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Acidic pH increases cGMP accumulation through the OGR1/phospholipase C/Ca^{2+}/neuronal NOS pathway in N1E-115 neuronal cells

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ABSTRACT

Neuronal NO synthase (nNOS)-mediated cGMP accumulation has been shown to affect a variety of neuronal cell activities, regardless of whether they are detrimental or beneficial, depending on the amount of their levels, under the physiological and pathological situations. In the present study, we examined the role of proton-sensing G protein-coupled receptors (GPCRs), which have been identified as new pH sensors, in the acidic pH-induced nNOS/cGMP activity in N1E-115 neuronal cells. In this cell line, ovarian cancer G protein-coupled receptor 1 (OGR1) and G protein-coupled receptor 4 (GPR4) mRNAs are expressed. An extracellular acidic pH increased cGMP accumulation, which was inhibited by nNOS-specific inhibitors. Acidic pH also activated phospholipase C/Ca^{2+} pathways and Akt-induced phosphorylation of nNOS at S1412, both of which have been shown to be critical regulatory mechanisms for nNOS activation. The acidic pH-induced activation of the phospholipase C/Ca^{2+} pathway, but not Akt/nNOS phosphorylation, was inhibited by small interfering RNA specific to OGR1 and YM-254890, an inhibitor of $G_{q/11}$ proteins, in association with the inhibition of cGMP accumulation. Moreover cGMP accumulation was inhibited by 2-aminoethoxydiphenyl borate, an inhibitor of inositol 1,4,5-trisphosphate channel; however, it was not by wortmannin, a phosphatidylinositol 3-kinase inhibitor, which inhibited Akt/nNOS phosphorylation. In conclusion, acidic pH stimulates cGMP accumulation preferentially through the OGR1/$G_{q/11}$ proteins/phospholipase C/Ca^{2+}/nNOS in N1E-115 neuronal cells. Akt-mediated phosphorylation of nNOS, however, does not appreciably contribute to the acidification-induced accumulation of cGMP.

1. Introduction

Extracellular acidic pH has been shown to take place with ischemia and neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease, in which lactate and by-products of glycolysis are accumulated [1]. In the ischemic situation, for example, a lack of blood supply causes hypoxia and the inhibition of aerobic respiration and, thereby, increases lactic acid production through glycolysis, causing a decrease in pH to 6.1–6.8 [2,3]. Acidic pH is thought to influence mitochondrial function and finally leads to neuronal cell death [4]. Recent studies have suggested that acid-sensing ion channels (ASICs) mediate neuronal cell death in the severe extracellular acidic pH of 6.0 to 5.0, which was associated with an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) [1]. Such a high proton concentration also stimulates sensory neurons, resulting in nociception through ASICs and transient receptor potential V1 (TRPV1) channels [5].

In addition to the ionotropic channels, which are stimulated by a severe acidic pH of 6.0 to 4.0, depending on their subtypes, recent studies have shown that OGR1 family G-protein coupled receptors (GPCRs), including ovarian cancer G protein-coupled receptor 1 (OGR1), G protein-coupled receptor 4 (GPR4), G2 accumulation (G2A), and T cell death-associated gene 8 (TDAG8), sense moderate extracellular pH of 8.0 to 6.0, resulting in the stimulation of intracellular signaling pathways [6–8]. For example, OGR1 is coupled to $G_{q/11}$ proteins and phospholipase C/Ca^{2+} signaling pathways, and TDAG8 and GPR4 are coupled to G_s proteins and adenylyl cyclase/cAMP pathways [6,8,9]. A moderate pH of higher than 6.0 has also shown to modulate a variety of neuronal cell activities [10], including neurotransmitter release [11] and prevention of neuronal cell death [12,13]. However, the mechanisms underlying acidic pH-induced actions are poorly understood.

As for the beneficial neuronal cell activities, the roles of nNOS/NO/cGMP pathways have been extensively examined in neuronal cells: moderate NO production through nNOS mediates neuronal cell survival

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[14], neurite extension [15–17], neuronal glucose homeostasis [18], memory [19], and so on, although high NO production causes neuronal cell death and pathological situations [20–22]. Extracellular acidic pH effects on cGMP accumulation have not been previously reported, even for cell types other than neuronal cells. In the present study, therefore, we examined whether acidic pH regulates nNOS/cGMP activity and, if so, the roles of proton-sensing GPCRs and their signaling pathways, focusing especially on Akt and Ca^{2+} signaling pathways, both of which have been known to be critical for neuronal cell activities, including nNOS regulation [22].

2. Materials and methods

2.1. Materials

Lipopolysaccharide (LPS), wortmannin, and anti-β-actin antibody were purchased from Sigma-Aldrich (St. Louis, MO); N^{ω}-propyl-L-arginine (N-PLA), (4S)-*N*-(4-Amino-5[aminoethyl]aminopentyl)-*N*′-nitroguanidine, TFA (nNOS inhibitor I) were from Calbiochem-Novabiochem Co. (San Diego, CA); [*myo*-2-^{3}H] inositol (23.0 Ci/mmol) was from American Radiolabeled Chemicals, Inc. (St. Louis, MO); 2-aminoethoxydiphenyl borate (2-APB) was from Enzo Life Sciences, Inc. (Farmingdale, NY); anti-phosphorylated S1417 nNOS (PAI-032) antibody (for mouse S1412) and BCA Protein Assay were from Thermo Scientific (Rockford, IL); anti-nNOS (#4234), anti-eNOS (#9572), anti-phosphorylated Akt Ser473 (#9271) and anti-Akt (#9272) antibodies were from Cell Signaling Technology (Beverly, MA); anti-iNOS antibody (SA-200) was from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA); Cyclic GMP EIA Kit was from Cayman Chemical Co. (Ann Arbor, MI); Fura-2/acetoxymethylester (Fura-2/AM) was from Dojindo (Tokyo, Japan); SUMITOMO Nerve-Cell Culture System was from Sumitomo Bakelite Co., Ltd. (Tokyo, Japan); small interfering RNA (siRNA) specific for mouse OGR1 (si-OGR1, L-056512-00) and non-silencing RNAs (si-NS, D-001206-13) were from Dharmacon, Inc. (Lafayette, CO); Lipofectamine RNAiMAX Reagent was from Invitrogen (Carlsbad, CA) and RT-PCR probes specific for G2A (Mm00490809 and Hs00203431), GPR4 (Mm00558777 and Hs00947870), OGR1 (Mm01335272 and Hs00268858), TDAG8 (Mm00433695 and Hs01087326) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 4352932E and 4352934E) were from Applied Biosystems (Foster City, CA). YM-254890 was a gift from Dr. M. Taniguchi of Astellas (Tsukuba, Japan). MG6 cells (RCB 2403) were provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. The sources of all other reagents were the same as described previously [23–26].

