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ORIGINAL PAPER

Protective effect of Homer 1a on tumor necrosis factor-α with cycloheximide-induced apoptosis is mediated by mitogen-activated protein kinase pathways

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Abstract Although Homer 1, of the postsynaptic density, regulates apoptosis, the signaling mechanisms are not fully elucidated. In this study, we found that tumor necrosis factor-α (TNF-α)/cycloheximide (CHX) treatment transiently increased Homer 1a (the short variant of Homer 1), but did not affect Homer 1b/c (the long variant of Homer 1). Overexpression of Homer 1a blocked TNF-α/CHX-induced apoptotic cell death, whereas inhibition of Homer 1a induction enhanced the pro-apoptotic effect of TNF-α/CHX treatment. Moreover, brain-derived neurotrophic factor, as a potential activator of endogenous Homer 1a, inhibited apoptotic cell death after TNF-α/CHX treatment through induction of Homer 1a. Since three major mitogen-activated protein kinase (MAPK) pathways have important roles in apoptosis, we examined if Homer 1a is involved in the effects of MAPK pathways on apoptosis. It was shown that inhibition of the ERK1/2 pathway increased the expression and the protective effect of Homer 1a, but inhibition of the p38 pathway produced the opposite effect. Cross-talk among MAPK pathways was also associated with the regulation of Homer 1a during apoptotic cell death. Blocking the p38 pathway increased the activity in the ERK1/2 pathway, while inhibition of ERK1/2 pathway abolished the effect of p38 inhibitor on Homer 1a. Furthermore, Homer 1a reversely affected the activation of MAPK pathways. These findings suggest that Homer 1a plays an important role in the prevention of apoptotic cell death and contributes to distinct regulatory effects of MAPK pathways on apoptotic cell death.

Keywords Homer protein · Postsynaptic density · Mitogen-activated protein kinase · Apoptosis · Brain-derived neurotrophic factor

Peng Luo and Yongbo Zhao have contributed equally to this work.

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Introduction

Homer 1, also called vesl-1, is a gene encoding an Enabled/vasodilator-stimulated phosphoprotein (Ena/VASP) homology 1 (EVH1) family-related protein that belongs to the Homer family of postsynaptic density (PSD) proteins [1, 2]. Homer 1a (also called vesl-1S) is a short variant of Homer 1 with two conserved domains, the EVH1 domain and a proline-containing motif (P motif). As a long variant of Homer 1, Homer 1b/c (also called vesl-1L) has a coiled-coil structure at carboxy-terminal domain along with the EVH1 domain and P motif [2–5]. The differences between Homer 1a and Homer 1b/c in structure define the roles that the two Homer 1 variants play in regulating postsynaptic structure and signal transduction. Homer 1b/c is able to form dimers via its carboxy-terminal domain, and it binds different target proteins, such as metabotropic glutamate receptors (mGluRs) and inositol

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1,4,5-trisphosphate receptor (IP_3R) [3, 6], which are functionally associated with each other, through its EVH1 domain. Homer 1a can also bind the same proteins as Homer 1b/c via its conserved domain, but the linkage of two target proteins cannot occur because Homer 1a lacks a coiled-coil structure. Therefore, Homer 1a serves as a dominant-negative protein in the regulation of Homer 1b/c-associated protein–protein interactions and its downstream signaling pathway [7].

Under physiological conditions, Homer 1 regulates the alteration of intracellular calcium (Ca^{2+}) [8–10] and synaptic plasticity [11–14]. Furthermore, Homer 1 is involved in neuropathic and inflammatory pain [15, 16], mental retardation syndromes [17, 18], Alzheimer's disease [19, 20], addiction [21, 22], and traumatic brain injury [23]. However, the function of Homer 1 in apoptotic cell death is controversial. In rat neurons, the formation of the mGluR-Homer 1b/c- phosphoinositide 3 kinase enhancer (PIKE) complex leads to activation of phosphoinositide 3 kinase (PI3K) and prevents apoptosis [24]. Moreover, Hippi, as a cell death-promoting protein, induces apoptosis in striatal neurons by complexing with Hip1 and its pro-apoptotic effect can be prevented when Homer 1c was co-expressed in cultured striatal neurons [25]. On the contrary, Homer 1 increases the susceptibility to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptotic cell death in HEK293 cells [26]. Down-regulation of Homer 1b/c inhibits glutamate-induced excitotoxicity and apoptosis in cortical neurons [27]. The role of Homer 1a in apoptotic cell death has not been previously described.

Tumor necrosis factor-α (TNF-α) is widely used as a potent apoptotic cell death inducer. However, TNF-α alone cannot induce apoptosis in some cell lines. Cycloheximide (CHX), a protein synthesis inhibitor, is sometimes required for TNF-α-induced apoptotic cell death [28–30]. Through TNF receptors, TNF-α can trigger activation of mitogen-activated protein kinase (MAPK) pathways, such as the extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK), and the p38 pathways [31–33]. Although activation of the ERK1/2 signaling pathway has been shown to regulate induction of Homer 1a [34, 35], the relationship between MAPK pathways and Homer 1 in apoptosis is unclear.

Here, we tested the function of Homer 1a in apoptotic cell death. We showed that Homer 1a expression was induced by TNF-α/CHX-induced apoptotic cell death while the expression of Homer 1b/c was constant. We further demonstrated that up-regulation of Homer 1a inhibited TNF-α/CHX-induced apoptotic cell death, whereas down-regulation of Homer 1a blocked its protective effect and increased apoptotic cell death. The expression and function of Homer 1a is regulated via MAPK pathways. Moreover, Homer 1a reversely affected the activation of MAPK pathways. These findings suggested that Homer 1a has potential anti-apoptotic effects on PC12 cells, thus providing a promising new strategy for the treatment of neurological diseases.

