Neuroscience 218 (2012) 295–304

IMMUNOHISTOCHEMICAL DEMONSTRATION OF INCREASED PROSTAGLANDIN $F_{2\alpha}$ LEVELS IN THE RAT HIPPOCAMPUS FOLLOWING KAINIC ACID-INDUCED SEIZURES

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Abstract—Prostaglandin (PG) $F_{2\alpha}$ is one of the major prostanoids biosynthesized by cyclooxygenases (COXs) from arachidonic acid. Although it has been reported that there is a selective surge in $PGF_{2\alpha}$ production in the hippocampus during kainic acid (KA)-induced seizure activity, the precise intra-hippocampal distribution of $PGF_{2\alpha}$ has not been elucidated due to the paucity of effective histological techniques for detecting PGs in tissues. We investigated the tissue distribution of $PGF_{2\alpha}$ in the rat hippocampus 30 min after KA injection by developing fixation and immunohistological-staining methods. To detect $PGF_{2\alpha}$ directly on histological sections, we used systemic perfusion fixation with water-soluble carbodiimide fixative, followed by immersion of the brains in Zamboni's fixative. We then performed immunofluorescence staining with anti-$PGF_{2\alpha}$ antibody, with negative control experiments used to confirm the staining specificity. Definitive immunolabeling for $PGF_{2\alpha}$ was evident most markedly in pyramidal cells of the hippocampal cornu Ammonis (CA) 3 sector and neurons of the hilus in KA-treated rats. Immunolabeling for $PGF_{2\alpha}$ was also evident in granule cells of the dentate gyrus. Double immunfluorescence staining revealed that $PGF_{2\alpha}$-immunopositive neurons expressed cytosolic phospholipases A_2, COX-2, and FP receptor. These results suggest that the major source of $PGF_{2\alpha}$ production immediately after KA injection was neurons of the hippocampal CA3 sector, hilus and dentate gyrus. These neurons exert $PGF_{2\alpha}$-mediated functions via FP receptors in an autocrine/paracrine manner and may play pathophysiological roles in the acute phase (30 min) of excitotoxicity.

Key words: prostaglandin $F_{2\alpha}$, water-soluble carbodiimide, kainic acid, excitotoxicity, hippocampus.

INTRODUCTION

Prostaglandin (PG) $F_{2\alpha}$ is one of the major prostanoids formed from the metabolism of arachidonic acid by cyclooxygenase (COX). $PGF_{2\alpha}$ exerts its physiological effects through binding to the FP receptor, a G-protein-coupled receptor that signals G_q-mediated increases in intracellular calcium (Smith et al., 1991; Narumiya, 2009; Woodward et al., 2011). $PGF_{2\alpha}$ exerts biological functions in the central nervous system (CNS), such as the induction of postsynaptic depolarization in cerebellar Purkinje cell dendrites (Kimura et al., 1985), induction of *c-fos* mRNA in dentate gyrus (DG) neurons (Lerea et al., 1997), and spinal cord-associated allodynia (Kunori et al., 2009).

A rapid and large increase in the amount of $PGF_{2\alpha}$ has been demonstrated by biochemical analyses in human neuropathological conditions (Egg et al., 1980; Ellis et al., 1981) and experimental animal models for excitotoxicity (Crockard et al., 1982; Baran et al., 1987; Naffah-Mazzacoratti et al., 1995; Yoshikawa et al., 2006). Although $PGF_{2\alpha}$ is the most abundant PG in the intact rat brain, its physiological functions remain undetermined. $PGF_{2\alpha}$ may play a role in certain pathological conditions of the brain (Yoshikawa et al., 2006). It has been reported that $PGF_{2\alpha}$ is produced by vascular cells, cytoplasm of hippocampal neurons, and interfascicular oligodendrocytes in rat brain following global ischemia (Ogawa et al., 1987). PGE_2, PGD_2, and $PGF_{2\alpha}$ exacerbate hypoxic neuronal injury in neuron-enriched primary cultures (Li et al., 2008). In these previous studies, how-

