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Homer1/mGluR1-mediated ER stress contributes to lysophosphatidic acid-induced neurotoxicity in cortical neurons

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ABSTRACT

Lysophosphatidic acid (LPA) is a glycerophospholipid that can be detected in serum, saliva and cerebrospinal fluid. However, the effect of LPA on neuronal death and survival has not been fully determined. In the present study, we investigated the potential neurotoxic effect of LPA in primary cultured cortical neurons. Treatment with LPA (0.5, 1 and 5 μM) markedly decreased neuronal viability, increased lactate dehydrogenase (LDH) release and promoted apoptosis in cortical neurons. The results of western blot showed that LPA increased the expression of endoplasmic reticulum (ER) stress associated factors, and the protein misfolding inhibitor 4-phenylbutyric acid (4-PBA) attenuated LPA-induced toxicity. In addition, treatment with LPA did not alter the expression and distribution of Homer1 in cortical neurons. The protein levels of metabotropic glutamate receptor 1 (mGluR1), but not metabotropic glutamate receptor 5 (mGluR5), were significantly increased by LPA at 12 and 24 h after treatment. Knockdown of Homer1 using specific siRNA partially prevented the LPA-induced neurotoxicity and ER stress. Furthermore, the results of Ca^{2+} imaging showed that treatment with LPA induced intracellular Ca^{2+} release, which could be partially prevented by 4-PBA and downregulation of Homer1. The LPA-induced intracellular Ca^{2+} release was associated with ER Ca^{2+} release through the Homer1-mGluR1 pathway. In summary, our results showed that LPA treatment induced ER stress and apoptosis in cortical neurons, and its neurotoxicity was partially mediated by Ca^{2+} release from the ER via the Homer1/mGluR1 pathway.

1. Introduction

Lipids and their derivatives are key molecules that not only act as energy suppliers, but also as the structure support of cellular membrane. Lysophosphatidic acid (LPA) is a glycerophospholipid that can be generated from many precursors, such as phosphatidic acid, and plays an important role in neuronal development and pathology (Frisca et al., 2012). LPA is released from various types of cells after injury, and detectable levels of LPA can be found in serum, saliva and cerebrospinal fluid (Aoki et al., 2008). Previous studies have shown that high concentrations of LPA induced apoptosis and necrosis in rat embryonic hippocampal neurons, whereas low levels of LPA could attenuate neuronal apoptosis after serum starvation or β-amyloid peptide treatment in cortical neurons (Steiner et al., 2000; Zheng et al., 2005). However, the mechanisms of LPA-induced effects on neuronal death and survival are not fully understood.

Ca^{2+} homeostasis is of pivotal interest for neurons due to its role in gene transcription, protein phosphorylation, neurotransmission, neuronal survival and death. Intracellular Ca^{2+} overload, defined as the high concentrations of cellular Ca^{2+} over a longer period of time, is a detrimental mechanism underlying multiple neurological disorders (Heyes et al., 2015; Lory and Mezghrani, 2010). To maintain Ca^{2+} homeostasis, the flow of Ca^{2+} is precisely regulated by the uptake of extracellular Ca^{2+} and the release of Ca^{2+} from intracellular stores, such as the endoplasmic reticulum (ER) (Verkhratsky, 2002, 2004). Not surprisingly, disruption of Ca^{2+} homeostasis in the ER results in the activation of ER stress coping response, which can improve cell survival through the unfolded protein response (UPR) or kill the stressed cells via apoptotic cell death (Marciniak and Ron, 2006). Accumulating evidence suggest that ER stress is linked to neuronal injury in several neurological disorders (Lindholm et al., 2006; Paschen, 2004; Sanderson et al., 2015).

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Metabotropic glutamate receptors (mGluRs) are G-protein-coupled receptors expressed mainly on neurons and glial cells in CNS. Activation of the group I mGluRs, including mGluR1 and mGluR5, increases intracellular Ca^{2+} level through a classical G_q protein-mediated mechanism, which causes the receptor-dependent Ca^{2+} release from the ER (Bardoul et al., 1998; Kaur et al., 2005). Homer proteins, including Homer1, Homer2 and Homer3, are a family of postsynaptic scaffolding proteins that play important roles in synapse function, receptor trafficking and Ca^{2+} homeostasis (Jardin et al., 2013; Xiao et al., 2000). They share a conserved N-terminal Ena/VASP homology 1 (EVH1) domain which links cell surface proteins to other PSD proteins, including shank, preso and Arc, as well as other Ca^{2+} regulating proteins, such as mGluRs (Hayashi et al., 2009; Sala et al., 2005).