Materials and methods

Cell lines and reagents

Rat PC12 cells (adrenal gland; pheochromocytoma) were obtained from the Institute of Biochemistry and Cell Biology, SIBS, CAS. PC12 cells were cultured in Dulbecco's modified Eagle's medium (Gibicon) plus 10 % fetal bovine serum (Hyclone Laboratories, Logan, UT). Rat recombinant TNF-α and BDNF were obtained from PeproTech Inc. PD98059 was obtained from Invitrogen. Cycloheximide and Dimethyl sulfoxide were obtained from Sigma. SP600125 and SB203580 were obtained from Calbiochem.

Antibodies

Primary antibodies to Homer 1a and Homer 1b/c were obtained from Santa Cruz Biotechnology Inc. Antibodies to caspase-3, cleaved-caspase-3, ERK1/2, phospho-ERK1/2, p38 MAPK, phospho-p38 MAPK, JNK, and phospho-JNK were obtained from Cell Signaling Technology. Antibody to β-actin was obtained from Sigma. Antibody to tubulin was obtained from Bioworlde. The secondary antibodies for Western blot were HRP conjugated anti-rabbit, anti-mouse, and anti-goat IgG (Santa Cruz Biotechnology). The secondary antibodies for immunofluorescence were Alex 488 donkey-anti-goat and Alex 594 donkey-anti-rabbit (Invitrogen).

Western blot analysis

After various treatments, PC12 cells in 6 cm dishes were washed with ice-cold PBS for three times and lysed with a lysis buffer with protease inhibitor mixture tablets and phosphatase inhibitor mixture tablets PhosSTOP (Roche Applied Science). Protein concentration of the supernatant was determined by using BCA protein kit. The proteins were separated by 10–15 % and 10 % SDS-PAGE gels and transferred to nitrocellulose membranes (Invitrogen). The membranes were soaked in 5 % nonfat milk solution in tris-buffered saline with 0.05 % Tween 20 (TBST) for 1 h at room temperature and then incubated overnight at 4 °C with the appropriate primary antibody (Homer 1a, 1:200; Homer 1b/c, 1:1,000; caspase-3, 1:1,000; cleaved-caspase-3, 1:1,000; ERK1/2, 1:1,000; phospho-ERK1/2, 1:500; p38 MAPK, 1:500; phospho-p38 MAPK, 1:200; JNK, 1:800; phospho-JNK, 1:500; β-actin, 1:2,500; and tubulin, 1:1,200). Membranes were washed in TBST and incubated

for 1 h at room temperature with the secondary antibodies diluted in blocking buffer. Immunoreactivity was detected by SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). The membranes were stripped for 15 min at room temperature with Re-blot Plus Strong Solution (Chemicon) and retested with tubulin or β-actin antibodies to normalize for protein loading. The optical densities of the bands were quantified by using an image analysis system with ImageJ (Scion Corporation).

Immunocytochemistry

After fixed with 4 % paraformaldehyde for 15 min at room temperature, PC12 cells were washed with phosphate-buffered saline (PBS) and permeabilized with 0.2 % Triton X-100, followed incubation of primary antibodies overnight at 4 °C. Primary antibodies were diluted as follow: Homer 1a (1:20) and Homer 1b/c (1:100). Then, cells were incubated with secondary antibodies (Alexa 488 donkey-anti-goat, Invitrogen, 1:300; Alexa-594-conjugated goat-anti-mouse, 1:300) for 2 h. Cultures were dehydrated with ethanol and mounted with 4,6-diamidino-2-phenylindole (DAPI) for nuclear staining (Sigma). Images were captured using an Olympus fluorescence microscope (Japan, Tokyo). All images of one experiment were acquired using the same exposure time to allow comparisons of relative levels of immunoreactivity between the different treatment conditions. At least six images of each group were taken by an evaluator blinded to the experimental conditions.

Cell viability assay

Cell viability assay was performed by using The Cell Proliferation Reagent WST-1 (Roche) following the manufactory protocol. Culture cells (at a concentration of $0.5–5 \times 10^4$) in microplates (tissue culture grade, 96 wells, flat bottom) in a final volume of 100 µl/well culture medium. After treatment, 10 µl Cell Proliferation Reagent WST-1 was added to each well and incubate for 4 h at 37 °C and 5 % CO_2. Add 100 µl/well culture medium and 10 µl Cell Proliferation Reagent WST-1 as used in the experiment into one well (e.g., 100 µl culture medium plus 10 µl Cell Proliferation Reagent WST-1). Use this background control (absorbance of culture medium plus WST-1 in the absence of cells) as a blank position for the ELISA reader. Shake thoroughly for 1 min on a shaker and measure the absorbance of the samples against a background control as blank using a microplate (ELISA) reader.

Hoechst and propidium iodide (PI) stain

Following exposure of PC12 cells to the various treatments, cell death rate was quantified and presented as percent of dead cells. Cell death rate was determined as the ratio of live-to-dead cells compared with the percent cell death in control wells to account for cell death attributable to mechanical stimulation of the cultures. Quantification of cell death rate was determined by staining treated cultures with 5 µM Hoechst 33342 and 2 µM PI (Sigma) for 10 min. Culture plates were placed on a mechanized stage of a Nikon microscope and photomicrographs were collected by a blinded observer. The numbers of total and dead (PI positive) cells were counted by automated computer assisted software (Image-Pro Plus version 6.0, Media Cybernetics, Inc). The raw counts are presented in an Excel file for generation of percent cell death and statistical analysis.

