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Mass spectrometric determination of prostanoids in rat hypothalamic paraventricular nucleus microdialysates

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ABSTRACT

The hypothalamic paraventricular nucleus (PVN) is one of the most important autonomic control centers in the brain. Several kinds of prostanoids, such as prostaglandin (PG) E_2, are considered to act in the PVN as mediators of autonomic responses. In the present study, we used liquid chromatography ion trap tandem mass spectrometry (LC–ITMS^n) to simultaneously quantify four prostanoids, thromboxane (Tx) B_2, PGE_2, PGD_2 and 15-deoxy-$\Delta^{12,14}$ (15d)-PGJ_2 in PVN microdialysates from urethane-anesthetized rats. The quantification limits were estimated to be 0.05 ng/mL for TxB_2, 0.025 ng/mL for PGE_2, 0.1 ng/mL for PGD_2, and 0.5 ng/mL for 15d-PGJ_2. The RSD% obtained from all prostanoids was <15%, indicating an acceptable level of reproducibility. LC–ITMS^n analysis of rat PVN microdialysates revealed that TxA_2 may play an important role in adrenomedullary outflow evoked by centrally administered *N*-methyl-D-aspartate, corticotrophin-releasing factor and glucagon-like peptide-1. This is the first study to use LC–ITMS^n to analyze prostanoid levels in rat PVN microdialysates. This LC–ITMS^n method will be useful for investigating the potential involvement of prostanoids in brain function.

1. Introduction

Prostanoids are a naturally occurring subclass of eicosanoids that are generated from arachidonic acid through oxidative pathways and act as lipid mediators. These cyclooxygenase metabolites include prostaglandins (PG) and thromboxanes (Tx), which are released in response to a variety of physiological and pathological stimuli in almost all organs, including the brain (Milatovic et al., 2011). In the brain, several observations raised the possibility that endogenously synthesized prostanoids might act as neuro-mediators in the central regulation of a variety of functions, such as autonomic responses, activation of the hypothalamic–pituitary–adrenal axis, social behavior, and learning and memory formation (Furuyashiki and Narumiya, 2011). Since prostanoids play major roles in many physiological processes in both health and disease (Miller, 2006), it is very important to investigate the prostanoid-dependent mechanisms.

We previously demonstrated the involvement of TxA_2 in brain function, particularly in the hypothalamic paraventricular nucleus (PVN) during *N*-methyl-D-aspartate (NMDA)-induced adrenomedullary outflow (Okada et al., 2000; Okada and Yamaguchi, 2010). In that study we measured TxB_2, a stable metabolite of TxA_2, using microdialysis and enzyme-linked immune-sorbent assay (ELISA). By pretreatment with cyclooxygenase inhibitor and TxA_2 synthase inhibitor, we also pharmacologically demonstrated that TxA_2 in the brain is involved in the centrally administered corticotropin-releasing factor (CRF)- and glucagon-like peptide-1 (GLP-1)-induced adrenomedullary outflow (Okada et al., 2003a; Arai et al., 2008). However, in these studies, we did not measure endogenously synthesized TxA_2 in the PVN.

In vivo microdialysis, which is one of the most useful techniques for neuroscience study, enables us to monitor the release of prostanoids in the brain (Anderzhanova and Wotjak, 2013). Quantification of the prostanoids in the microdialysates has been mainly carried out by immunological assays (Patel et al., 1992; Lazarewicz and Salinska, 1995; Okada et al., 2000). However, there are application limits to conventional immunological methods such as ELISA and radioimmunoassay (RIA). First, these antibody-based methods cannot simultaneously quantify different compounds in the same samples. Second, they have inherent drawbacks in that they might have undesirable cross-reactions in complex samples.

Liquid chromatography–tandem mass spectrometry (LC–MS/MS) has been widely used in recent bioanalytical work, since it is a powerful analytical technique that combines the resolving power of liquid chromatography with the detection specificity of mass spectrometry. LC–MS/MS analysis has been applied to profile arachidonic acid metabolites including PGE_2 and TxB_2 in various biological samples such as cell cultures, brain tissue, synovial fluid and human serum (Bell-Parikh et al., 2003; Yue et al., 2007; Ferreiro-Vera et al., 2011; Furugen et al., 2011). In addition, Schmidt et al. (2005) described LC–MS/MS analysis of prostanoids in microdialysis samples.

