ORIGINAL ARTICLE

Acid-sensing Ion Channels Activation and Hypoxia Upregulate Homer1a Expression

Jing-Jing Su,¹ Hui Pan,¹ Hou-Guang Zhou,² Yu-Ping Tang,² Qiang Dong² & Jian-Ren Liu¹¹ Department of Neurology, Shanghai Ninth People's Hospital, Shanghai Jiao Tong University, Shanghai, China² Department of Neurology, Huashan Hospital, Fudan University, Shanghai, China**Keywords**

Acid-sensing ion channels; Akt; ERK1/2; Homer1a; Hypoxia.

Correspondence

Qiang Dong, Department of Neurology, Huashan Hospital, Fudan University, Shanghai 200040, China.

Tel.: +862164174579;

Fax: +862164174579;

E-mail: qdong@shmu.edu.cn

and

Jian-Ren Liu, Department of Neurology, Shanghai Ninth People's Hospital, Shanghai Jiao Tong University, Shanghai 200011, China.

Tel.: +862163081475;

Fax: +862163081475;

E-mail: liujianren0571@hotmail.com

Received 22 May 2013; revision 18 October 2013; accepted 19 October 2013

SUMMARY

Background: Recent studies have indicated that dynamic alterations in the structure of postsynaptic density (PSD) are involved in the pathogenesis of many central nervous system disorders, including ischemic stroke. Homer is the newly identified scaffolding protein located at PSD and regulates synaptic function. Homer1a, an immediate early gene, has been shown to be induced by several stimulations, such as glutamate, brain-derived neurotrophic factor, and trauma. However, whether acidosis mediated by acid-sensing ion channels (ASICs) and hypoxia during cerebral ischemia can change Homer1a expression remains to be determined. **Results:** We investigated that acidosis and hypoxia selectively and rapidly upregulated Homer1a expression, but not Homer1b/c in cultured cortical neurons. We also found that Homer1a exhibited induction expression in brain cortex of the middle cerebral artery occlusion (MCAO) rats. Additionally, acid-evoked Homer1a mRNA induction depended on extracellular signal-regulated kinase1/2 (ERK1/2) and Akt activity, and ASIC1a-mediated calcium influx whereas hypoxia depended only on ERK1/2 activity. Also, we demonstrated that continuous acidosis and hypoxia resulted in pronounced cell injury and Homer1a knockdown with small interfering RNA aggravated this damage induced by 3 h acid and hypoxia incubation in neuro-2a cells. **Conclusion:** Homer1a might act as an activity-dependent regulator responding to extracellular stimuli during cerebral ischemia.

doi: 10.1111/cns.12206

The first two authors contributed equally to this work.

Introduction

The postsynaptic density (PSD) is a specialized structure which is present in the postsynaptic membrane in the central nervous system (CNS) and enriched in excitatory glutamate receptors, cytoskeletal and scaffolding proteins. It was previously reported that protein-protein interactions and gene induction expression enable the PSD apparatus to become a dynamic structure. Dynamic alterations of PSD are suggested to be involved in the regulation of synaptic function and plasticity, and be implicated in the development of many CNS disorders, such as ischemic stroke [1]. Emerging evidence suggests that cerebral ischemia and reperfusion induces biological and morphological alterations of PSD [2]. Takagi et al. [1] reported that transient global ischemia resulted in altered interaction between the postsynaptic protein PSD-95 and ionotropic glutamate receptor NMDA (N-methyl-D-aspartate).

Another study revealed that PSD-95 inhibitor that disrupted PSD-95/MNDA interaction provided neuroprotection by reducing infarct volumes and improving neurobehavioral functions [3]. These findings demonstrate that the dynamic alterations in the PSD during cerebral ischemia are related to synaptic disturbance and neuronal death [4]. Thus, understanding the molecular mechanisms of dynamic modifications in the PSD fraction is assumed to contribute to developing an appropriate strategy for the treatment of cerebral stroke.

Homer family proteins, the recently discovered scaffolding proteins, are concentrated at PSD fraction and encoded by three genes, including Homer1, 2, and 3. This family of proteins consists of two major groups: the long- and short-form proteins. The long form of Homer proteins such as Homer1b/c is constitutively expressed and encodes a C-terminal coiled-coil (CC) domain that mediates self-multimerization. Conversely, the short form of pro-

tein Homer1a identified as an immediate early gene (IEG) lacks the CC domain and possesses a characteristic repeat of the AU-rich motif, which is implicated in the destabilization of short-lived mRNAs [5]. All of the Homer proteins share a conserved N-terminal Ena/VASP homology 1 (EVH 1) domain that directly interacts with group I metabotropic glutamate receptors (mGluRs), inositol 1,4,5-triphosphate receptor (IP3R), and indirectly with NMDA receptor through Shank family proteins, and facilitates the assembly of signaling complexes [6–8]. Homer1a has also been shown to be rapidly transcribed followed by the neural activity, such as glutamate, brain-derived neurotrophic factor (BDNF), and trauma [9,10]. Increased Homer1a functions as a negative regulator that disassembles multimerization and uncouples protein signaling complexes mediated by Homer proteins [5]. Indeed, these results imply that Homer1a plays an essential role in the activity-dependent synaptic reorganization [11]. Additionally, we have earlier demonstrated that the dynamic alterations of PSD are implicated in the pathological mechanisms of cerebral ischemia and acidosis mediated by acid-sensing ion channels (ASICs) and hypoxia are the well established features of ischemic stroke [1,12]. Hence, it is of great interest to investigate the regulation of Homer1a expression after exposure to acid and hypoxia.