A previous study showed that LPA induced growth cone collapse through inducing the release of Ca^{2+} from ER in dorsal root ganglion (DRG) neurons (Elmes et al., 2004). In this study, we investigated the neurotoxic effect of LPA in primary cultured cortical neurons. We also investigated the underlying molecular mechanisms with focus on intracellular Ca^{2+} homeostasis and ER stress.

2. Materials and methods

2.1. Primary culture of cortical neurons

Healthy pregnant Sprague Dawley (SD) rats (day 15) were obtained from the Experimental Animal Center of the Fourth Military Medical University, and the experimental protocol was approved by the Animal Use and Care Committee of the Xi'an Jiaotong University. Cortical neurons were prepared using the method as previously described (Chen et al., 2012b). Briefly, pregnant animals were killed by cervical dislocation and the embryo cortex was isolated. Cortical tissues were washed by cold PBS and mechanically minced into small pieces (approximately 1 mm^3). Then, cortical tissues were digested by 0.25% trypsin at 37 °C for 15 min, and the pellet was resuspended in Primary Neuron Basal Medium supplemented with 2% B27. The re-suspended cell suspension was filtered using a cell strainer (70 μm), and cells were seeded in culture vessels precoated with poly-L-lysine (PLL, #P1399, Sigma, 0.5 mg/ml). Half of the culture medium was replaced by fresh Neurobasal medium every other day, and neurons on in vitro days 8–10 were used for following experiments. All efforts were carried out to reduce the number of animals used and to minimize their suffering.

2.2. Neuronal viability

WST-1 assay was performed to measure neuronal viability of cortical neurons, and the assay kit was obtained from Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). Cortical neurons were seeded in 96-well plates and treated with LPA. After addition of 20 μL WST-1 solution into each well, the neurons were incubated for further 3 h. The absorbance of each well was determined at a wavelength of 450 nm using Molecular Devices (SpectraMax M5, USA).

2.3. LDH release

Neuronal toxicity was determined by LDH release assay using a commercially available kit obtained from Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). After treatment with LPA, culture medium was collected, and the absorbance was determined at 490 nm.

2.4. TUNEL staining

TUNEL staining was performed to detect apoptotic neurons, which exhibit characteristic nuclear fragmentation and are labeled with terminal deoxynucleotidyl transferase (TdT). Briefly, cortical neurons were seeded onto the PLL-precoated coverslips and treated with LPA. After been fixed by 4% formaldehyde for 15 min at room temperature, neurons were incubated with the fluorescein TUNEL reagent mixture at 37 °C for 1 h (Roche, Penzberg, Germany). The number of TUNEL-positive cells was counted, and the ratio of TUNEL-positive cells to total cells was calculated as apoptotic rate.

2.5. Immunocytochemistry

Cortical neurons on PLL-precoated coverslips were rinsed with PBS and fixed by 4% formaldehyde for 15 min at room temperature. The cells were incubated with the primary antibodies against Homer1 (#8231, 1:50, Cell Signaling) or MAP-2 (sc-135979, 1:200, Santa Cruz) overnight at 4 °C, and then stained with Alexa 488-conjugated (green, 1:500) or Alexa 594-conjugated (red, 1:500) secondary antibodies for 1 h at 37 °C. Finally, the coverslips were mounted with mounting medium with DAPI and examined under a fluorescence microscopy.

2.6. Short interfering RNA (siRNA) and transfection

The Homer1 specific targeted siRNA (Si-Homer1) and control siRNA (Si-control) were synthesized from Jikai Bioengineering Institute (Beijing, China). The siRNA molecules were transfected using Lipofectamine RNAiMax reagent (Invitrogen, CA, USA) according to the manufacturer's instructions.

2.7. Ca^{2+} imaging

To determine the cytosolic Ca^{2+} concentration, cortical neurons were loaded with the labeled Ca^{2+} indicator Fura-2 AM in an HBSS solution. Neurons on coverslips were excited at 345 and 385 nm using a confocal laser scanning microscope, and the emission fluorescence at 510 nm was recorded. The fluorescence values were then plotted against time.

2.8. Western blot analysis

The standard western blot procedure was performed to detect the expression of the proteins using the following primary antibodies: glucose-regulated protein 78 (GRP78, sc-13539, Santa Cruz, 1:1000), C/EBP homologous protein (CHOP, sc-71136, Santa Cruz, 1:800), metabotropic glutamate receptor 1 (mGluR1, sc-293437, Santa Cruz, 1:400), metabotropic glutamate receptor 5 (mGluR5, Santa Cruz, 1:600), Cleaved-caspase-3 (#9664, Cell Signaling, 1:300), Homer1 (#8231, Cell Signaling, 1:200), β-actin (#3700, Cell Signaling, 1:2000).