Lentivirus construction and transfection

The coding sequence of Homer 1a was amplified by RT-PCR. The primer sequences were: forward, 5′-GAGGATCCC CGGGTACCGGTCGCCACCATGGGGGAGCAACCTA TCTTC-3′; reverse, 5′-TCATCCTTGTAGTCGCTAGCCT TAATCATGATTGCTGAATTGAATG-3′. The PCR fragments and the pGC-FU-EGFP-3FLAG plasmid (Shanghai GeneChem) were digested with Age I/Nhe I and then ligated with T4 DNA ligase to produce pGC-FU-Homer 1a-3FLAG. To generate the recombinant lentiviral vector expressed Homer 1a (LV-H1a), 293T cells were co-transfected with of the pGC-FU-EGFP-3FLAG plasmid (Shanghai GeneChem) (20 µg) with a cDNA encoding Homer 1a, pHelper1.0 plasmid (15 µg), and pHelper 2.0 plasmid (10 µg) by using Lipofectamine 2000 (Invitrogen) (100 µl). After 48 h, supernatant was harvested from and the viral titer was calculated by transducing 293T cells. As a control, we also generated a lentiviral vector that expresses GFP alone (LV-Con). Following 72 h transfection, PC12 cells were subjected to various measurements.

Short interfering RNA and transfection

The sequence of Homer 1a short interfering RNA (siRNA) was as follows: 5′-GCATGCAGTTACTGTATCT-3′. Control siRNA was 5′-UUCUCCGAACGUGUCACGU-3′, which should not knockdown any known proteins. The above siRNA molecules were chemically synthesized by Shanghai Genechem Company. The Homer 1a specific siRNA and control siRNA were transfected with Lipofectamine 2000 (Invitrogen) in 6-well plates. Following 48 h transfection, PC12 cells were subjected to various measurements.

Statistical analysis

All of the experiments were performed a minimum of three times. Statistical evaluation was done with GraphPad Prism

software, version 5.0 (GraphPad, San Diego, CA). Significant differences between experiments were assessed by univariate ANOVA (more than two groups) followed by Bonferroni's multiple comparisons or unpaired t test (two groups).

Results

TNF-α induces apoptotic cell death in PC12 cells

PC12 cells were incubated in the presence of CHX (10 μg/ml), TNF-α (10 ng/ml), or TNF-α (10 ng/ml) with CHX (10 μg/ml) (TNF-α/CHX) for 24 h. The cell viability and cytotoxicity were assessed at different time points (control, 1, 3, 6, 12, and 24 h). Effects on cell viability were not observed after exposure to CHX at any time point, while cell viability only slightly decreased at 24 h after exposure to TNF-α (Fig. 1a). A time-dependent cell death was observed after exposure to TNF-α/CHX. Cell viability decreased at 6, 12, and 24 h (Fig. 1a). Cytotoxicity assessment (PI stain) indicated that TNF-α/CHX induced cell death after 6 h (Fig. 1b). These results suggested that TNF-α significantly induced cell death of PC12 cells in the presence of CHX.

We then examined TNF-α/CHX-induced apoptosis in PC12 cells. PC12 cells analyzed with Hoechst stain showed the typical morphology of apoptosis (chromatin condensation and nuclear fragmentation) (Fig. 1c). A time-dependent activation of caspase-3 was also observed in cells treated with TNF-α/CHX (Fig. 1d, e). These results indicated that TNF-α/CHX treatment could induce apoptotic cell death in PC12 cells.

Time-dependent regulation of Homer 1a during apoptotic cell death in PC12 cells

To confirm the expression of Homer 1 during apoptosis, we examined endogenous levels of Homer 1a and Homer 1b/c protein in PC12 cells at different time points during their treatment with TNF-α/CHX. The level of Homer 1a protein increased significantly within 30 min of the start of TNF-α/CHX treatment (Fig. 2a, b). Homer 1b/c protein levels did not change significantly following TNF-α/CHX treatment (Fig. 2a, c). To clarify the role of TNF-α and CHX in inducing the expression of Homer 1, PC12 cells were treated with CHX, TNF-α, and TNF-α/CHX. Neither TNF-α treatment nor CHX treatment alone significantly induced the expression of Homer 1a and Homer 1b/c (Fig. S1).

