B. A guide cannula is attached to the tip of a manipulator of the instrument.

   3. Under anesthesia, attach an ear bar to the mouse so that its sinciput is horizontally held.
   4. Before the surgery, wet the mouse head thoroughly with physiological saline to avoid the hairs adhering to scissors and disturbing the operation. Alternatively, you can shave the scalp, although this is not necessary for the following operation.
   5. Remove the scalp at the top of the head around the bregma with scissors in a circle of at most 3 mm in diameter so that the skull becomes visible.
   6. Carefully remove the periosteum and aponeurosis covering the skull using tweezers and scissors until the film disappears.
   7. Wipe the skull with cotton wool, and let the surface dry for about 10 min.

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8. The bregma is difficult to visualize when the surface is wet. Until the bregma appears, make a hole of approximately 1 mm in diameter in the posterior part of the exposed skull using a dental drill to attach an anchor screw to fasten the guide cannula. The screw should be attached near the guide cannula to be set.
9. Move the manipulator just as the tip of the guide cannula comes over the bregma (Figure 2), and then record the vertical and horizontal tick marks of the stereotaxic instrument.

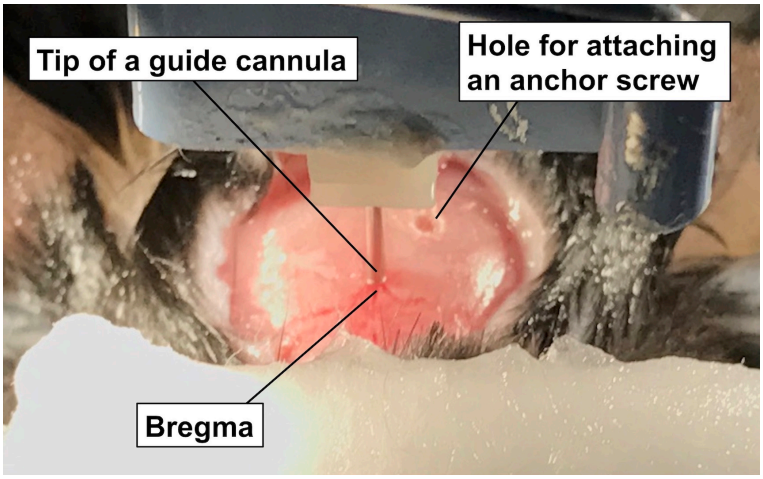

Figure 2. Setting the tip of the guide cannula onto the bregma of the skull. The photo represents the tip of a guide cannula on the bregma.

10. Move the tip of the guide cannula from the bregma, 0.9 mm forward, 0.1 mm right, and mark the position with oil-based ink. To drill a hole at the position, the guide cannula needs to be detached; therefore, it is recommended that the position set is marked with an oil-based ink before the cannula is removed.
11. Use a dental drill to make a hole of about 1 mm in diameter at the marked point.
12. Move the cannula down, touch it to the surface of the brain (at the level of the dura mater), and read the scale of the depth.
13. Move the cannula further down slowly to a depth of 4.0 mm from the surface. This operation usually takes at least 1 min. Wipe the blood off with absorbent cotton when the surgical area bleeds during the operation. If bleeding does not stop, keep cotton where the blood is coming out from until it stops. If the mice bleed by more than a tenth of whole blood, usually estimated as 1/13 of the body weight, discontinuation of the operation should be considered.
14. Fasten the cannula to the skull with instant adhesive temporarily.
15. When the instant adhesive hardens, use dental cement to seal the gap between the guide cannula and the skull.
16. When the cement is set, detach the cannula from the manipulator.
17. Using a precision screwdriver, attach an anchor screw to the hole made in Step A8.