2.9. Statistical analysis

Data are presented as means ± SEM and the statistical evaluation was performed by Student's *t*-test or one-way analysis of variance. Statistical difference was accepted when $p < 0.05$.

3. Results

3.1. LPA induces neurotoxicity in cortical neurons

To investigate the neurotoxicity of LPA in vitro, cortical neurons were treated with different concentrations of LPA. The results of WST-1 assay showed that LPA (0.5, 1 and 5 μM) significantly decreased neuronal viability in neurons (Fig. 1A). In addition, LPA was found to increase LDH release in a dose-dependent manner (Fig. 1B), although 0.1 μM LPA had no effect ($P > 0.05$). TUNEL staining was performed to detect neuronal apoptosis (Fig. 1C), and the results showed that LPA at the concentration of 0.5, 1 or 5 μM markedly increased the apoptotic rate at 24 h in cortical neurons (Fig. 1D).

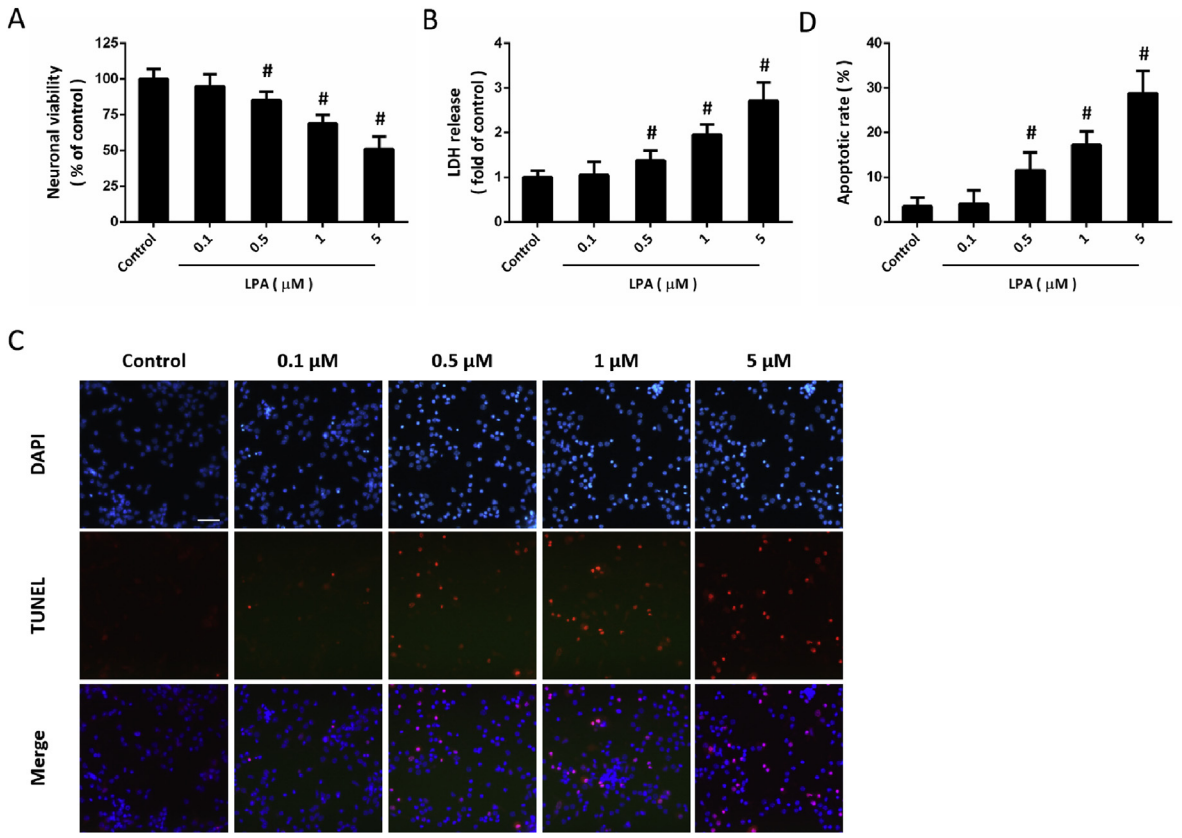

Fig. 1. LPA induces neurotoxicity in cortical neurons. (A) WST-1 assay shows that LPA decreased neuronal viability in a dose-dependent manner. (B) LDH release assay shows that LPA increased LDH release in a dose-dependent manner. (C and D) TUNEL staining (C) and quantification (D) show that LPA induced apoptosis in cortical neurons. Scale bar, 50 μm. Data are shown as mean ± SEM (n = 6). $^{\#}p < 0.05$ vs. Control group.