This study sought to investigate the effects of acidosis mediated by ASICs activation and hypoxia on Homer1a expression and probe the underlying mechanisms of this induction. To eliminate the potential secondary activation of glutamate and voltage-dependent calcium channels, blockers for NMDA and AMPA receptors and voltage-gated calcium channels were present in the acid-treated cells [12,13]. Here, we observed that acidosis and hypoxia displayed the capability to significantly upregulate Homer1a levels in cultured cortical neurons. Homer1a was also largely induced after reperfusion in middle cerebral artery occlusion (MCAO) rats. Additionally, acidosis-mediated Homer1a mRNA induction may depend on more signaling pathways than hypoxia, and exposure to acid and hypoxia resulted in robust cell insults which were exacerbated after downregulating Homer1a expression in neuro-2a cells. Thus, Homer1a might function as an activity-dependent factor in response to extracellular stimuli during cerebral ischemia.

Materials and methods

Materials

Amiloride, CNQX, MK801 and nimodipine were purchased from Tocris Bioscience (Bristol, UK). The One Step RNA PCR Kit (AMV) was obtained from TaKaRa (Otsu, Shiga, Japan). Rabbit anti-Homer antibody was obtained from Proteintech (Wuhan, China). Psalmotoxin 1 (PcTX1) was obtained from Peptide Company (Sunnyvale, CA, USA). Rabbit anti-p-ERK1/2 and anti-p-Akt antibodies, Horseradish peroxidase (HRP)-conjugated with goat antirabbit secondary antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). PD98059 and LY294002 were obtained from Cell Signaling Technology (Beverly, MA, USA). Lactate dehydrogenase (LDH) Detection Kit was obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The Cell Counting Kit-8 was obtained from Dojindo Molecular Technologies (Gaithersburg, Japan). FuGENE 6 Trans-

fection Reagent was obtained from GE Healthcare (Fairfield, CT, USA).

Cells Cultures and Transfection

Sprague–Dawley rats and neuro-2a cells were purchased from Shanghai Institute of Chinese Academy of Science. All the procedures complied with the standards for the *Guide for Care and Use of Laboratory Animals* and were approved by the local animal ethics committee. Rat primary cortical neurons and neuro-2a cells were cultured as previously described [12,14].

The small interfering RNA (siRNA) duplexes targeting mouse Homer1a mRNA were synthesized in (Invitrogen Grand Island, NY, USA). The control siRNAs with a nontargeting sequence for mouse were purchased from Invitrogen. The siRNAs were transfected with FuGENE 6 Transfection Reagent according to the manufacturer's instructions.

Hypoxia and Acidosis Insults

The original oxygen and glucose-containing media was removed from the dishes. Cells were incubated with deoxygenated glucose-free ECF at pH 7.4 in an anaerobic chamber whereas cells-treated with acid were incubated with ECF at pH 6.0 in a normoxic incubator. MES was used for more reliable pH buffering. All treatments were terminated after 90 min, adding the original media and incubating the cultures in the normoxic incubator. ECF contained (in mM) 140 NaCl, 5.4 KCl, 25 HEPES, 20 glucose, 1.3 CaCl₂, 1.0 MgCl₂, 0.0005 TTX (pH 7.4), 320–335 mOsm.

Middle Cerebral Artery Occlusion

Middle cerebral artery occlusion (MCAO) was performed as previously described [15].

Drug Treatments

To suppress the potential secondary activation of glutamate NMDA and AMPA receptors and voltage-gated calcium channels, MK-801 (10 μ M), CNQX (20 μ M), and nimodipine (5 μ M) were added together to the culture medium in acid-treated cells [12,13]. To determine whether ASIC1a-mediated calcium influx played a vital role in Homer1a mRNA induction, PcTX1 (100 ng/mL), a specific ASIC1a blocker, amiloride (100 μ M), a nonspecific ASICs blocker, or EGTA (2 mM), a calcium chelator, was applied 30 min before and the duration of acidosis insults. To study the role of ERK1/2 and PI3K-Akt signaling cascades blockade in the effects of acidosis and hypoxia on Homer1a mRNA induction, the cells subjected to acidosis or hypoxia were pre-incubated with MEK (Mitogen-activated protein kinase/ERK kinase) inhibitor PD98059 (10 μ M) or PI3K inhibitor LY294002 (10 μ M) for 30 min.

Reverse Transcriptase-Polymerase Chain Reaction

Homer1 mRNA expression was determined by quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) accord-

ing to the applied instructions. The following primers were used: for Homer1a, forward 5'-ACCAGAAAGTATCAATGGGACA-3' and reverse 5'-GCACAGCGTTTG CTTGACTA-3'; for Homer1b/c, forward 5'-ACACTGTTTATGGACTGGGATT-3' and reverse 5'-GCACAGCGTTTGCTTGACT A-3'.