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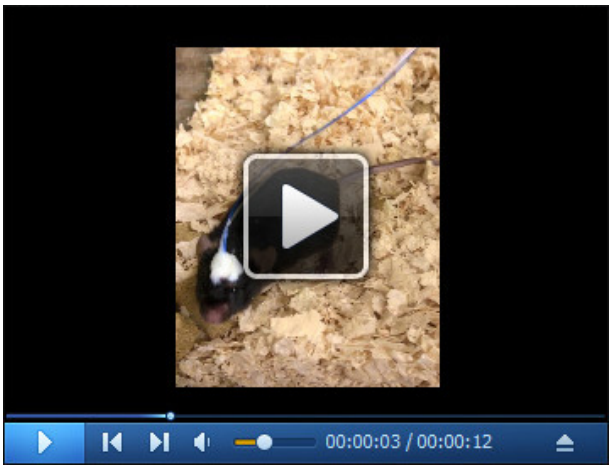
18. Coat the skull and anchor screw firmly with dental cement, and then fasten the cannula with the anchor screw. Please make sure the guide cannula cannot be moved; it may come off or move when the mouse moves freely, resulting in inaccurate measurements.
19. Once the cement is dry, apply woodworking glue to cover the surface of the cement. It usually takes approximately 10 and 5 min for the cement and glue to harden, respectively.
20. When the glue has hardened, release the mouse to its cage.
21. The next day, anesthetize the mice with pentobarbital and use tweezers to pull off the dummy probe that was inserted inside the guide cannula.
22. Insert a fresh microdialysis probe, and fix it with dental cement. Before insertion, rinse the probe according to the manufacturer's instruction. In our case, the tip of the probe was dipped in 70% EtOH solution and infused with KRPB for 5 min at a flow rate of 10 µl/min. Then, the tip of the probe was transferred into KRPB and infused with KRPB for another 5 min at the same flow rate. Probes were usually prepared on the day of use.
23. Keep the mice in their cages for another 5 days to recover from postoperative damage. If the mice lose body weight of 25% or more after the operation, the experiment is not recommended due to severe damage to the brain.
24. Attach the mouse to a free-moving device, transfer it to an acrylic cage, and infuse KRPB at a flow rate of 2 µl/min. The dialysate sample should be collected every hour in 0.6-ml microtubes kept on ice (Video 1).



Video 1. Collection of dialysate from free-moving mice. The mouse can freely move in the acryl cage during KRPB infusion. KRPB flows from a blue tube, and dialysate samples are collected from a colorless tube. (This video was made at Kanazawa Univ. according to guidelines from the Kanazawa Univ. on Animal Care and approved by the Animal Research Ethics Board of Kanazawa University under protocols # AP-143148, AP-153511, AP-163750.)

25. Two hours after initiation of infusion, administer LPS (100 µg/kg body) intraperitoneally to the mouse using an insulin syringe.

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26. Six hours after administration, euthanize the mouse by cervical dislocation, and isolate the brain. Fix the brain overnight at 4 °C in 4% PFA solution.
27. Collect the probe, dip it in 10 ng/ml PGE_2/KRPB solution, and infuse KRPB at 37 °C for 1 h at a flow rate of 2 µl/min. Store the collected dialysate sample at -30 °C until use. We confirmed that PGE_2 in the dialysate is relatively stable by storage at -30 °C for at least one week.
28. Embed the brain tissue fixed with 4% PFA in paraffin, and prepare brain sections attached on a glass slide.
29. To remove paraffin from the sections, dip them in fresh xylene for 4 min in a glass container, and repeat this step 4 times.
30. Dip the sections into absolute EtOH, followed by 95%, 90%, 80%, and 70% EtOH solution for 4 min each to remove xylene from them.
31. Stain nuclei with hematoxylin for 1 min.
32. After washing with tap water for 5 min, to visualize the cytoplasm, stain the sections with eosin for 40 s. Wash off extra eosin with tap water and dip the slides in 80% solution, followed by 95% EtOH and absolute ethanol for 10 s each to make them dehydrated.
33. Dip them into fresh xylene for 3 min, repeat this procedure three times, and then seal the sections using a mounting agent and cover glass.
34. Confirm the insertion position of the probe in the brain with a stereomicroscope (Figure 3). Hematoxylin and eosin (HE) staining makes it easy to observe the place of probe insertion.

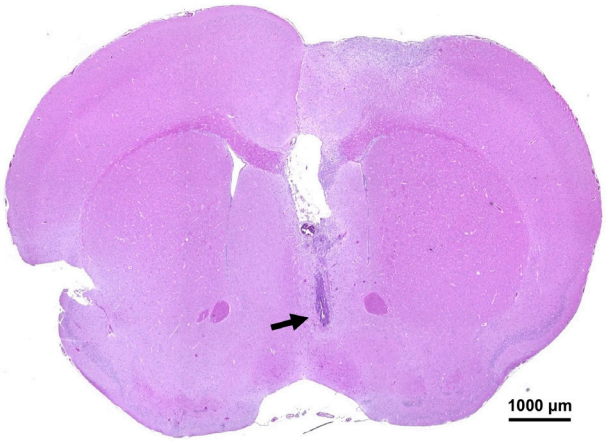

Figure 3. HE staining in the mouse brain after the microdialysis experiment. The histological image of the mouse brain coronal section is shown after microdialysis probe insertion. The arrow indicates the place of an edge of the inserted microdialysis probe. The indicated bar represents 1,000 µm.