3.2. LPA induces ER stress in cortical neurons

To investigate the potential effect of LPA on ER stress, cortical neurons were treated with 5 μM LPA, and the expression of ER stress-associated proteins was detected by western blot (Fig. 2A). The results showed that LPA increased the expression of GRP78 (Fig. 2B) and cleaved-caspase-12 (Fig. 2D) at 12 and 24 h, while elevated the protein level of CHOP from 6 h to 24 h (Fig. 2C). Next, neurons were treated with the protein misfolding inhibitor 4-PBA (2 mM) to confirm the potential involvement of ER stress. The results showed that the LPA-induced loss of neuronal viability (Fig. 2E) and increase of LDH release (Fig. 2F) were both partially prevented by 4-PBA (Huang et al., 2017).

3.3. Effects of LPA on Homer1 and mGluR1 expression

Due to the important role of Homer1-mGluR1 pathway in the regulation of ER Ca2+ release in neurons, we further investigate the effect of LPA on Homer1 and mGluR1 expression. Immunostaining was performed to investigate the effect of LPA on Homer1, and no significant alteration in fluorescence intensity and distribution was observed (Fig. 3A). Congruently, the results of western blot showed that the expression of Homer1 protein did not change within 24 h after LPA treatment (Fig. 3B). In addition, we also detected the expression of group I mGluRs proteins, including mGluR1 and mGluR5, in neurons (Fig. 3C). The results showed that LPA increased mGluR1 expression at 12 and 24 h after LPA exposure (Fig. 3D), but had no effect on mGluR5 protein levels (Fig. 3E).

3.4. Homer1/mGluR1 mediates the LPA-induced ER stress

To further elucidate the role of Homer1 in LPA-induced neurotoxicity, cortical neurons were transfected with Si-Homer1, and the results showed that the expression of Homer1 was significantly inhibited by Si-Homer1 transfection (Fig. 4A). After transfection, we repeated western blot assay at 24 h after LPA exposure (Fig. 4B). The results showed that LPA-induced expression of GRP78 (Fig. 4B), CHOP (Fig. 4D), as well as the cleavage of caspase-12 (Fig. 4D) were attenuated by Homer1 knockdown. As shown in Fig. 4F and G, similar results on neuronal viability and LDH release were also observed. In addition, we used the mGluR1 antagonist 1-aminoindan-1,5-dicarboxylic acid (AIDA) and the mGluR5 antagonist 2-Methyl-6-phenylethynyl-pyridine (MPEP) to determine the role of group I mGluRs (Marchetti et al., 2003). We found that the LPA-induced loss of neuronal viability (Fig. 4F) and LDH release (Fig. 4G) were reduced by AIDA, but not by MPEP.

3.5. LPA disrupts intracellular Ca^{2+} homeostasis via Homer1/mGluR1

Intracellular Ca^{2+} release has been demonstrated to contribute to neuronal injury induced by various toxic agents. Thus, we performed Ca^{2+} imaging in Ca^{2+} free buffer solution in cortical neurons. LPA induced a significant increase in intracellular Ca^{2+} levels, which was attenuated by the protein misfolding inhibitor 4-PBA (Fig. 5A). Transfection with Si-Homer1 markedly suppressed the Ca^{2+} response induced by LPA compared to Si-control group (Fig. 5B). Furthermore, the LPA-induced intracellular Ca^{2+} release was nullified by the mGluR1 antagonist AIDA (100 μM), but not by the mGluR5 antagonist MPEP (3 μM, Fig. 5C). As shown in Fig. 5D, the LPA-induced Ca^{2+} response was attenuated by the inositol triphosphate receptor (IP_3R) inhibitor xestospongin (Xes, 800 nM), but the ryanodine receptor (RyR) inhibitor ryanodine (100 μM) had no such effect (Huang et al., 2017).