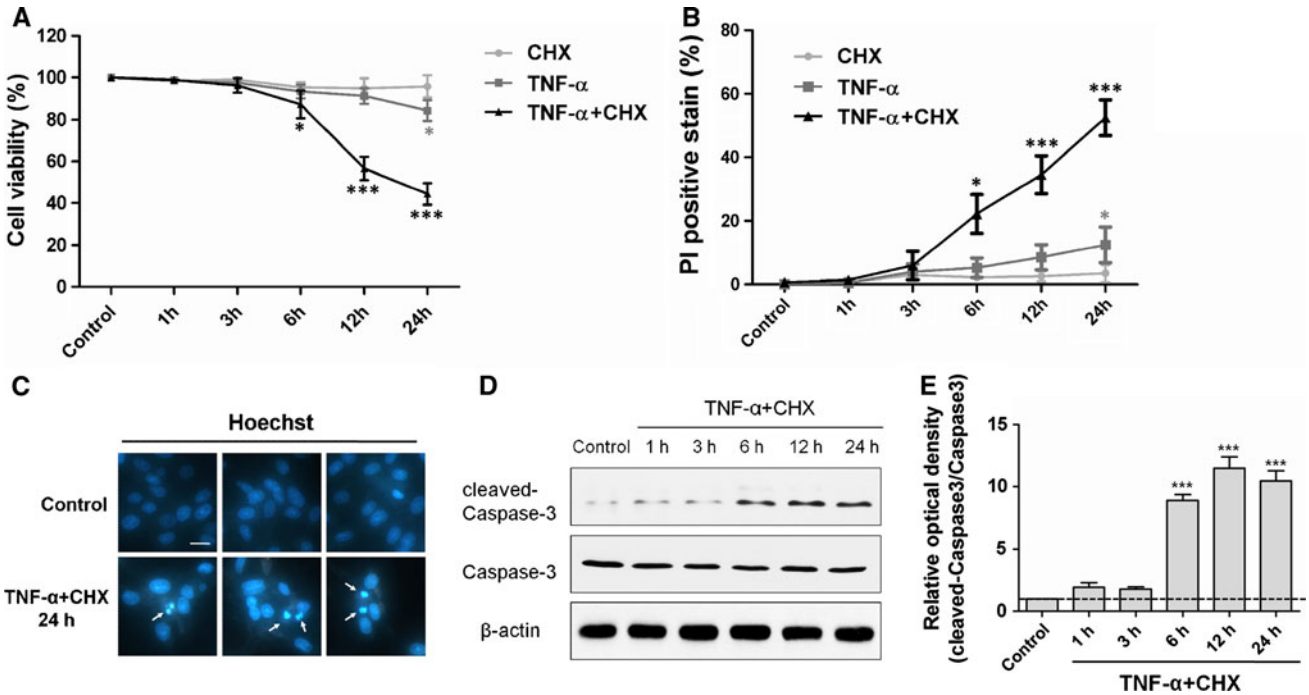

Fig. 1 TNF-α/CHX-induced apoptotic cell death in PC12 cells. PC12 cells were treated with CHX, TNF-α, and TNF-α/CHX for the time indicated, respectively. **a** Cell viability was assayed by WST. **b** The cytotoxicity was calculated by the ratio of PI positive cells and total cells. **c** The morphological change of nuclei was detected by Hoechst stain. *Scale bar* = 20 μm. **d** Western blot analysis for pro-apoptotic protein. PC12 cells were treated with TNF-α/CHX for time indicated. **e** The quantitative analysis of **d** was shown in the *bar graphs*. The *bars* are the means and SE of five independent experiments. * $p < 0.05$ and *** $p < 0.001$ by using Bonferroni's test after ANOVA

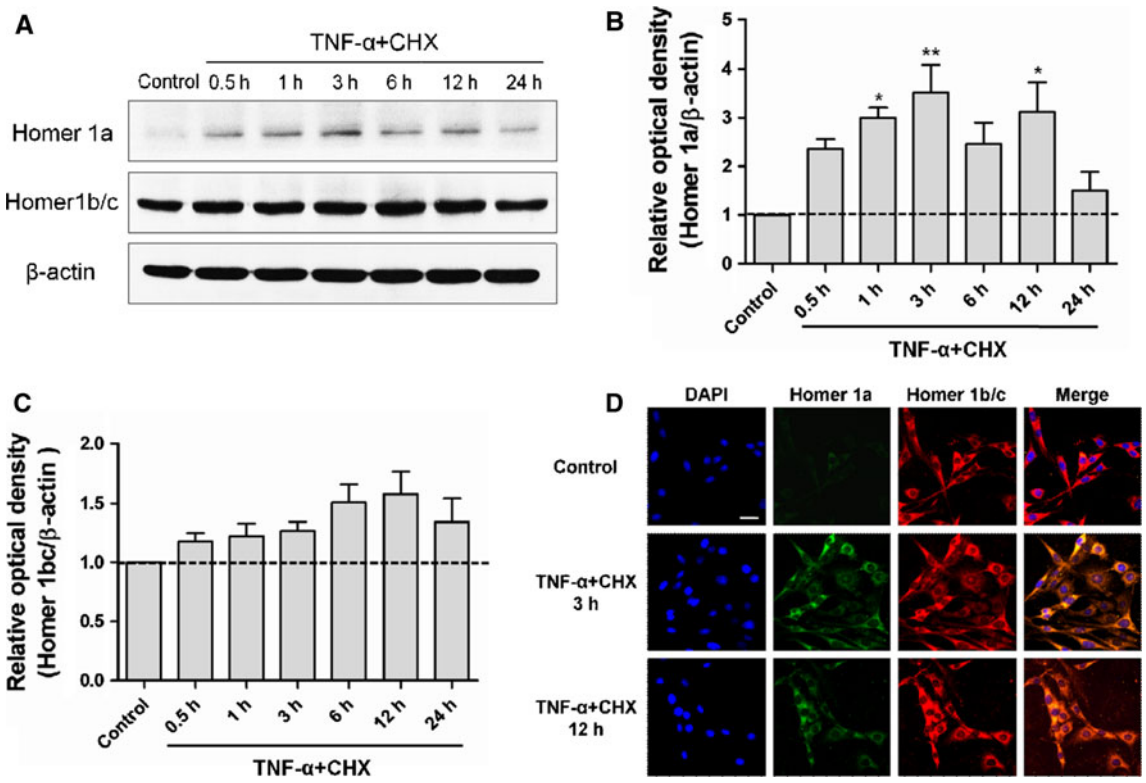

Fig. 2 The expression of Homer 1a and Homer 1b/c after TNF-α/CHX treatment. **a** Western blot analysis for Homer 1a and Homer 1b/c after treatment with TNF-α/CHX for time indicated. β-actin was used as loading control. **b**, **c** The quantitative analysis of **a** was shown in the *bar graphs*. **d** Immunofluorescence stain for Homer 1a and Homer 1b/c. *Scale bar* = 40 μm. After treatment of TNF-α/CHX for indicated time, PC12 cells were stained by Homer 1a and Homer 1bc. The nuclei were stained by DAPI. The images indicated that both Homer 1a and Homer 1b/c were located at cytoplasm and overlaid. The *bars* are the means and SE of five independent experiments. * $p < 0.05$ and ** $p < 0.01$, by using Bonferroni's test after ANOVA