2.2. Cell culture

N1E-115 cells, a mouse neuroblastoma cell line, were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with heat-inactivated 10% fetal bovine serum. For analyses of cell response, the cells were plated on culture dishes with poly-D-lysine, and then cultured in fresh medium without serum for another day. SH-SY5Y cells (from ECACC, EC94030304), a human neuroblastoma cell line, were grown in DMEM/Ham's F12 supplemented with 15% fetal bovine serum. Cortical neurons were isolated and cultured according to the instruction manual of SUMITOMO Nerve-Cell Culture System. In brief, the cerebral cortex of mouse at embryonic days 15–18 was cut into pieces and incubated for 20 min at 37 °C in Enzyme Solution containing papain. Dissociated cells were cultured for 5 days in Neuron Culture Medium supplemented with glial conditioned medium but without serum. Human umbilical vein endothelial cells (HUVECs) (passage number between 5 and 12) were cultured, as previously described [27]. MG6 cells were a microglial cell line with human *c-myc* from C57BL/6 mouse, and were grown in DMEM supplemented with 10% FBS, 100 μM β-mercaptoethanol and 10 μg/ml insulin as described in [28]. The MG6 cells were treated with LPS (1 μg/ml) 24 h before preparation of protein extracts for NOS analysis. For preparation of cortical neurons, the study was carried out in strict accordance with the guidelines of the Animal Care and Experimentation Committee of Gunma University, and all animals were bred in the Institute of Animal Experience Research of Gunma University. The protocol was approved by the Animal Care and Experimentation Committee of Gunma University (Permit Number: 11-019).

2.3. cGMP measurement

N1E-115 cells on 12-well plates were washed and pre-incubated for 10 min at 37 °C in a HEPES-buffered medium composed of 20 mM HEPES (pH 7.6), 134 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 2.0 mM $CaCl_2$, 2.5 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 5 mM glucose, and 0.1% BSA. The medium was then replaced with the same medium (0.2 ml) containing 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) under an appropriate pH. After an incubation at 37 °C for the indicated time, the reaction was terminated by the addition of 0.5 N HCl (25 μl). The cells were immediately frozen and store at −20 until cGMP assay. Cyclic GMP in the acid fraction was measured according to the instruction manual of cGMP EIA Kit. The protein content of the adherent cells was determined with the BCA Protein Assay.

2.4. Measurement of $[Ca^{2+}]_i$

The cells on 10-cm dish were gently harvested from dishes with PBS containing 0.05% trypsin–EDTA. The cells were incubated with 1 μM Fura-2/AM and $[Ca^{2+}]_i$ was estimated from the changes in the intensities of 540 nm fluorescence obtained by the two excitations (340 nm and 380 nm), which were monitored by the CAF-110 fluorometer (JASCO, Tokyo, Japan), as described previously [29].

2.5. Measurement of [^{3}H]inositol monophosphate (IP) production

The cells were cultured on 12-well dishes in the growth medium, and then the medium was changed to TCM199 (Sigma-Aldrich) containing 2 μCi/ml [^{3}H]inositol and 0.1% BSA and incubated for 24 h. After being washed once with the HEPES-buffered medium containing 0.1% BSA, the cells were preincubated for 10 min with 10 mM LiCl and incubated for further 15 min with the same medium under an appropriate pH to measure [^{3}H]IP, as described previously [30]. Data were normalized to 10^5 dpm of the radioactivity of unstimulated cells, and expressed as percentage of the basal values at pH 7.6.

2.6. Estimation of nNOS and Akt activation

Anti-phosphorylated antibodies against nNOS (phospho S1412 nNOS and nNOS) and Akt (phospho S473 Akt and Akt) were used for estimation of their activation. The serum-starved cells were incubated at 37 °C in the HEPES-buffered medium containing 0.1% BSA together with test substances under an appropriate pH for the indicated times. Reactions were terminated by washing twice with ice-cold phosphate-buffered saline (PBS) and adding a lysis buffer composed of 50 mM HEPES, pH 7.0, 150 mM NaCl, 0.1% Nonidet P-40, 1% phosphatase inhibitor cocktail, and 1% proteinase inhibitor cocktail (Sigma-Aldrich). The recovered lysate was centrifuged at 14,000 ×*g* for 20 min. The supernatant was resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the protein bands were detected by the alkaline phosphatase method, as described previously [23].

2.7. Transfection of siRNA

The siRNA targeted for OGR1 receptor (si-OGR1) or non-silencing RNA (si-NS) was transfected into N1E-115 cells using Lipofectamine RNAiMAX Reagent as described previously [25]. In brief, the cells were