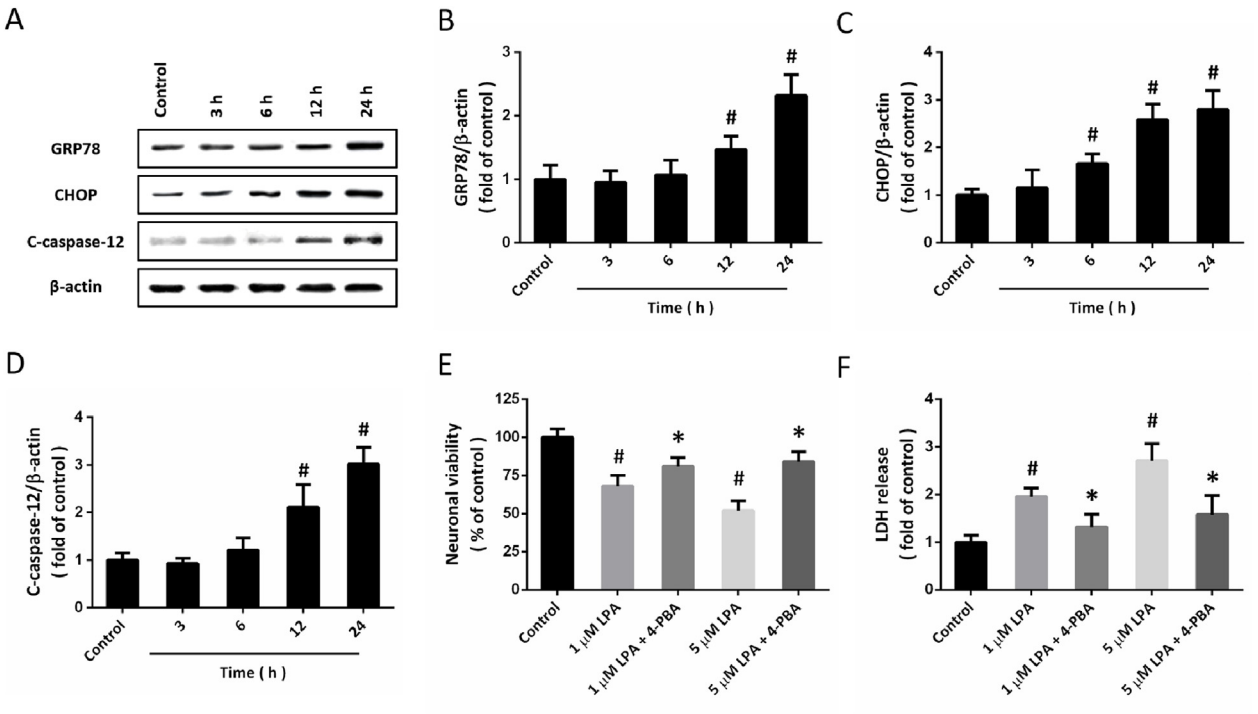

Fig. 2. LPA induces ER stress in cortical neurons. (A–D) Western blot (A) and quantification show that LPA increased the expression of GRP78 (B), CHOP (C) and cleaved-caspase-12 (D) in a time-dependent manner. (E) WST-1 assay shows that 4-PBA preserved neuronal viability after LPA treatment. (F) LDH release shows that 4-PBA reduced LDH release after LPA treatment. Data are shown as mean ± SEM (n = 6). $^{\#}p < 0.05$ vs. Control group. $^{*}p < 0.05$ vs. LPA group.