To clarify the distribution of Homer 1a and Homer 1b/c during apoptosis, we detected the localization of Homer 1a and Homer 1b/c at different time points during treatment with TNF-α/CHX. PC12 cells showed strong Homer 1b/c immunoreactivity at each time points in the cytoplasma without significant alteration. Homer 1a immunoreactivity was also observed in the cytoplasma and changed at different time points. The overlay of Homer 1a and Homer 1b/c was demonstrated with Homer 1a and Homer 1b/c double staining (Fig. 2d).

Induction of Homer 1a reduces apoptotic cell death

We found that expression of Homer 1a was significantly increased during TNF-α/CHX-induced apoptotic cell death. To investigate the biological functions of Homer 1a in apoptotic cell death, PC12 cells were transfected with lentiviral vector expressed Homer 1a (LV-H1a) or control lentiviral vector (LV-Con) (Fig. 3a, b, and S2). Then, we evaluated the effect of the overexpression of Homer 1a on TNF-α/CHX-induced apoptotic cell death. After treatment with TNF-α/CHX for 12 h, we found that overexpression of Homer 1a by transfection with LV-H1a improved the viability of PC12 cells (Fig. 3c), reduced cytotoxicity (Fig. 3d), and inhibited the activation of caspase-3 (Fig. 3e, f). These results suggested up-regulation of Homer 1a by transfection inhibited TNF-α/CHX-induced apoptotic cell death.

On the other hand, we also examined the effect of down-regulation of Homer 1a on PC12 cells after TNF-α/CHX treatment. PC12 cells were transfected with Homer 1a specific siRNA (si-H1a) and control siRNA (si-Con) (Fig. 4a, b). Down-regulation of Homer 1a attenuated viability of PC12 cells (Fig. 4c), increased cytotoxicity (Fig. 4d), and elevated the activation of caspase-3 (Fig. 4e, f) after TNF-α/CHX treatment. These results indicated that down-regulation of Homer 1a aggravated the apoptotic cell death, which meant that Homer 1a played a protective role in TNF-α/CHX-induced apoptotic cell death.

Homer 1a is involved in the BDNF protection against apoptosis

Because induction of Homer 1a reduced the apoptotic cell death, we investigated whether some protective agents could inhibit TNF-α/CHX-induced apoptotic cell death by induction of Homer 1a. In previous studies, BDNF has been used as an activator of endogenous Homer 1a. Here,

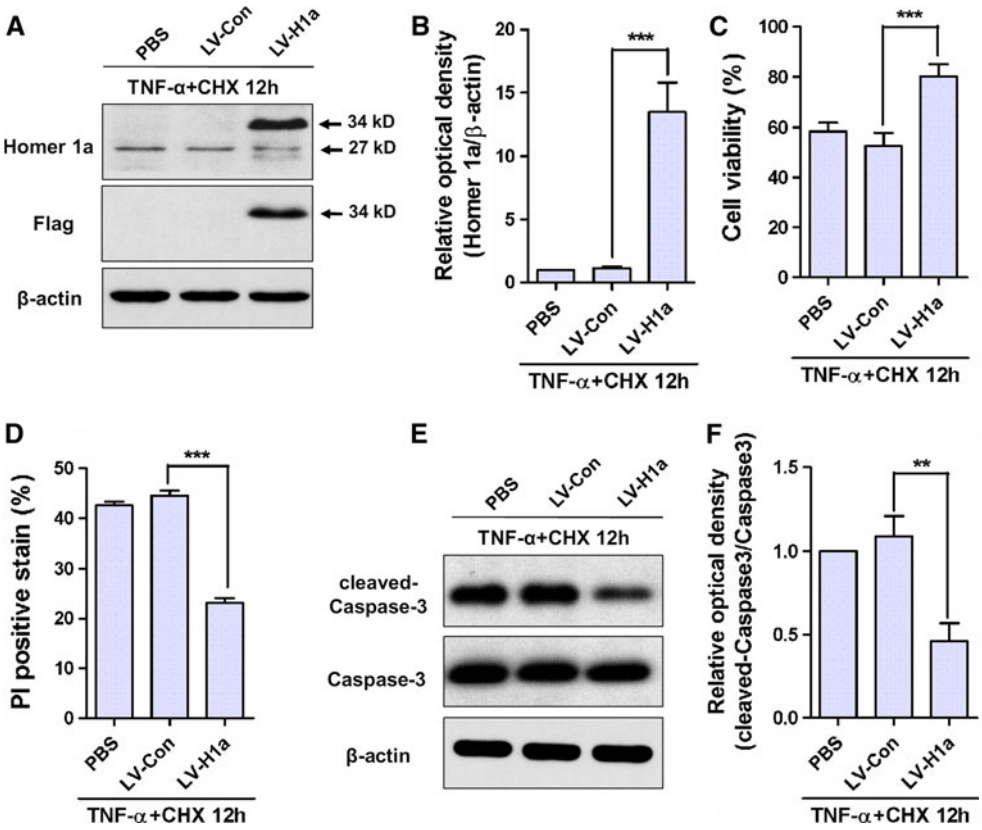

Fig. 3 Up-regulation of Homer 1a prevents the apoptotic cell death induced by TNF-α/CHX. PC12 cells were infected with LV-H1a vectors expressed Homer 1a. LV-Con was used as a vehicle control. **a** Western blot analysis for Homer 1a and Flag after treatment with TNF-α/CHX for 12 h. β-actin was used as loading control. **b** The quantitative analysis of **a** was shown in the *bar graphs*. **c**, **d** After treatment with TNF-α/CHX for 12 h, cell viability and cytotoxicity were measured by WST and PI staining. **e** Western blot analysis for cleaved-caspase-3 and caspase-3. **f** The quantitative analysis for **e** was shown in the *bar graphs*. The *bars* are the means and SE of five independent experiments. ** $p < 0.01$ and *** $p < 0.001$ by using *t* test