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Abbreviations: ANOVA, analysis of variance; AOI, areas of interest; BSA, bovine serum albumin; CA, cornu Ammonis; CNPase, 2′,3′-cyclic nucleotide 3′-phosphodiesterase; CNS, central nervous system; COX, cyclooxygenase; $cPLA_2$, cytosolic phospholipase A_2; DG, dentate gyrus; DMSO, dimethylsulfoxide; ELISA, enzyme-linked immunosorbent assays; FP receptor, Prostaglandin $F_{2\alpha}$ receptor; GFAP, glial fibrillary acidic protein; Iba1, ionized calcium binding adaptor molecule 1; KA, kainic acid; LDG, lower blade of dentate gyrus; NGS, normal goat serum; NMDA, N-methyl-D-aspartate; PBS, phosphate-buffered saline; PBS-T, phosphate-buffered saline containing 0.05% Tween 20; PG, prostaglandin; Prostamide, prostaglandin ethanolamide; UDG, upper blade of dentate gyrus; WSC, water-soluble carbodiimide.

0306-4522/12 $36.00
http://dx.doi.org/10.1016/j.neuroscience.2012.05.013

ever, the authors were not able to determine the precise distribution of $PGF_{2\alpha}$ or to quantify relative changes in the amount of tissue $PGF_{2\alpha}$ within the hippocampus following experimentally induced injury. There are no previous reports showing the colocalization and/or relationship of cellular $PGF_{2\alpha}$ with PG-synthesizing enzymes or receptors.

In the present study, we investigated the intra-hippocampal distribution of $PGF_{2\alpha}$ during the acute phase (30 min) of kainic acid (KA)-induced excitotoxic injury and used a semi-quantitative histological-staining method to determine changes in the amount of $PGF_{2\alpha}$. Our histological method for detecting PG in tissues allowed double immunofluorescence staining to show the colocalization of cellular $PGF_{2\alpha}$ with PG-synthesizing enzymes, as well as its relationship with the FP receptor. We demonstrate that KA-induced immediate production of $PGF_{2\alpha}$ in the hippocampus is most prominent in neurons of the cornu Ammonis (CA) 3 sector and hilus, and that $PGF_{2\alpha}$ may exert its pathophysiological effects in an autocrine/paracrine manner.

EXPERIMENTAL PROCEDURES

Reagents

$PGF_{2\alpha}$, $PGF_{1\alpha}$, $PGF_{3\alpha}$, PGD_2, PGE_2, 6-keto $PGF_{1\alpha}$, 9α,11β-PGF_2, 8-iso $PGF_{2\alpha}$, and prostaglandin ethanolamide (prostamide) $F_{2\alpha}$ were purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). 1-Ethyl-3-(dimethylaminopropyl) carbodiimide chloride (water-soluble carbodiimide, WSC) was purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Fatty acid free-bovine serum albumin (BSA) was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). N-hydroxysuccinimide, indomethacin, and KA were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Conjugation of PGs with BSA

$PGF_{2\alpha}$–BSA was prepared according to a previously described procedure (Auletta et al., 1974), with modification. $PGF_{2\alpha}$ (5 mg) dissolved in 0.5 ml dimethylformamide and BSA (20 mg) dissolved in 1 ml distilled water were mixed in an 8-ml glass vessel and allowed to conjugate by addition of WSC (20 mg) dissolved in 0.5 ml distilled water at 4 °C with shaking for 20–24 h. After conjugation, the reaction mixture was dialyzed overnight against phosphate-buffered saline (PBS) at 4 °C. The $PGF_{2\alpha}$–BSA solution was diluted with PBS to a final volume of 10 ml and stored at −20 °C. Other PG-related substances ($PGF_{1\alpha}$, $PGF_{3\alpha}$, PGD_2, PGE_2, 6-keto $PGF_{1\alpha}$, 9α,11β-PGF_2, 8-iso $PGF_{2\alpha}$, and prostamide $F_{2\alpha}$) were conjugated with BSA in the same manner.

Preparation of a $PGF_{2\alpha}$ affinity column

An affinity column for $PGF_{2\alpha}$ was prepared with an NH_2-activated cellulose-bead column. The NH_2-activated cellulose-bead column was prepared from Cellufine GCL-2000 (Chisso Corp., Tokyo, Japan) according to previously described methods (Matsumoto et al., 1979; Yamamoto et al., 2007). $PGF_{2\alpha}$ (1 mg dissolved in 0.5 ml dimethylformamide) and WSC (5 mg dissolved in 0.5 ml distilled water) were mixed and applied to an NH_2-activated cellulose-bead column. The conjugation was allowed to proceed at 4 °C with shaking for 20–24 h. After conjugation, the $PGF_{2\alpha}$-column was washed with 10-ml 0.1 M sodium acetate buffer (pH 4.0) containing 0.5 M NaCl and equilibrated in PBS.

