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Protein tyrosine kinase regulates α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor trafficking induced by acute hypoxia in cultured brainstem neurons

H. Wang[1], L.C. Yu[2] and Y.C. Li[3]

[1]Shandong Key Laboratory of Robotics and Intelligent Technology, Shandong University of Science and Technology, Qingdao, China
[2]Laboratory of Neurobiology, School of Life Science, Peaking University, Beijing, China
[3]Department of Neurobiology & Anatomy, Drexel University College of Medicine, Philadelphia, PA, USA

Corresponding author: Y.C. Li
E-mail: jnpywr@163.com

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ABSTRACT. This study was performed to investigate the modulation effect of protein tyrosine kinase on postsynaptic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor trafficking induced by acute hypoxia in cultured brainstem neurons. The cultured neurons were exposed to 1% O_2 and the expression of AMPA receptor subunit GluR2 on the cell surface was significantly increased, while total GluR2 was not markedly changed. Furthermore, the hypoxia-

induced increase in GluR2 expression on the cell surface was partially blocked by the protein tyrosine kinase membrane-permeable inhibitor genistein. In contrast, both the protein tyrosine kinase agonist nerve growth factor and protein tyrosine phosphatase inhibitor vanadate promoted the hypoxia-induced increase of GluR2 expression on cell surface. Moreover, GluR2 could be phosphorylated by tyrosine under normoxia and hypoxia conditions *in vitro* on brainstem neurons, and tyrosine phosphorylation of GluR2 was significantly stronger under hypoxia conditions. Our results indicate that acute hypoxia induces the AMPA receptor subunit GluR2 to rapidly migrate to the cell membrane to modify the strength of the synapse. This study indicates that tyrosine phosphorylation of the receptor is an important pathway regulating the rapid migration of GluR2 in the postsynaptic domain induced by hypoxia.

Key words: Brainstem neuron; Protein tyrosine kinase; GluR2; Hypoxia; α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

INTRODUCTION

The ionotropic glutamate α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor consists of four types of subunits: GluR1, -2, -3, and -4 (Hollmann and Heinemann, 1994). Many studies have shown that the AMPA receptor mediates fast excitatory synaptic transmission and is involved in the response to hypoxia. Antagonists of the AMPA receptor have been shown to attenuate injuries to neuron or glia cells during brain hypoxia (Follett et al., 2000). Hypoxia respiratory responses were inhibited after the AMPA receptor was blocked (Whitney et al., 2000). Microinjection of an AMPA receptor agonist in the nucleus tractus solitaries increased ventilation by 50% under sustained hypoxia, while an AMPA receptor antagonist abolished this effect. Expression of phosphorylated AMPA receptor protein subunits increased in nucleus of the solitary tract of chronic sustained hypoxia rats (Pamenter et al., 2014). However, the mechanisms and modulation of postsynaptic AMPA receptor trafficking during hypoxia are unclear. Receptor trafficking is an important mechanism underlying the modification of AMPA receptor activity. Recent studies demonstrated that AMPA receptors could move into (internalization) and out of (insertion) the postsynaptic membrane (Lüscher et al., 1999; Malinow, 2003). Since AMPA receptors are expressed at high levels in the brainstem, which is sensitive to hypoxia, the present study examined the influence of acute hypoxia on the modulation of postsynaptic AMPA receptors in cultured brainstem neurons.

AMPA internalization or insertion is likely regulated by serine/threonine phosphorylation of AMPA receptor subunits (Boehm et al., 2006; Lee et al., 2010). A recent study showed that tyrosines on the AMPA receptor could be phosphorylated by Src family protein tyrosine kinases (PTK) (Beretta et al., 2009; Zhang et al., 2013). Therefore, in this study, we also explored whether insertion of the AMPA receptor subunit GluR2 into the cell surface under acute hypoxia *in vitro* was regulated by tyrosine kinase phosphorylation.

MATERIAL AND METHODS

Cell preparation

All animal procedures were conducted according to the Guide for the Care and Use of Laboratory Animals published by the US NIH (8th edition, revised in 2011, page 121). The brainstems of newborn Sprague Dawley rat (Peking University, Beijing, China) were peeled and trypsinized for 25 min and then subdivided into several smaller pieces. Cells were dissociated by gentle trituration through glass Pasteur pipettes of successively smaller tip diameters and plated onto glass coverslips treated with poly-L-lysine. Next, the cells were cultured in DMEM containing 10% fetal bovine serum (Gibco, Grand Island, NY, USA) for 10-14 days. Additionally, at 48 h, 10 μM Ara-C was supplemented to inhibit glial cell growth; thereafter, half of the medium was replaced twice per week.