we found that endogenous expression of Homer 1a was significantly increased after administration of BDNF at concentrations of 50, 100, and 200 ng/ml (Fig. 5a, b). The expression of Homer 1b/c was not affected by BDNF (Fig. 5a, c). These results suggested that BDNF activated the expression of Homer 1a in a concentration-dependent manner in PC12 cells.

Since BDNF induced the expression of Homer 1a, we speculated that Homer 1a might contribute to the anti-apoptotic effect of BDNF (Fig. S3). To test our speculation, following transfection with si-H1a or si-Con, PC12 cells were pretreated with BDNF and then treated with TNF-α/CHX. In agreement with our speculation, we found that viability of PC12 cells transfected with si-H1a was lower than that of PC12 cells transfected with si-Con (Fig. 5d), while cytotoxicity and expression of active caspase-3 of PC12 cells transfected with si-Con were higher than that of PC12 cells transfected with si-Con (Fig. 5e–g). These results indicated that BDNF could inhibit TNF-α/CHX-induced apoptotic cell death by induction of Homer 1a.

TNF-α-mediated activation of MAPK pathways regulates apoptotic cell death

MAPK pathways, such as ERK1/2, JNK, and p38, are thought to play an important role in the regulation of apoptotic cell death. We examined the changes in MAPK pathways after TNF-α/CHX treatment and found that the ERK1/2, JNK, and p38 pathways showed activity at different time points (Fig. 6a–d). To elucidate the effects of TNF-α and CHX on the activation of MAPK pathways, we observed the activation of ERK1/2, JNK, and p38 at 6 and 12 h after treatment with CHX, TNF-α, and TNF-α/CHX. We found that CHX treatment did not induce significant activation of MAPK pathways, while TNF-α treatment, like TNF-α/CHX treatment, activated MAPK pathways at both time points (Fig. S4). These results showed that TNF-α was mainly responsible for the activation of MAPK pathways.

Next, we examined the effects of MAPK inhibitors on TNF-α/CHX-induced apoptotic cell death. After 12 h exposure to TNF-α/CHX, we found that pretreatment with PD98059 improved cell viability and reduced cytotoxicity. Pretreatment with SB203580 attenuated cell viability and increased cell death (Fig. 6e, f). The results of an assay measuring active-caspase-3 indicated that PD98059 reduced the activation of caspase-3, while SB203580 increased the activation of caspase-3 (Fig. 6g, h). Furthermore, pretreatment with SP600125 did not affect the TNF-α/CHX-induced apoptotic cell death. These results suggested that activation of ERK1/2 pathway contributed to the apoptotic cell death after TNF-α/CHX treatment, while activation of p38 pathway inhibited TNF-α/CHX-induced apoptotic cell death.