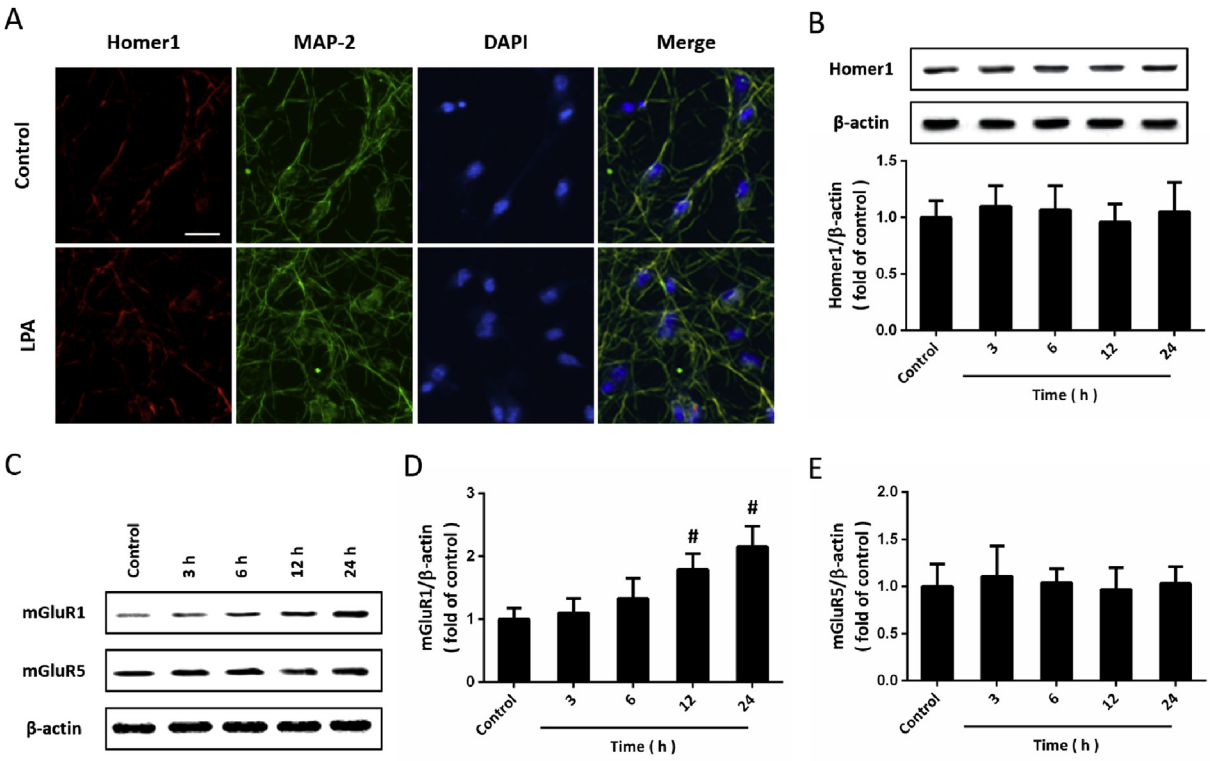

Fig. 3. Effects of LPA on Homer1 and mGluR1 expression. (A) Immunostaining with Homer1 and MAP-2 antibodies shows that the distribution of Homer1 was unaffected by LPA. (B) Western blot shows that LPA treatment did not alter the expression of Homer1 up to 24 h. (C–E) Western blot (C) and quantification show that LPA increased the expression of mGluR1 (D), with no effect on mGluR5 expression (E). Scale bar, 50 μm. Data are shown as mean ± SEM (n = 6). $^{\#}p < 0.05$ vs. Control group.

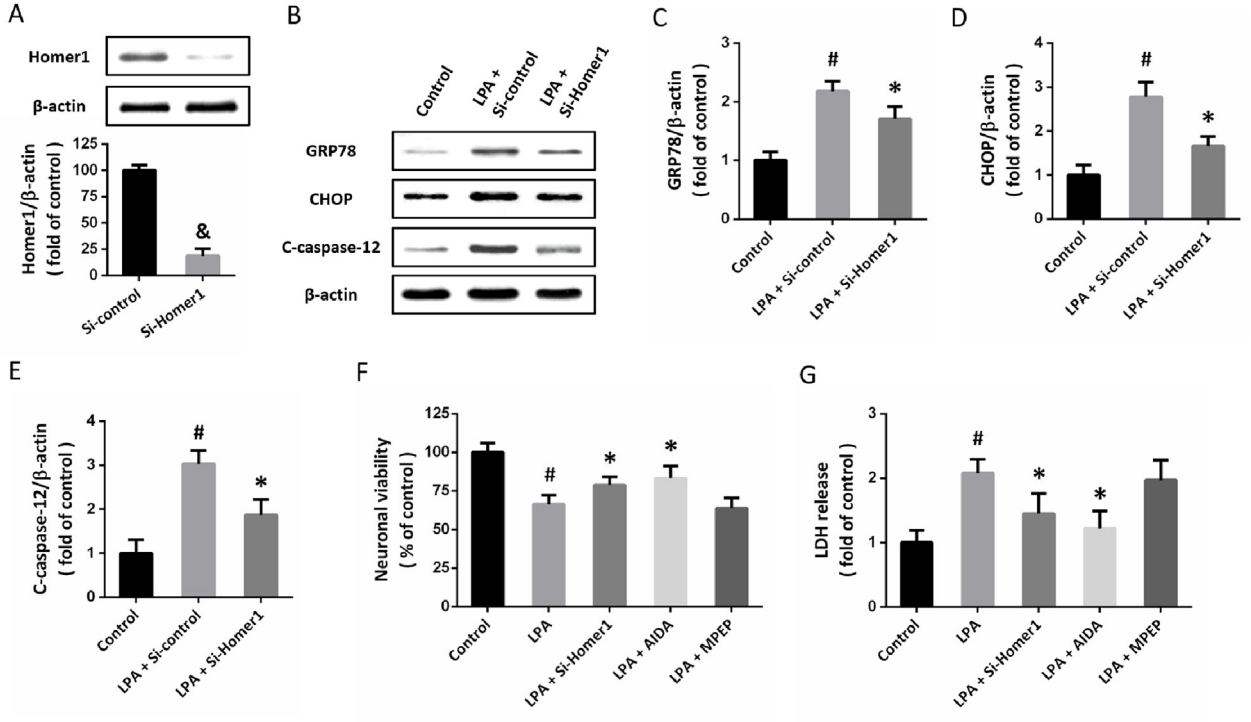

Fig. 4. Homer1/mGluR1 mediates the LPA-induced ER stress. (A) Western blot shows that transfection with Si-Homer1 effectively reduced the expression of Homer1 in cortical neurons. (B–E) Western blot (B) and quantification show that knockdown of Homer1 partially reversed the LPA-induced expression of GRP78 (C), CHOP (D) and cleaved-caspase-12 (E). (F) WST-1 assay shows that knockdown of Homer1 and the mGluR1 antagonist AIDA preserved neuronal viability after LPA treatment. (G) LDH release shows that knockdown of Homer1 and the mGluR1 antagonist AIDA reduced LDH release after LPA treatment (F). Data are shown as mean ± SEM (n = 6). $^{\#}p < 0.05$ vs. Control group. $^{*}p < 0.05$ vs. LPA group.