Western Blotting

Cells were lysed, separated by 10% SDS-PAGE, and subsequently transferred onto a polyvinylidene difluoride membrane. The polypeptides were exposed to rabbit anti-Homer (1:1000), anti-p-ERK1/2 (1:2000), or anti-p-Akt (1:2000) antibodies for 2 h and goat antirabbit secondary antibody (1:1000) for 1 h.

Cell Injury Assay-LDH Measurement

Cell injury was determined by the measurement of LDH release in various treatments. The amount of LDH released into the medium was determined using the LDH Detection Kit as previously described [12].

Cell Viability Assay

Cell viability was assayed with the Cell Counting Kit-8 (CCK-8) according to the supplier recommendations. The results were expressed as the percentage of viable cells relative to control group.

Three dishes were used for each group in the experiments of induction expression whereas eight dishes were used in LDH release and cell viability assay. Each experiment was conducted for three or eight times, respectively.

Statistical Analysis

Results were expressed as mean $\pm$ SD. Statistical analyses were performed by one-way ANOVA, followed by *post hoc* tests for multiple comparisons. Unpaired Student's *t*-tests were used for comparisons of two groups only. $P < 0.05$ was considered to be statistically significant.

Results

Acidosis Preferentially and Substantially Upregulates Homer1a mRNA Levels

Previous studies have demonstrated that Homer1a as an IEG can be regulated in an activity-dependent manner [10]. Acidosis and hypoxia have been shown to be the common features of ischemia stroke [12]. To explore the potential role of Homer1a in cerebral ischemia, in the present study, we first sought to determine whether acidosis had an effect on Homer1a expression. Rat cortical neurons were treated with acid for 90 min in the presence of blockers for NMDA and AMPA receptors and voltage-gated calcium channels. Homer1a mRNA expression was examined at the time points after 90 min acid incubation. As shown in Figure 1A, B, Homer1a mRNA level was significantly upregulated instantly after acid stimulation. This induction peaked at 3 h and gradually restored to the normal level at 12 h after 90 min acid stimulation.

Conversely, no significant changes in Homer1b/c mRNA level were observed despite a fairly small increase at 1 h after acid treatment. The results from Western blot analysis indicated that treatment of cells with 90 min acid markedly increased Homer1a protein levels which peaked at 3 h and gradually returned to the basal level at 24 h after acidosis (Figure 1C, D). These results demonstrated that acidosis preferentially and substantially upregulated Homer1a mRNA and its translation product in cultured cortical neurons.

We next addressed Homer1a mRNA levels after continuous acid incubation. Homer1a mRNA was dramatically induced as early as 0.5 h acid stimulation. This induction peaked at 3 h and sustained at higher levels than the basal level up to 12 h stimulation (Figure 1E, F). These data indicate that continuous acid stimulation resulted in a much higher and longer Homer1a induction compared with 90 min acid treatment.

Hypoxia Transiently Upregulates Homer1a mRNA Levels

It is well established that hypoxia is another common phenomenon in brain ischemia [12]. We next addressed whether hypoxia had a similar ability to upregulate Homer1a level in cultured neurons. As shown in Figure 2A, B, Homer1a mRNA transiently peaked after hypoxia treatment for 90 min, and thereby gradually fell back to the basal level at 12 h reoxygenation. Similar to acid incubation, levels of Homer1b/c were not strikingly altered after hypoxia treatment. Western blot analysis showed that 90 min hypoxia treatment rapidly promoted Homer1a protein levels which peaked at 3 h reoxygenation (Figure 2C, D). The results from continuous hypoxia treatment displayed that Homer1a mRNA was strikingly increased at 0.5 h hypoxia stimulation. The levels of Homer1a mRNA were still significantly higher than the basal level at 12 h stimulation (Figure 2E, F). Collectively, these findings demonstrated that hypoxia could selectively and transiently induce Homer1a expression, which was consistent with the results from acid stimulation.

Homer1a mRNA Induction in Rat Brain Cortex Following MCAO

We next investigated Homer1a expression in brain cortex of MCAO rats followed by reperfusion. As shown in Figure 3A, Homer1a mRNA produced significant induction which peaked at 1 h reperfusion and sustained at higher levels than the basal level up to 24 h reperfusion. These results further provided evidence for Homer1a induction in the *in vivo* ischemic animal models.

Homer1a mRNA Induction by ASIC1a Channel-mediated Calcium Influx

We observed that acidosis and hypoxia could selectively and rapidly upregulate Homer1a expression. But little is known about the mechanisms underlying activity-dependent Homer1a mRNA induction by the two distinct stimuli in cultured cortical neurons. We first gained insights into how acidosis enhanced Homer1a mRNA expression. A body of studies has demonstrated that calcium influx is implicated in the activity-dependent gene