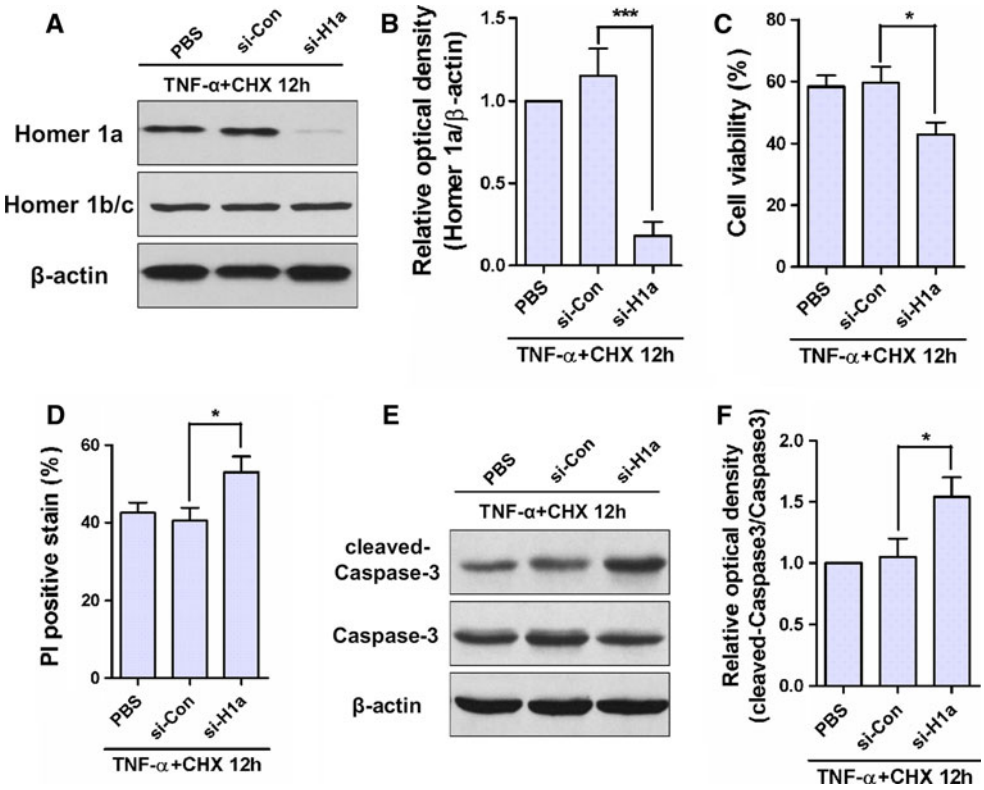

Fig. 4 Down-regulation of Homer 1a aggravates the apoptotic cell death induced by TNF-α/CHX. PC12 cells were infected with si-H1a. si-Con was used as a vehicle control. **a** Western blot analysis for Homer 1a and Homer 1b/c after treatment with TNF-α/CHX for 12 h. β-actin was used as loading control. **b** The quantitative analysis of **a** was shown in the *bar graphs*. **c**, **d** After treatment with TNF-α/CHX for 12 h, cell viability and cytotoxicity were measured by WST and PI staining. **e** Western blot analysis for cleaved-caspase-3 and caspase-3. **f** The quantitative analysis for **e** was shown in the *bar graphs*. The *bars* are the means and SE of five independent experiments. * $p < 0.05$ and *** $p < 0.001$ by using *t* test

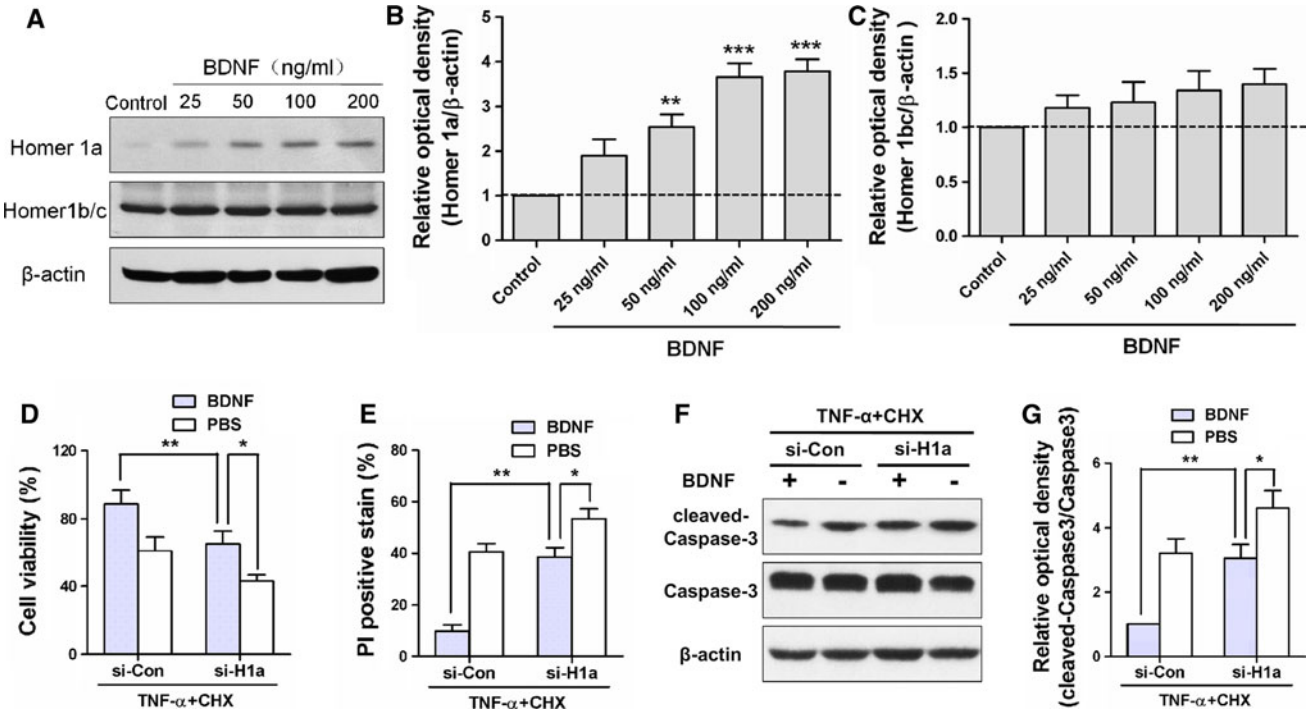

Fig. 5 Induction of endogenous Homer 1a by BDNF attenuates the TNF-α/CHX-induced apoptotic cell death. PC12 cells were incubated with different concentration of BDNF (BD: 25, 50, 100, and 200 ng/ml). **a** Western blot analysis for Homer 1a and Homer 1b/c. β-actin was used as a loading control. **b**, **c** The quantitative analysis for **a** was shown in the *bar graphs*. PC12 cells were transfected with si-H1a or si-Con and then treated with BDNF (BD, 200 ng/ml). **d**, **e** After treatment with TNF-α/CHX for 12 h, cell viability and cytotoxicity were measured by WST and PI staining. **f** Western blot analysis for cleaved-caspase-3 and caspase-3. **g** The quantitative analysis for **f** was shown in the *bar graphs*. The *bars* are the means and SE of five independent experiments. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ by using Bonferroni's test after ANOVA