4. Discussion

The object of this study was to use the leverage of an in vitro model of primary cultured cortical neurons to determine the molecular mechanisms underlying the LPA-induced neuronal injury. The results showed that (a) LPA induces neurotoxicity in a dose-dependent manner in cortical neurons; (b) LPA activates ER stress associated factors; (c) LPA increases the expression of mGluR1, but not mGluR5 or Homer1; (d) knockdown of Homer1 attenuates the LPA-induced ER stress and neuronal injury; and (e) mechanistically, LPA promotes intracellular Ca^{2+} release through the Homer1/mGluR1 pathway.

Under physiological conditions, relative low level of LPA regulates vascular development, neurogenesis, immunity, reproduction and would healing. However, significant increased levels of LPA can be observed in various pathological conditions, including cancer, atherosclerosis and brain injury (Eder et al., 2000; Siess et al., 1999). A previous study showed that the levels of LPA ranged from 10 pmol/mg to 49 pmol/mg in atheromatous plaques and from 1.2 pmol/mg to 2.8 pmol/mg in normal arterial tissue (Tigyi et al., 1995). It is known that this increased expression pattern after injury can be a detrimental

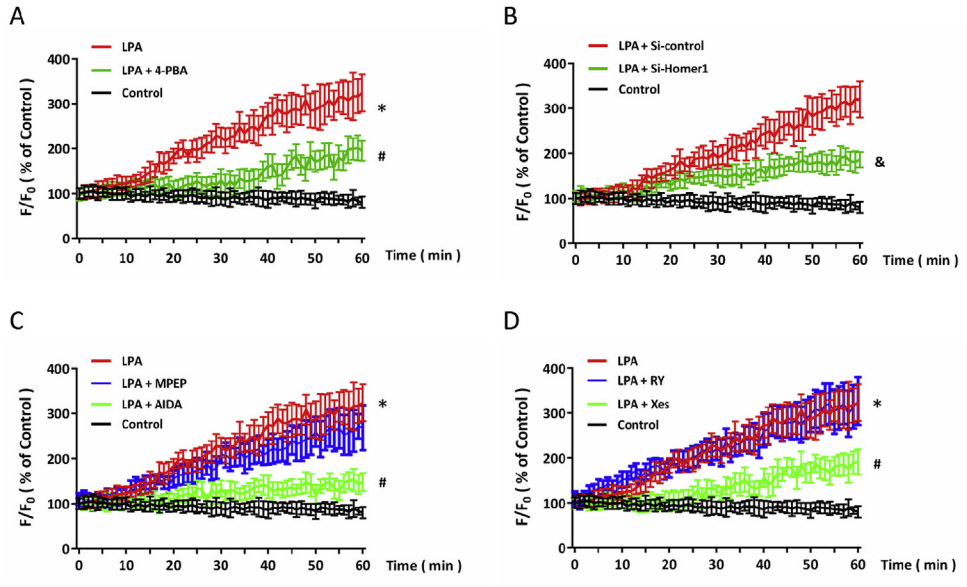

Fig. 5. LPA disrupts intracellular Ca^{2+} homeostasis via Homer1/mGluR1. Ca^{2+} imaging was performed under Ca^{2+}-free solution to determine intracellular Ca^{2+} release. (A) Ca^{2+} imaging shows that 4-PBA attenuated LPA-induced intracellular Ca^{2+} release in cortical neurons. (B) Ca^{2+} imaging shows that downregulation of Homer1 attenuated LPA-induced intracellular Ca^{2+} release. (C) Ca^{2+} imaging shows that AIDA, not MPEP, reversed LPA-induced regulation of intracellular Ca^{2+} release. (D) Ca^{2+} imaging shows that Xes, not RY, reversed LPA-induced regulation of intracellular Ca^{2+} release. Data are shown as mean ± SEM (n = 6). $^{*}p < 0.05$ vs. Control group, $^{\#}p < 0.05$ vs. LPA group, $^{\&}p < 0.05$ vs. LPA + Si-Homer1 group.

signaling, or may be an endogenous protective mechanism. Congruently, the exact effect of LPA on neuronal apoptosis and survival is contradictory. A recent study demonstrated that LPA mediated neuronal cell death through activation of asparagine endopeptidase in cerebral ischemia-reperfusion injury (Wang et al., 2018). The authors showed that the toxic effects could be observed at higher than 0.25 μM in neurons and at higher than 12 μM in PC12 cells. In the present study, our results showed that LPA at the concentrations of 0.5–5 μM significantly decreased neuronal viability and increased neuronal apoptosis in cortical neurons. All these data suggested that high concentrations of LPA induced neuronal injury in primary cultured neurons.