In vitro hypoxia model

Culture plates were placed into special chambers equipped with a thermostat housing for incubation at 37°C under hypoxic conditions while gassing the chamber with 1% O_2 5% CO_2, and 94% N_2.

Fluorescence immunostaining

The density of cultured cells was 100,000. Neurons were fixed in 4% paraformaldehyde and incubated with 10% normal donkey serum in phosphate-buffered saline (PBS) containing 0.3% Triton X-100 for 20 min at room temperature. Next, the cells were incubated with GluR2 antibody (kindly provided by Wang Y.T., University of British Columbia, diluted with PBS) overnight at 4°C. The neurons were immunostained in Cy2 conjugated donkey anti-rabbit secondary antibody (1:200, Jackson, Philadelphia, PA, USA) for 1 h in the dark. Fluorescence images were acquired using a confocal microscope (Zeiss, Göttingen, Germany).

Cell enzyme-linked immunosorbent assay

The density of cultured cells was 1000 / μL. Cells on 96-well plates were treated with different drugs (100 μM genistein; 100 μM daidzein; 50 mg/mL NGF; 100 μM vanadate) before hypoxia, and then washed 3 times with PBS and fixed with 4% paraformaldehyde for 20 min. For total labeling, cells were incubated with 0.3% Triton for 30 min to permeabilize the membrane. Next, the cells were incubated with 10% normal horse serum for 30 min and then incubated with GluR2 antibody (1:1000, Chemicon International, Temecula, CA, USA) diluted with PBS-TX (PBS containing 0.1% TritonX-100) or PBS overnight at 4°C. The cells were washed 3 times with PBS and incubated with biotin-conjugated second antibody (1:200, Beijing Zhongshanjinqiao, Beijing, China). After washing 3 times with PBS, the avidin-biotin complex elite kit (Vector Laboratories, Burlingame, CA, USA) was added and washed 1 h later. Horseradish-peroxidase substrate TMD (Sigma, St. Louis, MO, USA) was added to produce a color reaction that was stopped with $2NH_2SO_4$. The optical density was read on a microplate reader (Bio-Rad, Hercules, CA, USA) at 450 nm. The surface expression of GluR2 subunits was presented as the percentage normalized to the normoxia control group (cells without hypoxia or drug treatment).

Immunoprecipitation

Cells were lysed with RIPA lysis buffer containing fresh protease inhibitor (1 mM Na_3VO_4, 10 µg/mL aprotinin, 10 µg/mL leupeptin, and 200 mM phenylmethylsulfonyl fluoride). Samples were incubated with 30 µL Protein G beads (Santa Cruz Biotechnology, USA) and centrifuged at 1000 *g* for 5 min. The supernatant was equally divided in two parts. One part was incubated with 2 µg GluR2 antibody, while the other was treated with 2 µg IgG overnight at 4°C. Next, 30 µL Protein G beads were supplemented into the samples, incubated, and collected by centrifugation. Subsequently, 20 µL loading buffer was added and the samples were boiled for 5 min. The protein was separated on 4-12% gradient Tris-glycine gels (Novex, San Diego, CA, USA) and transferred onto 0.2-mm nitrocellulose membranes. The membranes were blocked with 5% fat-free dry milk in Tris-buffered saline-Tween 20 and then incubated with primary antibodies, including anti-GluR2 (1:500) and anti-phosphotyrosine (1:500; Boster, Pleasanton, CA, USA). The membrane was washed and incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000, Zhongshan, Guangdong, China) for 1 h at room temperature. The immunoblot bands were detected by enhanced chemiluminescence (Amersham, Amersham, UK) and exposed to X-rays films.

Statistics

Data are reported as means ± SD. Differences between groups were determined by the Student *t*-tests (STATISTICA, 99 Edition, StatSoft, Inc., Tulsa, OK, USA) and $P \leq 0.05$ was considered to indicate significant differences.