Figure 1 Preferential induction of Homer1a expression after acid stimulation. Cells were treated with acid for 90 min or continuous acid incubation. Homer1 expression was examined for the indicated times. **(A, B)** Acidosis selectively upregulated Homer1a mRNA levels, but not Homer1b/c mRNA, after 90 min acid incubation. **(C, D)** Acid treatment for 90 min transiently promoted Homer1a protein expression. **(A–D)** The induction peaked at 3 h after 90 min acid stimulation and gradually fell back to the basal level. **(E, F)** Homer1a was induced more quickly and longer after continuous acid incubation, peaked at 3 h and gradually decreased. $n = 3$. Homer1a expression was compared. * $P < 0.05$ for acid exposure at the corresponding time point versus normal control or 3 h versus 6 h and 3 h versus 12 h (protein expression) or 3 h versus 12 h and 6 h versus 12 h (continuous acid incubation). ** $P < 0.01$ for acid exposure at the corresponding time point versus normal control. *** $P < 0.01$ for 3 h versus 6 h and 3 h versus 12 h (90 min acid incubation). To suppress the activation of N-methyl-D-aspartate (NMDA) and AMPA receptors and voltage-gated calcium channels, MK-801 (10 μ M), CNQX (20 μ M), and nimodipine (5 μ M) were together added to the culture medium.

Figure 2 Temporal induction of Homer1a expression after hypoxia treatment. **(A–F)** Treatment of cells with hypoxia for 90 min or continuous hypoxia transiently increased Homer1a mRNA and protein expression. However, hypoxia stimulation had no induced effects on the Homer1b/c levels **(A, B)**. **(A–F)** This induction gradually restored after the peak level. $n = 3$. Homer1a expression was compared. * $P < 0.05$ for hypoxia exposure at the corresponding time point versus normal control or 3 h versus 6 h and 6 h versus 12 h (continuous hypoxia). ** $P < 0.01$ for hypoxia exposure at the corresponding time point versus normal control or 3 h versus 6 h and 3 h versus 12 h (protein expression) or 3 h versus 12 h (continuous hypoxia). # $P < 0.05$ for 0 h versus 3 h or 3 h versus 12 h (90 min hypoxia). ## $P < 0.01$ for 0 h versus 12 h (90 min hypoxia).

Figure 3 (A) Homer1a mRNA induction in rat brain cortex after middle cerebral artery occlusion (MCAO). Quantitative analysis showed that focal cerebral ischemia resulted in Homer1a induction which peaked at 1 h reperfusion. (B, C) Homer1a mRNA induction by ASIC1a-mediated calcium influx. Cells were pre-incubated for 30 min in the presence of 100 μ M amiloride, 100 ng/mL Psalmotoxin 1 (PcTx1), or 2 mM EGTA, and further incubated with acid treatment for 3 h in the presence of blockers for N-methyl-D-aspartate (NMDA) and AMPA receptors and voltage-gated calcium channels. Levels of Homer1 mRNA were quantified by RNA blotting. Blockade of ASIC1a channel by amiloride or PcTx1 and chelation of extracellular calcium by EGTA nearly abolished the acid-induced Homer1a mRNA increase. $n = 3$. * $P < 0.05$ for reperfusion time point after MCAO versus control. ** $P < 0.01$ for 1 h and 3 h reperfusion versus control or 24 h and 48 h reperfusion versus 1 h reperfusion or Acid alone (3 h) versus control or Acid + Ami, Acid + PcTx1, and Acid + EGTA versus Acid alone. Ami, Amiloride.

expression in neurons [16,17]. Sato et al. [10] reported that glutamate stimulation of mice cerebellar granule cells upregulated Homer1a mRNA via influx of extracellular calcium mediated by the glutamate-dependent calcium channel NMDA. Thus, we examined the role of a glutamate-independent calcium channel ASIC1a in Homer1a mRNA induction. Cortical neurons were pretreated with the specific ASIC1a blocker PcTx1, nonspecific ASICs blocker amiloride, or calcium chelator EGTA for 30 min, and thereby incubated with acid application for 3 h in the setting of blockers for glutamate and voltage-gated calcium channels. As shown in Figure 3B, C, acid treatment substantially enhanced Homer1a mRNA levels. The addition of ASICs inhibitors markedly suppressed Homer1a mRNA induction by acid treatment. Importantly, chelation of extracellular calcium by EGTA nearly counteracted acid-mediated Homer1a mRNA increase. These results indicated that calcium influx mediated by the glutamate-independent calcium channel ASIC1a was at least partially responsible for Homer1a mRNA induction after acidosis.

Roles of ERK1/2 and PI3K/Akt Signaling Cascades in Homer1a mRNA Induction

Previous studies have reported that ERK1/2 signaling is implicated in Homer1a mRNA induction after glutamate stimulation [10]. Moreover, PI3K/Akt has also been reported to be involved in mGluR1s function which can be regulated by Homer proteins [18,19]. Thus, we further defined the roles of ERK1/2 and PI3K/Akt signaling cascades in Homer1a mRNA induction. We first monitored the expression of ERK1/2 and Akt phosphorylation by immunoblotting at the indicated times after continuous acid and hypoxia treatments. The results indicated that there was a slight inhibitory effect on the expression of p-ERK1/2 and p-Akt at 5 min acid stimulation. However, the expression of p-ERK1/2 and p-Akt was rapidly enhanced at 30 min acid treatment and thereby slowly decreased. It sustained at higher levels than the basal level up to 90 min (Figure 4A, B). The results from hypoxia stimulation showed that p-ERK1/2 induction peaked at 30 min, and gradually fell back to basal level at 6 h. In contrast, no marked change was observed in p-Akt expression after continuous hypoxia treatment (Figure 4C, D). These results suggested that ERK1/2 and Akt signaling might be involved in mechanisms of acidosis insults. However, hypoxia injury might require ERK1/2 activity.