ER stress is an elaborate cellular mechanism essential for cell function and survival. Under stress conditions, the accumulation of unfolded proteins activates the UPR to restore ER function, but apoptotic cell death occurs when the ER stress is prolonged or too severe (Chen et al., 2012b; Stefani et al., 2012). The UPR is mediated by several ER transmembrane receptors, most of which are maintained in an inactivate state via interaction with the ER chaperone GRP78 in resting condition (Gorman et al., 2012). In this study, the level of GRP78 was found to be increased by LPA treatment at 12 and 24 h. Congruently, we also observed increased expression of CHOP and augmented cleavage of caspase-12. CHOP is a member of the C/EBP family of transcription factors, and CHOP deficiency was found to provide resistance to ER stress-induced apoptosis (Li et al., 2014). Caspase-12 functions as an initiator caspase that activates other downstream caspases during ER stress (Szegezdi et al., 2003). To confirm the involvement of ER stress in our in vitro model, we repeated the neuronal viability and LDH release assay using the protein misfolding inhibitor 4-PBA. The protective effects of 4-PBA against the LPA-induced neuronal injury strongly supported that the ER stress-associated apoptosis is one of the molecular mechanisms underlying LPA-induced neurotoxicity.

Previous studies showed that the mGluR1 antagonist exerted protective effects against excitotoxic neuronal injury, but inhibition of mGluR5 could cause controversial effects in different experimental paradigms (Chen et al., 2012a, 2012c). In this study, we found that LPA increased the expression of mGluR1 at 12 and 24 h, but had no effect on mGluR5. Thus, we repeated neuronal viability and LDH release assay using the mGluR1 antagonist AIDA and the mGluR5 antagonist MPEP. As expected, the LPA-induced neuronal injury was significantly attenuated by AIDA, but not by MPEP. Intriguingly, the LPA-induced neuronal injury was not aggravated by MPEP, indicating that neither protective nor detrimental effects of mGluR5 was observed in our in vitro model. It is well known that the ER Ca^{2+} release mediated by activation of group I mGluRs could contribute to intracellular Ca^{2+} overload under neurological disorders (Zhang et al., 2015, 2018). Our results of Ca^{2+} imaging showed that the LPA-induced Ca^{2+} was sharply attenuated by AIDA, but not by MPEP treatment. All these data suggested that the mGluR1-dependent regulation of intracellular Ca^{2+} metabolism via ER Ca^{2+} release partially mediated the LPA-induced neurotoxicity.

Homer1, also known as Ves1, has been demonstrated to be a key Ca^{2+} regulator that binds and anchors the mGluRs, IP_3Rs, RyRs and canonical transient receptor potential (TRPC) channels (Grinevich et al., 2012; Luo et al., 2012). Knockdown of Homer1 was shown to protect dopamine neurons against MPP^+-induced intracellular Ca^{2+} overload through regulating L-type channels, TRPC channels and the IP_3R in the ER (Chen et al., 2013). A previous study showed that downregulation of Homer1b/c, a constitutively expressed long form of Homer1 protein, attenuated glutamate-induced excitotoxicity via ER and mitochondrial pathway in cortical neurons (Chen et al., 2012b). This protection was found to be associated with the reduced IP_3 content, ER Ca^{2+} release and group I mGluRs. In our present study, no obvious effect on Honer1 expression was found in neurons after LPA treatment. However, LPA-induced neuronal injury and ER stress were partially prevented by si-Homer1 transfection. The ER Ca^{2+} release after LPA treatment was attenuated by Homer1 knockdown and inhibition of mGluR1 (not mGluR5). All these data strongly indicated that LPA induced neuronal injury via the Homer1/mGluR1 pathway in our in vitro model.

In summary, our results showed that LPA treatment induced ER stress and apoptosis, which contribute to its neurotoxicity in cortical neurons. In addition, LPA exposure regulated intracellular Ca^{2+} homeostasis via the Homer1/mGluR1-mediated Ca^{2+} release from the ER.

Conflicts of interest

There is no conflict of interest.

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