RESULTS

Acute hypoxia increases expression of GluR2 on cell surface

Fluorescence immunostaining results (Figure 1A) revealed that GluR2 was expressed both on the plasma membrane surface and in the intracellular compartment under normoxia conditions. However, GluR2 expression in the plasma membrane increased after hypoxia for 1 or 2 h. A cell enzyme-linked immunosorbent assay showed that GluR2 subunits on the cell surface significantly increased to 137.15 ± 6.85% ($P < 0.01$, $N = 8$) and 141.22 ± 7.03% ($P < 0.01$, $N = 4$) after hypoxia for 1 and 2 h, respectively, compared with the control normoxia group (OD reading was normalized to 100%) (Figure 1B).

To further confirm that the increase in GluR2 on the plasma membrane surface was caused by the receptor moving from the intracellular compartment to the cell surface, total GluR2 protein was detected by cell-enzyme-linked immunosorbent assays under permeabilized conditions (Figure 1B). No significant changes were observed before or after hypoxia ($P > 0.05$, $N = 4$).

Influence of protein tyrosine kinase on increased cell surface expression of GluR2 caused by hypoxia

To confirm whether PTK played a role in hypoxia-induced GLuR2 insertion, cultured cells were pretreated with 100 µM genistein (a membrane-permeable inhibitor of PTK), 50

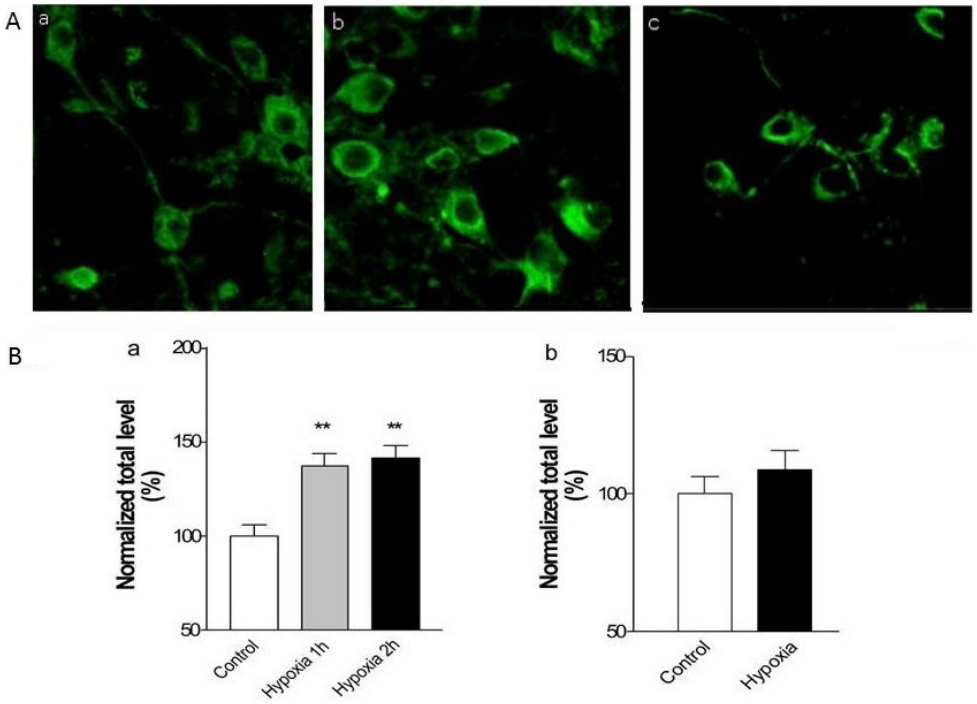

Figure 1. Hypoxic challenge-induced GluR2 inserted into plasma membrane. **A.** Distribution of AMPA receptor GluR2 without hypoxia challenge (**a**), after exposure to hypoxia for 1 h (**b**) and 2 h (**c**) **B.** Expression of GluR2 was quantitatively analyzed under non-permeablilized conditions (**a**) and permeabilized (**b**) conditions. Values are reported as means ± SD. *P < 0.05, **P < 0.01.

mg/mL NGF (PTK activator), or 100 μM vanadate (membrane-permeable protein tyrosine phosphatase inhibitor), and then exposed to hypoxia for 1 h. The results demonstrated that following genistein pretreatment, GluR2 on the neuron membrane was increased to 115.30 ± 5.630% after hypoxia and was decreased significantly compared to the results for hypoxia alone (*vs* 137.15 ± 6.850%) (P < 0.01, N = 8), but still higher than that under normoxia control conditions (115.30 ± 5.630% *vs* 100%) (P < 0.05, N = 8). With NGF or vanadate pretreatment, GluR2 on the membrane was increased to 155.11 ± 9.79% and 148.51 ± 8.41%, respectively. Compared to the results without pretreatment (*vs* 137.15 ± 6.85%), NGF and vanadate significantly enhanced hypoxia-induced GluR2 insertion (Figure 2).