B. Collection of hypothalamic tissues
   1. Administer LPS (100 µg/kg body) intraperitoneally to a mouse.
   2. At the designated period of time, euthanize the mouse by cervical dislocation.

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3. Immediately, decapitate the mouse, incise the scalp and avoid damage to the brain, expose the skull, and then isolate the whole brain. The dissection can be performed on tissue papers spread on ice.
4. Place the brain ventral side up on glass plates on ice to keep it relatively cold.
5. Dissect out the diencephalon by coronal cuts just in front of the optic chiasma and behind the mammillary body. Remove the arcuate nuclei by another coronal cut in the middle of the optic tract, from rostral to infundibulum. Please see the previous article (Salehi *et al*., 2012) for detailed procedures.
6. Cut the obtained brain block along the anterior commissure, and collect the ventral side of the anterior commissure as the hypothalamic tissue in a 1.5-ml microtube.
7. Freeze the hypothalamic tissue immediately in liquid nitrogen, and store in a -80 °C deep freezer until use. The frozen samples can be stored for at least one week, under which PGE_2 is relatively stable.

C. Collection of CSF

Note: CSF was collected based on the method reported previously (Liu et al., 2012).

1. Administer LPS (100 μg/kg body) intraperitoneally into a mouse.
2. At the designated period of time, anesthetize the mouse with pentobarbital (*i.p.* administration, 50 mg/kg body).
3. Under anesthesia, attach the mouse head to the stereotaxic instrument with its neck tilted forward as much as possible. Take caution not to break the neck by bending it too much. Further information is provided in the previous report (Liu *et al*., 2012).
4. Cut and remove the skin over the occipital region with scissors, and expose the dura of the cisternal region with tweezers.
5. Attach a glass micropipette horizontally to the manipulator of the stereotaxic instrument.
6. Insert the tip of the micropipette into the dura at a depth of approximately 1 mm, and then suck up the CSF into the micropipette. The volume of the collected CSF should be approximately 10 μl.
7. Transfer the collected CSF to a 1.5-ml microtube.
8. Freeze the CSF immediately in liquid nitrogen, and store in a -80 °C deep freezer until use. The frozen samples can be stored for at least one week, under which PGE_2 is relatively stable.

Part II: PGE_2 extraction

A. Interstitial fluid (The schematic figure is shown as Figure 4)

1. Transfer 100 μl of dialysate sample to a 1.5-ml microtube, and add 200 μl of 1.25 ng/ml d_4-PGE_2 (as an internal standard) and 15 μl of 10% formic acid aqueous solution (v/v). Set the final concentration of d_4-PGE_2 at 0.635 ng/ml by addition of 85 μl of de-ionized water. The

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concentration of 0.635 ng/ml corresponds to 5 ng/ml when the sample is set to 50 µl with the mobile phase, which is high enough not to be affected by daily fluctuation of the measurement.

2. Add 500 µl of ethyl acetate, and shake vigorously for 15 min.
3. Spin the sample by a benchtop centrifuge for 10 min at 21,000 *x g* at 4 °C.
4. Transfer the supernatant to a fresh 1.5-ml microtube, and evaporate the supernatant to dryness under reduced pressure at room temperature for 30 min with a Savant SpeedVac SPD2010.
5. Reconstitute the obtained residues with 50 µl of 25% acetonitrile solution containing 0.075% formic acid (v/v), and dissolve them as much as possible by shaking for at least 10 min.
6. Separate the undissolved materials or debris by centrifuge for 10 min at 21,000 *x g* at 4 °C, and then obtain the clear supernatant.
7. Apply the supernatant to LC-MS/MS to determine C_{isf} of PGE_2.