Preparation of anti-$PGF_{2\alpha}$ polyclonal antibody

Male Japanese White Rabbits (2.5 kg body weight) were purchased from Japan SLC, Inc. (Hamamatsu, Japan). All animals were handled in accordance with the Guide for Animal Experiments at Aichi Human Service Center and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised in 1996. An antiserum against $PGF_{2\alpha}$ was raised according to a previously described procedure (Aoki et al., 1987). The antiserum was precipitated with ammonium sulfate, subsequently resuspended in binding buffer (0.01 M sodium phosphate, 0.15 M NaCl at pH 7.0), and the crude IgG was passed three times through the $PGF_{2\alpha}$ column. The $PGF_{2\alpha}$ column was then washed with 10-ml binding buffer and 10-ml washing buffer (0.01 M sodium phosphate, 0.5 M NaCl at pH 7.0). Antibodies were eluted in 1-ml fractions with ActiSep elution medium (Sterogene Bioseparation, Inc., Arcadia, CA, USA) and the fractions were exchanged into PBS using a PD-10 column (Amersham Biosciences, Piscataway, NJ, USA). Protein containing fractions (measured by absorbance at 280 nm) were pooled, exchanged in a preservation buffer (10% normal goat serum (NGS) containing 0.02% NaN_3 in PBS), and stored at −20 °C until use.

Competitive enzyme-linked immunosorbent assays

Specificity of the anti-$PGF_{2\alpha}$ antibody was confirmed by competitive enzyme-linked immunosorbent assays (ELISA) according to a previously described procedure with some modification (Hayashi et al., 1987; Morikawa et al., 2007). Briefly, 96 well-microtest plates (Nunc-Immuno-Plate Maxisorp F96, Nalge Nunc, Roskilde, Denmark) were incubated with diluted antigen solutions (1.6 ng/ml) at 4 °C overnight. After rinsing with PBS containing 0.05% Tween 20 (PBS-T), plates were incubated with blocking buffer (5% skim milk in PBS-T) for 2 h at room temperature. Plates were then incubated with the mixtures of anti-$PGF_{2\alpha}$ antibody (4.5 ng/ml) and serial dilution (10^{-6} to 10^{1} μg/ml) of PG-related substances conjugated with BSA in blocking buffer at 4 °C overnight. After washing with PBS-T, plates were then incubated with horseradish peroxidase-conjugated anti-rabbit IgG (Medical & Biological Laboratories Co. Ltd., Tokyo, Japan) diluted 1:2500 in blocking buffer at room temperature for 3 h. The plates were then rinsed with PBS-T, and incubated with 0.1 M sodium citrate buffer (pH 4.0) containing 0.3 mg/ml of 2,2′-azinobis(3-ethylbenzothiazolin-6-sulfonic acid) diammonium salt (Wako, Osaka, Japan) and 0.003% H_2O_2 at room temperature for 20 min. Absorbance at 405 nm was immediately measured using a Multiskan JX plate reader (Thermo BioAnalysis Co., Tokyo, Japan). Cross reactivity was calculated as previously described (Hayashi et al., 1987).

Treatments of rats and tissue preparations

KA-induced seizure models were conducted as in our previous studies (Yoshikawa et al., 2006; Furukawa et al., 2011). Briefly, 3-week-old male Wistar rats were purchased from Japan SLC, Inc. (Hamamatsu, Japan) and divided into three groups: (1) Rats receiving 10 mg/kg indomethacin dissolved in dimethylsulfoxide (DMSO, 5 mg/ml) by an intraperitoneal (i.p.) injection, followed by an i.p. injection of 20 mg/kg KA (0.5 mg/ml saline) with a 2-h interval between injections [IND + KA group]. (2) Rats receiving volume-matched DMSO and 20 mg/kg KA with a 2-h interval [KA group]. (3) Vehicle-treated control rats receiving volume-matched DMSO and saline with a 2-h interval [Ctrl group]. Thirty minutes after the second injection, rats were deeply anesthetized with