To investigate whether the drugs had the same effects under normoxia conditions, the cells were treated with the drugs described above for 1 h in a CO_2 culture chamber (under normoxia conditions) before fixation. No significant changes were observed between pretreatment with drugs and no drug pretreatment (Figure 2).

Acute hypoxia enhances tyrosine phosphorylation of GluR2

The AMPA receptor subunit GluR2 was immunoprecipitated both under normoxia and hypoxia conditions, and then analyzed by immunoblotting with an anti-phosphotyrosine antibody. As shown in Figure 3, tyrosine phosphorylation of the GluR2 (wt 90 kDa) in the

control group (normoxia) and hypoxia group (hypoxia for 1 h) was detected. A significant increase in tyrosine phosphorylation of GluR2 occurred in the hypoxia group (Figure 3).

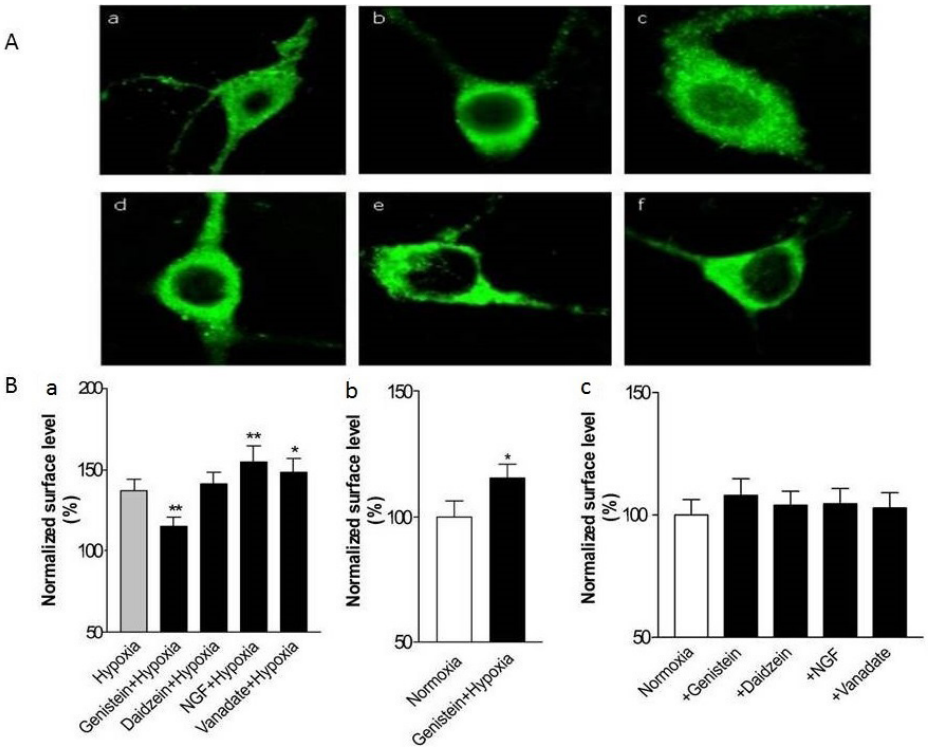

Figure 2. Effects of tyrosine on hypoxia-induced insertion of GluR2. **A.** (**a**) Without drug and hypoxia treatment. (**b**) Hypoxia for 1 h. (**c-f**) Pretreated with genistein, daidzein, vanadate, or NGF then hypoxia for 1 h. **B.** (**a-c**) PTK regulates GluR2 insertion under hypoxia conditions, but not under normoxia conditions. Values are reported as means ± SD. *P < 0.05, **P < 0.01.