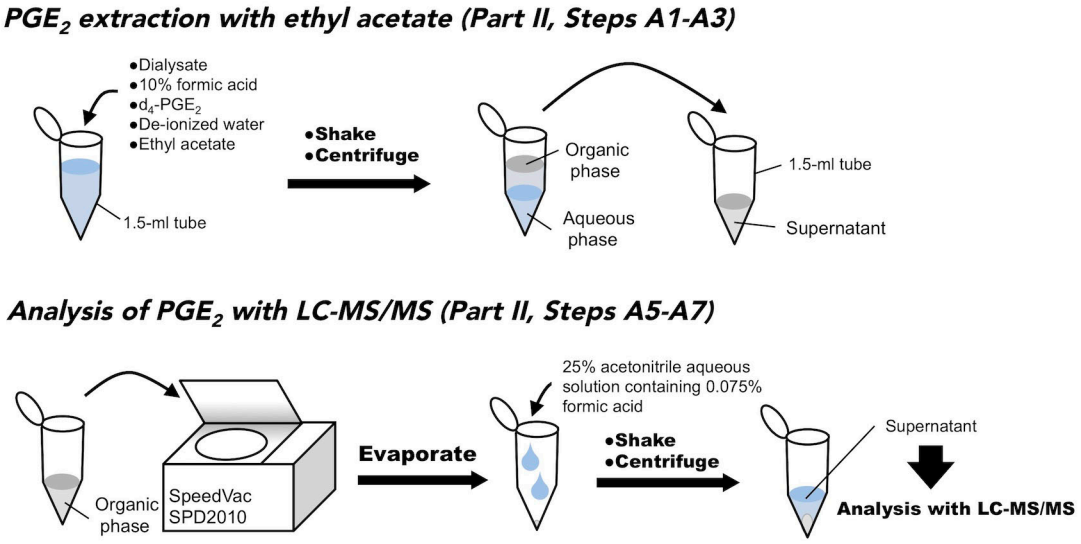

Figure 4. Schematic figure of PGE_2 extraction from the dialysate

B. Hypothalamic tissue (The schematic figure is shown as Figure 5)

1. Prepare a block of hypothalamic tissue, and weigh approximately 10 mg in a 1.5-ml microtube. Then, add 200 µl of PBS, 200 µl of 10% glycerol solution, 5 µl of 10 mg/ml BHT solution, and 10 µl of 100 ng/ml d_4-PGE_2 solution (to be 2.4 ng/ml at the final concentration). Homogenize the tissue, and shear deoxyribonucleotides for 10 s with an ultrasonic sonicator.
2. Transfer the homogenate to a 10-ml glass conical tube, and add 2.485 ml of 25% acetonitrile solution (v/v). Shake vigorously for 5 min.
3. Add 3 ml of absolute acetonitrile to a solid phase column (Oasis® MAX column cartridge), pressurize with the plunger of a 2.5 ml syringe, and pass through the column.
4. Add 3 ml of 25% aqueous acetonitrile solution (v/v) to the cartridge, and pass through the column.
5. Add 3 ml of the prepared homogenate to the cartridge, and pass through the column.

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6. Add 3 ml of 25% aqueous acetonitrile solution (v/v) to the cartridge, and pass through the column.
7. Add 3 ml of absolute acetonitrile to the cartridge, and pass through the column.
8. Add 1.3 ml of 1% formic acid acetonitrile solution (v/v), and collect the eluate into a 2.0-ml microtube.
9. Evaporate the eluate for 1 h at room temperature under reduced pressure using a Savant SpeedVac SPD2010.
10. Add 50 µl of 25% acetonitrile aqueous solution containing 0.075% formic acid (v/v), and shake for 10 min.
11. Separate the supernatant with a benchtop centrifuge for 10 min at 21,000 *x g* at 4 °C.
12. Apply the supernatant to LC-MS/MS to measure C_{hyp} of PGE_2.