Thus, we addressed whether the blockade of these signaling pathways by the specific inhibitors had effect on Homer1a mRNA induction after acid and hypoxia exposure. Cultured cells were treated with the MEK inhibitor PD98059 or PI3K inhibitor LY294002 together with acid or hypoxia stimulation for 3 h. We found that the blockade of MEK and PI3K/Akt signaling by PD98059 and LY294002, respectively, almost completely abrogated Homer1a mRNA induction by acidosis (Figure 5A, B). However, only the addition of MEK inhibitor PD98059 abolished hypoxia-evoked Homer1a mRNA induction (Figure 5C, D). Altogether, these data supported the view that acidosis-stimulated Homer1a mRNA induction involved ERK1/2 and PI3K/Akt signaling pathways whereas hypoxia-induced Homer1a mRNA upregulation was in dependence of ERK1/2 activity.

Figure 4 Extracellular signal-regulated kinase1/2 (ERK1/2) and Akt phosphorylation by acid and hypoxia stimulation. Cells were treated with continuous acid and hypoxia treatments for the indicated times, and cell lysates were immunoblotted with anti-p-ERK1/2 and anti-p-Akt antibodies. **(A, B)** The expression of p-ERK1/2 and p-Akt was rapidly induced at 30 min acid stimulation and thereby decreased slowly. **(C, D)** Hypoxia significantly induced p-ERK1/2 expression at 30 min and subsequently p-ERK1/2 expression was markedly decreased at 60 min and 90 min. However, hypoxia showed no remarkable changes in p-Akt levels. $n = 3$. * $P < 0.05$ for 60 min versus 90 min in p-ERK1/2 expression (continuous acid incubation). ** $P < 0.05$ for 30 min versus 90 min in p-ERK1/2 expression (continuous acid incubation). *** $P < 0.01$ for continuous acid time versus control in p-ERK1/2 and p-Akt expression or continuous hypoxia time versus control in p-ERK1/2 expression. ## $P < 0.01$ for 30 min versus 60 min and 60 min versus 90 min in p-ERK1/2 expression (continuous hypoxia). *** $P < 0.001$ for 30 min versus 90 min in p-ERK1/2 expression (continuous hypoxia). The acid-incubated cells were still treated with hypoxia.

Role of Homer1a in Cell Injury by Acidosis and Hypoxia

We had demonstrated acid and hypoxia-evoked Homer1a mRNA induction and the potential mechanisms underlying this induction. However, the role of Homer1a in cell injury triggered by acidosis and hypoxia remains to be determined. We first sought to examine LDH release and cell survival at the various time points after the two stimuli. As shown in Figure 6A, B, when compared with normal cells, neurons exposed to continuous acidosis and hypoxia displayed a time-dependent increase in LDH release and decrease in cell viability. More importantly, acidosis produced higher LDH release and lower cell survival than hypoxia at each

time point in cultured cells. Generally, these findings implied that cells subjected to acidosis and hypoxia display injured changes and acid-induced Homer1a mRNA expression was accompanied by more pronounced cell damage than hypoxia at the same time points.

Finally, we observed cell injury alterations after Homer1a knockdown using siRNA in neuro-2a cells exposed to acidosis and hypoxia. The data indicated that Homer1a downregulation largely enhanced acid- and hypoxia-induced LDH release when compared with the control siRNA-transfected cells at 3 h acid and hypoxia treatments (Figure 6C, D). These results suggested that Homer1a might play a beneficial role during the initial phase of acid- and hypoxia-mediated neuronal injury.

Figure 5 Blockade of Homer1a mRNA induction by inhibitors of Mitogen-activated protein kinase (MEK) or PI3K signaling. Cultured cells were pre-incubated with the MEK inhibitor PD98059 (10 μ M) or PI3K inhibitor LY294002 (10 μ M) for 30 min, and treated further with acid or hypoxia stimulation for 3 h. **(A, B)** The inhibition of MEK or PI3K/Akt signaling abrogated acid-evoked Homer1a mRNA induction. Blockers for major calcium entry pathways were present in the medium. **(C, D)** The blockade of MEK abolished the enhancement of Homer1a mRNA by hypoxia treatment. $n = 3$. $^{**}P < 0.01$ for Acid and Hypoxia alone versus control or Acid + PD and Acid + LY versus Acid alone or Hypoxia + PD versus Hypoxia alone in Homer1a expression. PD, PD98059; LY, LY294002.