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The PVN is one of the most important autonomic control centers in the brain, wherein several kinds of prostanoids such as TxA_2 and PGE_2 are considered to act as mediators of autonomic responses (Furuyashiki and Narumiya, 2011). However, endogenously synthesized prostanoids in the PVN were not measured in the previous studies, showing that the effects of prostanoids in the PVN on adrenomedullary outflow remained unclear. The objective of this research was to investigate whether centrally administered NMDA, CRF and GLP-1 can induce production of four prostanoids, TxB_2, PGE_2, PGD_2 and 15-deoxy-$\Delta^{12,14}$ (15d)-PGJ_2 after optimization of liquid chromatography–ion trap tandem mass spectrometry (LC–$ITMS^n$) conditions for quantification of four prostanoids.

2. Materials and methods

2.1. Reagents

NMDA was purchased from Tocris Bioscience (Bristol, UK). CRF and GLP-1 were purchased from Peptide Institute (Osaka, Japan). Methyl acetate and ethyl acetate were purchased from Kanto Chemical (Tokyo, Japan). LC–MS grade formic acid, HPLC grade acetonitrile, methanol and distilled water were purchased from Wako Pure Chemicals (Osaka, Japan). Urethane was obtained from Sigma-Aldrich (Tokyo, Japan). All prostanoids used as standards were obtained from Cayman Chemical Company (Ann Arbor, MI, USA).

2.2. Preparation of standards

Standard stock solutions (1 mg/mL) of individual TxB_2, PGE_2, PGD_2 and 15d-PGJ_2 were prepared in methyl acetate. The internal standard stock solutions (0.1 mg/mL) of individual TxB_2-d_4, PGE_2-d_4, PGD_2-d_4 and 15d-PGJ_2-d_4 were also prepared in methyl acetate. Serial dilutions of standard stock solutions with methyl acetate were performed for method validation.

2.3. Subjects and surgery

Fifteen male Wistar rats (360–400 g, Japan SLC, Hamamatsu, Japan) were used. Rats were maintained in a room under a constant day–night cycle for more than 2 weeks and had free access to food and water. Rats were anesthetized with urethane (1.0 g/kg body weight) intraperitoneal injection. The femoral vein and artery were cannulated for infusion of saline (1.2 mL/h) and blood sample collection, respectively. Plasma levels of noradrenaline and adrenaline were determined from the collected blood samples as described previously (Okada and Yamaguchi, 2010). The animal was then placed in a stereotaxic apparatus. A stainless guide cannula held on the tip of an L-shaped stainless steel cannula was implanted stereotaxically just above the right PVN, as previously reported (Okada et al., 2000). The stereotaxic coordinates of the implantation sites were placed 1.8 mm posterior to the bregma, 0.3 mm lateral to midline, and 7.0 mm ventral to dura, according to the rat brain atlas of Paxinos and Watson (1997). A microdialysis probe (0.22 mm outer diameter, 1 mm membrane length; Eicom, Kyoto, Japan) was then inserted into the guide cannula. The PVN was perfused with sterile Ringer's solution (147 mM NaCl, 4 mM KCl and 2.3 mM $CaCl_2$) at a flow rate of 2 μL/min using a microinfusion pump (EP-60, Eicom). Perfusion of sterile Ringer's solution for equilibration was continued for at least 3 h after probe insertion, after which the dialysates were collected at 20 min intervals. NMDA dissolved in sterile Ringer's solution was applied into the PVN through a microdialysis probe for 30 min. CRF and GLP-1 were dissolved in sterile Ringer's solution and injected slowly into the right cerebral ventricle in a volume of 5 μL with a 10 μL Hamilton syringe. Collected samples were frozen at −80 °C until analysis.

All experiments were conducted in compliance with the guidelines for the care and use of laboratory animals approved by the Aichi Medical University Graduate School of Medicine, Nagakute, Aichi, Japan.