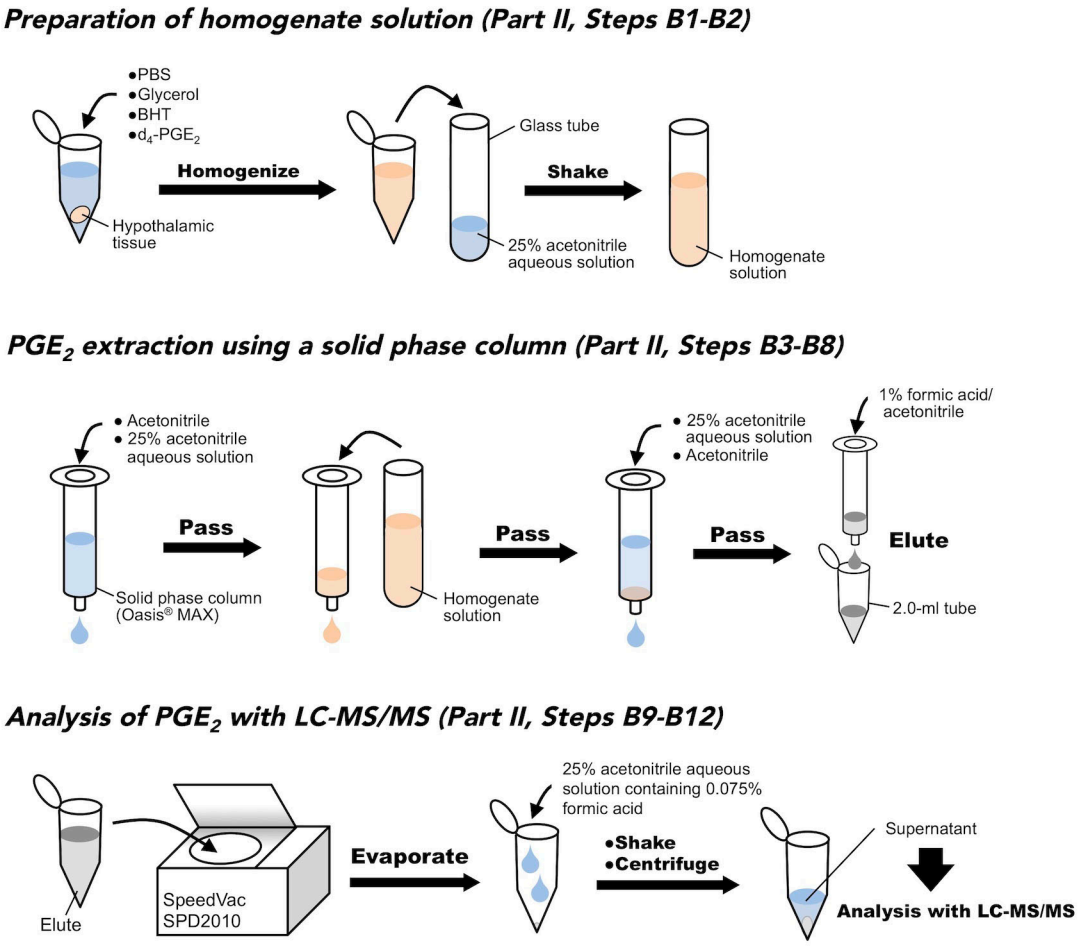

Figure 5. Schematic figure of PGE_2 extraction from hypothalamic tissues

C. CSF

1. Add 200 µl of PBS, 200 µl of 10% aqueous glycerol, 5 µl of 10 mg/ml BHT, and 10 µl of 100 ng/ml d_4-PGE_2 aqueous solution (to be 2.4 ng/ml at the final concentration) to a 10-ml glass conical tube containing 5 µl of CSF.

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2. Add 2.58 ml of 25% acetonitrile solution (v/v), and mix by vigorous shaking for 5 min.
3. Subsequent procedures are as described for the hypothalamic tissue (see Procedure B).

Part III: PGE_2 quantification by means of LC-MS/MS

The sample subjected to the extraction procedure is measured by LC-MS/MS. The measurement conditions are shown below. Molecular weights of PGE_2 and PGD_2 are identical, but they can be separated under the condition described below for LC-MS/MS analysis (Figure 6).

1. Analytical column: Capcell Pak C18 IF2 (2.1 mm inner diameter x 100 mm, 2 μm)
2. Mobile phase:
   a. Aqueous phase: 0.1% formic acid/water (v/v)
   b. Organic phase: acetonitrile
3. Gradient of (b) concentration:
   0-9 min: 15-50%
   9-10 min: 50-90%
   10-11 min: 90%
   11-12 min: 90-15%
   12-13 min: 15%
4. Flow rate: 0.4 ml/min
5. Injection volume: 30 μl
6. Q1, Q3: Mass-to-charges ratios (m/z) are set to determine PGE_2 and PGE_2-d_4. Q1 and Q3 are set to 351.00 and 271.35 for PGE_2 and 355.00 and 275.25 for PGE_2-d_4, respectively.