Discussion

Homer1a represents a member of the IEG family and has been established to be rapidly upregulated in response to extracellular stimuli that affect synaptic plasticity. NMDA receptor activation by glutamate and BDNF have been reported to enhance Homer1a expression in cultured mice cerebellar granule cells [10]. Similarly, cocaine administration strongly increases Homer1a levels in rat striatum neurons *in vivo* and *in vitro* and this event is mediated through the dopamine D1 receptor [20]. Likewise, seizure, long-term potentiation (LTP), and light stimulation have also been considered to have the potential to raise the transcription induction of Homer1a mRNA [21,22]. Consistent with previous studies, in this study, we demonstrated the selective and marked increases in the levels of Homer1a expression, but not Homer1b/c in cultured neu-

rons following acid and hypoxia exposure. In addition, we also observed that Homer1a was largely induced after reperfusion in MCAO rats. These results indicate that Homer1a may act as a neuronal activity marker in various pathological conditions.

A variety of studies have revealed that calcium ions, as a principal second messenger, plays a critical role in the activity-dependent gene expression in neurons [17]. The intracellular calcium is believed to have the ability to activate protein kinase signaling cascades, such as calmodulin-dependent protein kinases (CaMK) and protein kinase C (PKC), and bring about the activation of gene transcription in neurons [16,23]. Sato et al. [10] provided evidence that glutamate stimulation of mice cerebellar granule cells upregulated Homer1a mRNA via influx of extracellular calcium mediated by the glutamate-dependent calcium channel NMDA receptor. However, the effect of calcium influx mediated

Figure 6 Role of Homer1a in cell injury by acidosis and hypoxia. **(A, B)** Time-dependent increase in Lactate dehydrogenase (LDH) release and decrease in cell viability induced by continuous acid and hypoxia stimulation of cultured cells. Acidosis evoked cell damage more remarkably than hypoxia. **(C, D)** Homer1a downregulation aggravated cell insults. $n = 8$. * $P < 0.05$ for continuous acid and hypoxia time versus normal control (LDH release and cell survival) or siHomer1a-transfected cells versus control siRNA-transfected cells exposed to acid or hypoxia for 3 h. # $P < 0.05$ and ## $P < 0.01$ for acid versus hypoxia treatment at the same time points. ** $P < 0.01$ for continuous acid and hypoxia time versus normal control (LDH release and cell survival) or 3 h acid and hypoxia versus normal condition in control siRNA-transfected cells. *** $P < 0.001$ versus normal control in acid-treated cells (cell survival). MK-801, CNQX, and nimodipine were present in the ECF for the acid-incubated cells. si-Con, control siRNA; si-H1a, siHomer1a.

by the glutamate-independent calcium channel ASIC1a on Homer1a induction remains to be established. Here, we observed that the addition of specific ASIC1a blocker PcTX1, nonspecific ASICs blocker amiloride, or calcium chelator greatly suppressed acid-evoked Homer1a mRNA increase. These findings imply that glutamate-independent calcium channel ASIC1a-mediated calcium influx is indispensable for Homer1a mRNA induction after acid stimulation, which is consistent with previous reports [10].

Previous studies showed that ERK1/2 signaling was involved in the process of IEG expression induction including Homer1a when exposed to environmental stimuli in cultured cells [10,17,24]. The mechanisms underlying ERK1/2-mediated IEG induction have been well characterized in neurons *in vivo*. It has been proved that ERK1/2 signaling is transferred to cellular nucleus after serial phosphorylation events and the activated ERK1/2 potentiates its ability to regulate transcription activity of many IEGs [25]. It has been reported that the transcription factors cyclic AMP-responsive

element-binding protein (CREB) and Elk-1 are crucial for the ERK1/2-mediated IEG transcription activation. There are several response elements for CREB and Elk-1 in the promoter region of IEG c-fos. ERK1/2 has been addressed to function as a common component in two signaling pathways (ERK/Elk-1 and ERK/CREB) converging on the c-fos promoter in cultured neurons [26]. In this study, acidosis and hypoxia exhibited their potentials to increase p-ERK1/2 and Homer1a mRNA levels. The inhibition of ERK activity by MEK blocker PD98059 almost completely counteracted Homer1a induction triggered by acidosis and hypoxia. These data suggest that ERK activity is required for acid- and hypoxia-mediated Homer1a induction, consistent with previous reports [10]. More interestingly, our results from bioinformatics analysis showed that Homer1a promoter region contained the response elements for three transcription factors, such as CREB, activator protein-1 (AP-1), and nuclear factor-kappa B (NF- κ B) (data not shown). Therefore, further studies need to be done to

shed light on whether these transcription factors might be implicated in the regulation of Homer1a expression mediated by ERK1/2 during acid and hypoxia exposure.

PI3K/Akt is another important signaling pathway that participates in cerebral ischemia/reperfusion injury [27] and has also been reported to be involved in mGluR1s function which can be regulated by Homer proteins [18,19]. In addition, it has been confirmed that phosphoinositide 3 kinase enhancer (PIKE)-L, a new designated form of PI3K enhancer, binds to Homer1b/c and mGluR1s to form mGluR1-Homer-PIKE-L complex which leads to PI3K activation [28]. Based on these observations, we presumed that PI3K/Akt signaling cascades might be closely related to the function and regulation of Homer proteins including Homer1a. Here, we presented evidence that acidosis, but not hypoxia, significantly induced p-Akt dynamic expression. However, LY294002, a PI3K inhibitor, abolished the induction-promoting ability of acidosis on Homer1a. These results indicate that PI3K/Akt signaling is essential for Homer1a transcription activity induced by acidosis.