2.4. Extraction of PVN microdialysate prostanoids for LC–$ITMS^n$ analysis

Forty microliters of microdialysates were spiked with 10 μL of the internal standard mixture containing 2.5 ng/mL TxB_2-d_4, 1 ng/mL PGE_2-d_4, 5 ng/mL PGD_2-d_4 and 10 ng/mL 15d-PGJ_2-d_4 for calibration. Ethyl acetate (1 mL) was then added to the microdialysate. The solution was shaken for 30 s and centrifuged for 2 min at 10,000 ×*g*. The ethyl acetate phase containing the prostanoids was collected and dried with a centrifugal vacuum system. The residue was dissolved in 50 μL 25% methanol containing 0.1% formic acid, and then filtered with Millex®-HV (0.45 μm, Millipore Co., MA, USA). A 20 μL aliquot of the resulting sample was subjected to LC–$ITMS^n$ analysis. To measure prostanoid concentration of the microdialysates, the peak area ratio relative to the internal standard was calculated and determined from the corresponding calibration curve.

2.5. LC–$ITMS^n$ conditions

The LC separation was performed at room temperature with an ACCELA HPLC system (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a Synergi Hydro-RP column (150 × 2.0 mm i.d., 4.0 μm, Phenomenex, CA, USA). The mobile phases were 40% acetonitrile (A), and 100% methanol (B). The LC elution gradient employed was: 0 to 2.0 min 100% eluent A, 2.0 to 4.0 min eluent A decreased linearly to 50%, and then to 100% eluent B within 1.0 min. 100% Eluent B was held for 4.2 min. The eluent was then shifted back to 100% A, which was held for 3.7 min to equilibrate the column for the next sample. The flow rate was 0.2 mL/min.

Mass spectrometric analysis was accomplished using an LTQ Velos (Thermo Fisher Scientific) equipped with a heated electrospray ionization source. The negative ion mode was chosen for prostanoid detection. The source voltage was set at 3 kV, while the capillary temperature was set at 350 °C and the tube lens offset was set at 15 V. The sheath gas flow rate was 35 (arbitrary units) and the auxiliary gas flow rate was 10 (arbitrary units). Full scan experiments were performed in the range of *m/z* 50–1000. Total microscans were set at 1, and the maximum injection time was set at 10 ms. Subsequent MS/MS experiments were performed in the range of *m/z* 70–600. The maximum injection time was set at 100 ms.

2.6. Method validation

Prostanoid solutions for calibration were prepared daily from stock solutions by mixing and diluting with 25% methanol containing 0.1% formic acid. The calibration curve range for each prostanoid was adjusted according to its LC–MS responses (Table 1). Recovery was estimated by spiking 10 μL of known amounts of prostanoids at low (100 pg/mL for TxB_2, 40 pg/mL for PGE_2, 200 pg/mL for PGD_2 and 400 pg/mL for 15d-PGJ_2), intermediate (200 pg/mL for TxB_2, 80 pg/mL for PGE_2, 400 pg/mL for PGD_2 and 800 pg/mL for 15d-PGJ_2), and high (500 pg/mL for TxB_2, 200 pg/mL for PGE_2, 1000 pg/mL for PGD_2 and 2000 pg/mL for 15d-PGJ_2) concentrations and 10 μL of the internal standard mixture into 40 μL sterile Ringer's solution. Intra-day precision and accuracy were determined by measuring six experiments during one day. Inter-day precision and accuracy were determined by measuring on six different days.

Table 1
Linearities and limits of quantification for the analyzed standard prostanoids.

Prostanoids	Slope[a]	Intercept[a]	Linear range (ng/mL)	r2	LOQ (ng/mL)
TxB_2	2.46 ± 0.270	0.002 ± 0.027	0.05–1	0.999	0.05
PGE_2	7.43 ± 0.774	−0.031 ± 0.027	0.02–0.4	0.998	0.025
PGD_2	0.928 ± 0.165	−0.031 ± 0.020	0.1–2	0.999	0.1
15d-PGJ_2	0.926 ± 0.125	−0.081 ± 0.054	0.2–4	0.997	0.5

[a] Values are expressed as the mean ± S.D. (n = 5).