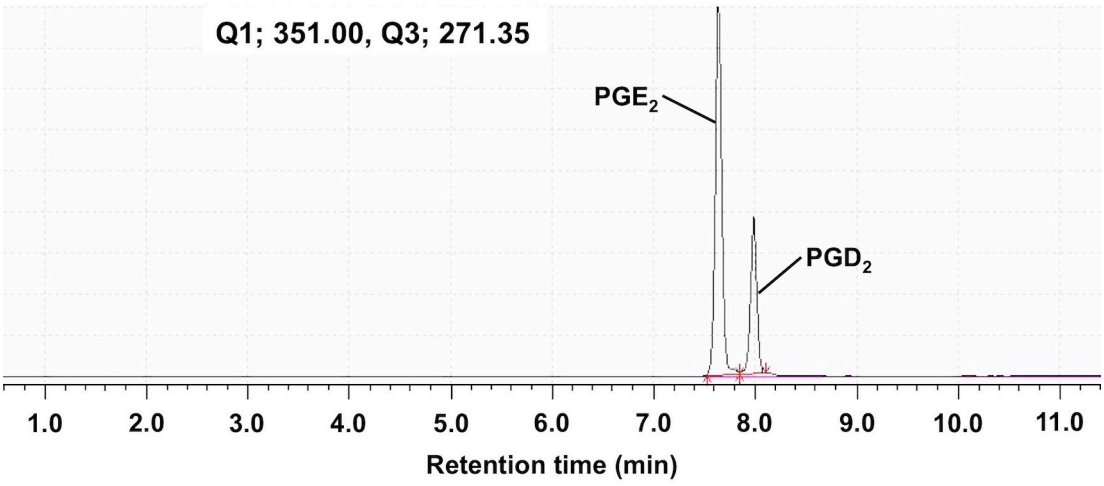

Figure 6. Chromatogram image of PGE_2 and PGD_2 in this protocol. PGE_2 and PGD_2 were detected at the same m/z Q1 (351.00) and Q3 (271.35). PGE_2 and PGD_2 were detected at retention times of 7.6 and 8.0 min, respectively.

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Data analysis

Calculation of interstitial PGE_2 concentration in the mouse hypothalamus:

The *in vitro* recovery rate (RR) of PGE_2 concentration in the microdialysis method was calculated by the following equation,

$$\mathrm{RR}\ (\%) = 100 \times \frac{C_{d,vitro}}{C_r}$$

where, $C_{d,vitro}$ and C_r represent the PGE_2 concentration in the dialysate sample and the PGE_2 concentration dissolved in KRPB, respectively. C_{isf} was estimated by dividing the concentration of PGE_2 in the dialysate sample collected from the mouse ($C_{d,vivo}$) by RR as described below:

$$C_{isf} = \frac{C_{d,vivo}}{RR}$$

Notes

Please refer to the original publication http://www.jneurosci.org/content/38/24/5584 for examples of the expected results of this protocol.

Recipes

1. Pentobarbital solution (6.48 mg/ml)
   a. Weigh 64.8 mg of pentobarbital sodium, and dissolve it in physiological saline
   b. Add sterilized physiological saline up to 10 ml
   c. Store the solution at room temperature under shading
2. 10% glycerol solution (v/v)
   Mix 100 µl of glycerol with 900 µl of de-ionized water, and store the solution at room temperature
3. Krebs-Ringer phosphate buffer (KRPB, total volume 100 ml; pH 7.4)
   a. Weigh 700.8, 17.9, 14.4, 10.8, 50.1, and 13.3 mg of NaCl powder (at a final concentration of 120 mM), KCl (2.4 mM), $MgSO_4$ (1.2 mM), NaH_2PO_4 (0.9 mM), $Na_2HPO_4 \cdot 12H_2O$ (1.4 mM), and $CaCl_2$ (1.2 mM), respectively
   b. Dissolve the powders in de-ionized water
   c. Adjust the pH to 7.4 with 5 N HCl
   d. Fill them with water up to 100 ml
   e. Filter the solution using a 0.2-µm filter to sterilize it
   f. Store the solution at room temperature until use
4. 0.5 M PB (pH 7.4)
   a. Weigh 6 g of NaH_2PO_4 powder, and dissolve it in 100 ml of de-ionized water (0.5 M)

b. Weigh 53.7 g of $Na_2HPO_4 \cdot 12H_2O$ powder, and dissolve it in 300 ml of de-ionized water (0.5 M)
c. Adjust the pH to 7.4 by adding 0.5 M NaH_2PO_4 solution to 0.5 M Na_2HPO_4
d. Store the solution at room temperature

5. 4% PFA solution (w/v, total volume of 200 ml)
   a. Boil 150 ml of de-ionized water using a microwave oven
   b. Add 120 µl of 5 N NaOH
   c. Add 8 g of PFA powder
   d. After PFA is dissolved, add 40 ml of 0.5 M PB
   e. Fill with de-ionized water to 200 ml
   f. Store the solution at 4 °C until use

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Competing interests

The authors have no conflicts of interest to disclose.

Ethics

All animal experiments were performed in accordance with the requirements of the Kanazawa University Institutional Animal Care and Use Committee (permit numbers: AP-143148, AP-153511, and AP-163750).

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