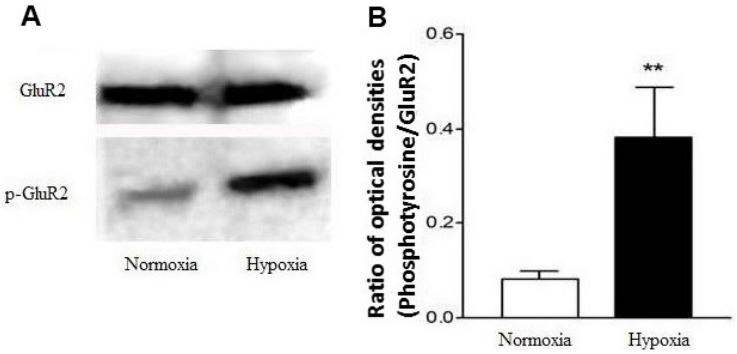

Figure 3. Effect of acute hypoxia on tyrosine phosphorylation of GluR2. **A.** Cultured brainstem cells with or without hypoxia challenge (1 h) were immunoprecipitated with antibodies specific for GluR2, and precipitates were analyzed by immunoblotting with antibodies for GluR2 (top) and anti-phosphotyrosine (bottom). **B.** Tyrosine phosphorylation of GluR2 was increased significantly under hypoxia conditions.

DISCUSSION

In this study, we demonstrated that AMPA receptor subunit GLuR2 on the neuron membrane was increased after hypoxic challenge, but total GluR2 protein levels in the neurons were not affected. This suggests that GLuR2 insertion was strengthened by acute hypoxia. This hypoxia-induced GluR2 insertion was attenuated by the PTK inhibitor genistein and strengthened by the PTK activators NGF and vanadate. Tyrosine-phosphorylated GLuR2 was increased significantly after hypoxia. We predict that PTK plays a role in regulating GLuR2 insertion under hypoxia conditions.

AMPA receptors control the majority of excitatory synaptic transmission (Wisden and Seeburg, 1993). It is well-known that both the change of transmitter release and the number of receptors clustered in the postsynaptic membrane can modify synaptic activity. Studies have indicated that postsynaptic AMPA receptors are dynamically recycled between the plasma membrane and intracellular pools (Man et al., 2000), which is thought to play a major role in tuning synaptic strength because postsynaptic AMPA receptors on the cell surface modulate the efficiency of synaptic transmission. An increase in AMPA receptors on the membrane surface can be achieved by either facilitating receptor insertion or inhibiting receptor internalization (Chung et al., 2000; Man et al., 2000). We detected an increase in GluR2 on the cell surface induced by acute hypoxia, indicating that GluR2 is involved in the functional response to hypoxia mediated by the AMPA receptor by insertion into the plasmalemma. Other AMPA receptor subunits, such as GluR1, may undergo similar processes.

Receptor phosphorylation is thought to be a common mechanism used to alter receptor function and modulate synaptic plasticity by changing channel open probability or open time. Numerous studies have found that the phosphorylation state of AMPA receptor subunits regulates membrane trafficking of the receptor (Malinow and Malenka, 2002; States et al., 2008). Our data showed that GluR2 insertion was strengthened after hypoxic challenge and that this phenomenon was attenuated when PTK was inhibited or was strengthened when PTK was activated. In addition, stronger tyrosine phosphorylation *in vitro* was detected on immunoprecipitated GluR2 under hypoxia compared to normoxia conditions. Direct receptor phosphorylation by PTK may be one of the mechanisms underlying the dynamic regulation of hypoxia-induced AMPA receptor insertion into the membrane, and the contribution of PTK does not fully account for the overall increases in GluR2 insertion under hypoxia conditions because genistein could not completely eliminate this effect. Thus, multiple kinase systems may be involved in altering GluR2 expression during hypoxia.

In previous studies, tyrosine phosphorylation of GluR2 was required for insulin-stimulated AMPA receptor endocytosis (Ahmadian et al., 2004). However, tyrosine phosphorylation of tyrosine 876 on GluR2 was only required for AMPA-induced GluR2 internalization, but enhanced insulin-stimulated AMPA receptor internalization was not detected (Hayashi and Huganir, 2004). These findings indicate that phosphorylating different tyrosine residues in the AMPA receptor subunit has different physiological functions. In this study, we did not explore which tyrosine phosphorylation residue was required for hypoxia-induced GluR2 insertion. In conclusion, the present study demonstrated that a rapid increase in AMPA receptor insertion may an efficient method of enhancing synaptic transmission under hypoxia conditions. Furthermore, tyrosine phosphorylation of GluR2 may contribute to hypoxia-induced AMPA receptor insertion.

Conflicts of interest

The authors declare no conflict of interest.

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