Previous study demonstrated that activity-induced Homer1a significantly decreased chronic inflammatory hyperalgesia [29]. However, the role of Homer1a induction in ischemic stroke remains elusive. It is well established that calcium signaling in response to extracellular stimuli plays a vital role in neuronal death during cerebral ischemia [30]. Furthermore, NMDA and mGluR1s are the well-known calcium channels responsible for calcium influx from extracellular space and intracellular calcium release, respectively [31]. Homer1a can keep calcium homeostatic through reflecting NMDA and mGluR1s signaling [7,32]. Together, we proposed that Homer1a induction might be associated with pathological process of cerebral ischemia. Here, these results showed that Homer1a knockdown markedly exacerbated cell damage at 3 h of acid and hypoxia stimulation, indicating that Homer1a might play a beneficial part in the early stage of acid and hypoxia insults. It has been identified that cerebral ischemia induces IEGs expression which is regarded as a brain plasticity response that enables neurons to cope with potentially harmful conditions [33]. Homer1a overexpression has been proved to

directly activate mGluR1s independent of their agonists in cultured neurons [34]. On the other hand, mGluR1s have also been shown to protect cultured neurons from apoptosis [18]. Therefore, the transient upregulation of Homer1a might be thought to initiate the protective mechanism against injury. In the present data, Homer1a expression peaked at 3 h after 90 min acid and hypoxia stimulation which might be implicated in early defense mechanism and plasticity response of brain. However, in our study, accompanied by continuous acid and hypoxia treatments, acidosis triggered more robust cell injury than hypoxia, and persistent Homer1a upregulation. It has been reported that ASICs activation by acidosis and hypoxia can regulate synaptic structure and induce cell death [2,35]. Furthermore, the upregulated Homer1a by acidosis and hypoxia is suggested to be involved in the activity-dependent synaptic reorganization [11]. Accordingly, we presumed that the late dynamic expression of Homer1a might be related to the final cell damage exposed to acidosis and hypoxia, which requires further deep investigation.

In summary, our study demonstrates that acidosis and hypoxia can remarkably induce Homer1a expression. Furthermore, acidosis might trigger more complex neuronal responses than hypoxia, including ERK1/2 and Akt activity, and ASIC1a-mediated calcium influx, to initiate Homer1a transcription relevant to cell ischemic injury, which might account for why PI3K/Akt signaling is involved in the acidosis-induced Homer1a alteration, but not hypoxia. These results demonstrate that Homer1a might serve as a key adaptor protein that links extracellular stimuli to intracellular response and pathological process of cerebral ischemia.

Acknowledgments

This work was supported by National Natural Scientific Foundation of China (81200941, 81271302).

Conflict of Interest

The authors declare no conflict of interest.

References

- Takagi N, Logan R, Teves L, Wallace MC, Gurd JW. Altered interaction between PSD-95 and the NMDA receptor following transient global ischemia. *J Neurochem* 2000;**74**:169–178.
- Kovalenko T, Osadchenko I, Nikonenko A, et al. Ischemia-induced modifications in hippocampal CA1 stratum radiatum excitatory synapses. *Hippocampus* 2006;**16**:814–825.
- Sun HS, Doucette TA, Liu Y, et al. Effectiveness of PSD95 inhibitors in permanent and transient focal ischemia in the rat. *Stroke* 2008;**39**:2544–2553.
- Hu BR, Park M, Martone ME, Fischer WH, Ellisman MH, Ziviv JA. Assembly of proteins to postsynaptic densities after transient cerebral ischemia. *J Neurosci* 1998;**18**:625–633.
- Xiao B, Tu JC, Petralia RS, et al. Homer regulates the association of group I metabotropic glutamate receptors with multivalent complexes of homer-related, synaptic proteins. *Neuron* 1998;**21**:707–716.
- Brakeman PR, Lanahan AA, O'Brien R, et al. Homer: a protein that selectively binds metabotropic glutamate receptors. *Nature* 1997;**386**:284–288.
- Tu JC, Xiao B, Yuan JP, et al. Homer binds a novel proline-rich motif and links group I metabotropic glutamate receptors with IP3 receptors. *Neuron* 1998;**21**:717–726.
- Tu JC, Xiao B, Naisbitt S, et al. Coupling of mGluR/Homer and PSD-95 complexes by the Shank family of postsynaptic density proteins. *Neuron* 1999;**23**:583–592.
- Huang WD, Fei Z, Zhang X. Traumatic injury induced homer-1a gene expression in cultured cortical neurons of rat. *Neurosci Lett* 2005;**389**:46–50.
- Sato M, Suzuki K, Nakanishi S. NMDA receptor stimulation and brain-derived neurotrophic factor upregulate homer 1a mRNA via the mitogen-activated protein kinase cascade in cultured cerebellar granule cells. *J Neurosci* 2001;**21**:3797–3805.
- Inoue Y, Udo H, Inokuchi K, Sugiyama H. Homer1a regulates the activity-induced remodeling of synaptic structures in cultured hippocampal neurons. *Neuroscience* 2007;**150**:841–852.
- Liu L, Zhang R, Liu K, et al. Tissue kallikrein protects cortical neurons against in vitro ischemia-acidosis/reperfusion-induced injury through the ERK1/2 pathway. *Exp Neurol* 2009;**219**:453–465.
- Xiong ZG, Zhu XM, Chu XP, et al. Neuroprotection in ischemia: blocking calcium-permeable acid-sensing ion channels. *Cell* 2004;**118**:687–698.
- Wang LJ, Colella R, Yorke G, Roisen FJ. The ganglioside GM1 enhances microtubule networks and changes the morphology of Neuro-2a cells in vitro by altering the distribution of MAP2. *Exp Neurol* 1996;**139**:1–11.
- Lunga EZ, Weinstein PR, Carlson S, Cummins R. Reversible middle cerebral artery occlusion without craniectomy in rats. *Stroke* 1989;**20**:84–91.
- Bito H, Deisseroth K, Tsien RW. Ca²⁺-dependent regulation in neuronal gene expression. *Curr Opin Neurobiol* 1997;**7**:419–429.
- Xia Z, Dudek H, Miranti CK, Greenberg ME. Calcium influx via the NMDA receptor induces immediate early gene transcription by a MAP kinase/ERK-dependent mechanism. *J Neurosci* 1996;**16**:5425–5436.
- Liu F, Gong X, Zhang G, Marquis K, Reinhart P, Andree TH. The inhibition of glycogen synthase kinase 3beta by a metabotropic glutamate receptor 5 mediated pathway confers neuroprotection to Abeta peptides. *J Neurochem* 2005;**95**:1363–1372.
- Ronesi JA, Huber KM. Homer interactions are necessary for metabotropic glutamate receptor-induced long-term

- depression and translational activation. *J Neurosci* 2008;**28**:543–547.
20. Zhang GC, Mao LM, Liu XY, et al. In vivo regulation of Homer1a expression in the striatum by cocaine. *Mol Pharmacol* 2007;**71**:1148–1158.
 21. Kato A, Ozawa F, Saitoh Y, Hirai K, Inokuchi K. vesl, a gene encoding VASP/Ena family related protein, is upregulated during seizure, long-term potentiation and synaptogenesis. *FEBS Lett* 1997;**412**:183–189.
 22. Park HT, Kang EK, Bae KW. Light regulates Homer mRNA expression in the rat suprachiasmatic nucleus. *Brain Res Mol Brain Res* 1997;**52**:318–322.
 23. Ghosh A, Greenberg ME. Calcium signaling in neurons: molecular mechanisms and cellular consequences. *Science* 1995;**268**:239–247.
 24. Kawamoto T, Togi K, Yamauchi R, et al. Endothelin-1 activates Homer 1alpha expression via mitogen-activated protein kinase in cardiac myocytes. *Int J Mol Med* 2006;**18**:193–196.
 25. Sgambato V, Pagès C, Rogard M, Besson MJ, Caboche J. Extracellular signal-regulated kinase (ERK) controls immediate early gene induction on corticostriatal stimulation. *J Neurosci* 1998;**18**:8814–8825.
 26. Vanhoutte P, Barnier JV, Guibert B, et al. Glutamate induces phosphorylation of Elk-1 and CREB, along with c-fos activation, via an extracellular signal-regulated kinase-dependent pathway in brain slices. *Mol Cell Biol* 1999;**19**:136–146.
 27. Carloni S, Girelli S, Buonocore G, Longini M, Balduini W. Simvastatin acutely reduces ischemic brain damage in the immature rat via Akt and CREB activation. *Exp Neurol* 2009;**220**:82–89.
 28. Rong R, Ahn JY, Huang H, et al. PI3 kinase enhancer-Homer complex couples mGluRI to PI3 kinase, preventing neuronal apoptosis. *Nat Neurosci* 2003;**6**:1153–1161.
 29. Tappe A, Klugmann M, Luo C, et al. Synaptic scaffolding protein Homer1a protects against chronic inflammatory pain. *Nat Med* 2006;**12**:677–681.
 30. Choi DW. Calcium-mediated neurotoxicity: relationship to specific channel types and role in ischemic damage. *Trends Neurosci* 1988;**11**:465–469.
 31. Masu M, Nakajima Y, Moriyoshi K, Ishii T, Akazawa C, Nakanashi S. Molecular characterization of NMDA and metabotropic glutamate receptors. *Ann NY Acad Sci* 1993;**707**:153–164.
 32. Bertaso F, Roussignol G, Worley P, Bockaert J, Fagni L, Ango F. Homer1a-dependent crosstalk between NMDA and metabotropic glutamate receptors in mouse neurons. *PLoS ONE* 2010;**5**:e9755.
 33. Back T. Pathophysiology of the ischemic penumbra—revision of a concept. *Cell Mol Neurobiol* 1998;**18**:621–638.
 34. Ango F, Prézeau L, Muller T, et al. Agonist-independent activation of metabotropic glutamate receptors by the intracellular protein Homer. *Nature* 2001;**411**:962–965.
 35. Zha XM. Acid-sensing ion channels: trafficking and synaptic function. *Mol Brain* 2013;**6**:1.