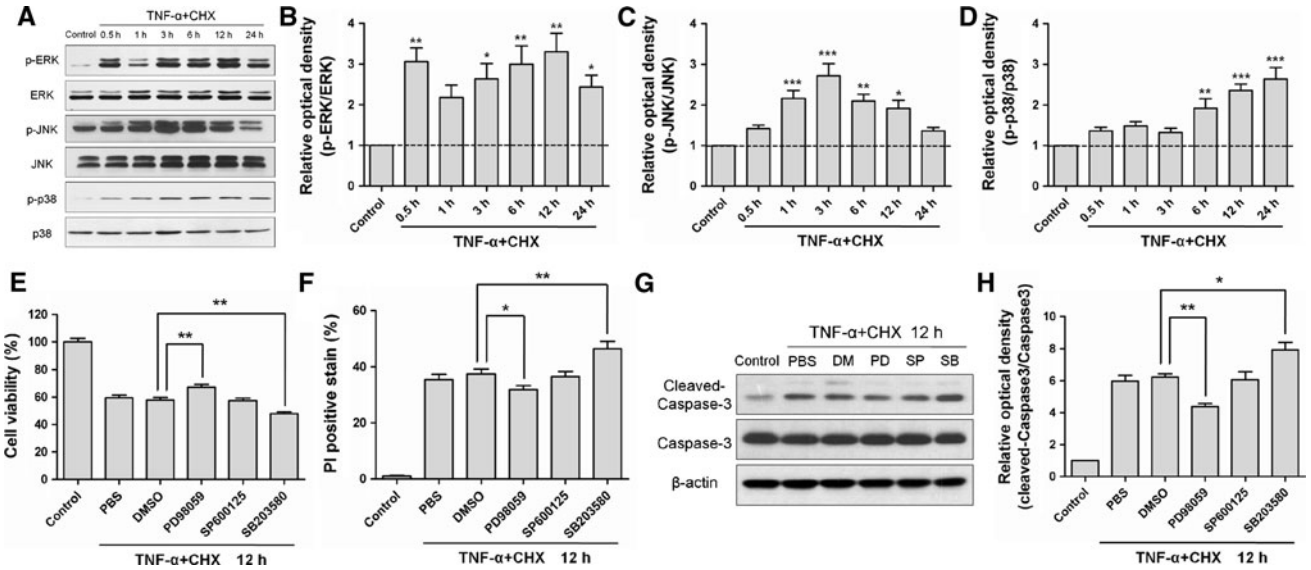

Fig. 6 MAPK pathways differentially regulates TNF-α/CHX-induced apoptotic cell death. **a** Western blot analysis for phosphorylation of ERK1/2, JNK, and p38 after TNF-α/CHX treatment for time indicated. The total ERK1/2 (ERK), total JNK (JNK), and total p38 (p38) were used as the loading control for phospho-ERK1/2 (p-ERK), phospho-JNK (p-JNK), and phosphor-p38 (p-p38) respectively. **b–d** The quantitative analysis for **a** was shown in the *bar graphs*. PC12 cells were respectively incubated with PD98059 (PD, 40 μM), SP600125 (SP, 20 μM), and SB203580 (SB, 20 μM) at 1 h before TNF-α/CHX treatment. DMSO (DM, 0.1 % V/V) was used as a vehicle control and PBS was used as a sham control. **e**, **f** after treatment with TNF-α/CHX for time indicated, cell viability and cytotoxicity were measured by WST and PI staining in PC12 cells. **g** Western blot analysis for cleaved-caspase-3 and caspase-3. **h** The quantitative analysis for **g** was shown in the *bar graphs*. The *bars* are the means and SE of five independent experiments. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ by using Bonferroni's test after ANOVA

Because the cross-talk among different MAPK pathways has been reported during apoptotic cell death, we hypothesized that interaction between MAPK pathways would be involved in differentially regulating apoptotic cell death. Here, we observed that the phosphorylation of ERK1/2 was elevated by the SB203580 after TNF-α/CHX treatment, but was not affected by SP600125. Activation of the JNK pathway was not influenced by either PD98059 or SB203580, and activation of the p38 pathway was not affected by either PD98059 or SP600125 (Fig. 7a–d). These results indicated that p38 pathway inhibited the activation of ERK1/2 pathway. Compared to pretreatment with SB203580 alone, pretreatment with SB203580 and PD98059 attenuated apoptotic cell death. Compared to pretreatment with PD98059 alone, however, pretreatment with PD98059 and SB203580 aggravated apoptotic cell death (Fig. 7e–h). These results showed that p38 pathway not only inhibited apoptotic cell death by reducing tha phosphorylation of ERK1/2, but also reduced the apoptotic cell death without involvement of ERK1/2 pathway.

Homer 1a is differentially regulated by MAPK pathways

. By using ERK1/2 inhibitor PD98059, JNK inhibitor SP600125, and p38 inhibitor SB203580, we examined the effects of MAPK pathways on Homer 1a and Homer 1b/c after TNF-α/CHX treatment. Pretreatment of cells with PD98059 increased the expression of Homer 1a, while pretreatment with SB203580 reduced the expression of Homer 1a. Pretreatment with SP600125 did not affect the expression of Homer 1a after TNF-α/CHX treatment (Fig. 8a, b). In addition, the expression of Homer 1b/c was not affected by these inhibitors (Fig. 8a, c). These results showed that activation of ERK1/2 pathway reduced the induction of Homer 1a, while activation of p38 pathway promoted the induction of Homer 1a.

Because the cross-talk among different MAPK pathways has been reported during apoptotic cell death, we hypothesized that interaction between MAPK pathways would be involved in differentially regulating Homer 1a. Compared to pretreatment with SB203580 alone, pretreatment with SB203580 and PD98059 increased the expression of Homer 1a. Compared to pretreatment with PD98059 alone, however, pretreatment with PD98059 and SB203580 did not change the induction of Homer 1a (Fig. 8d, e). These results indicated that the regulation of Homer 1a by p38 pathway was dependent on its cross-talk with ERK pathway.

Since ERK1/2 promoted the TNF-α/CHX-induced apoptotic cell death and inhibited the induction of Homer 1a, we speculated that ERK1/2 might affect apoptotic cell

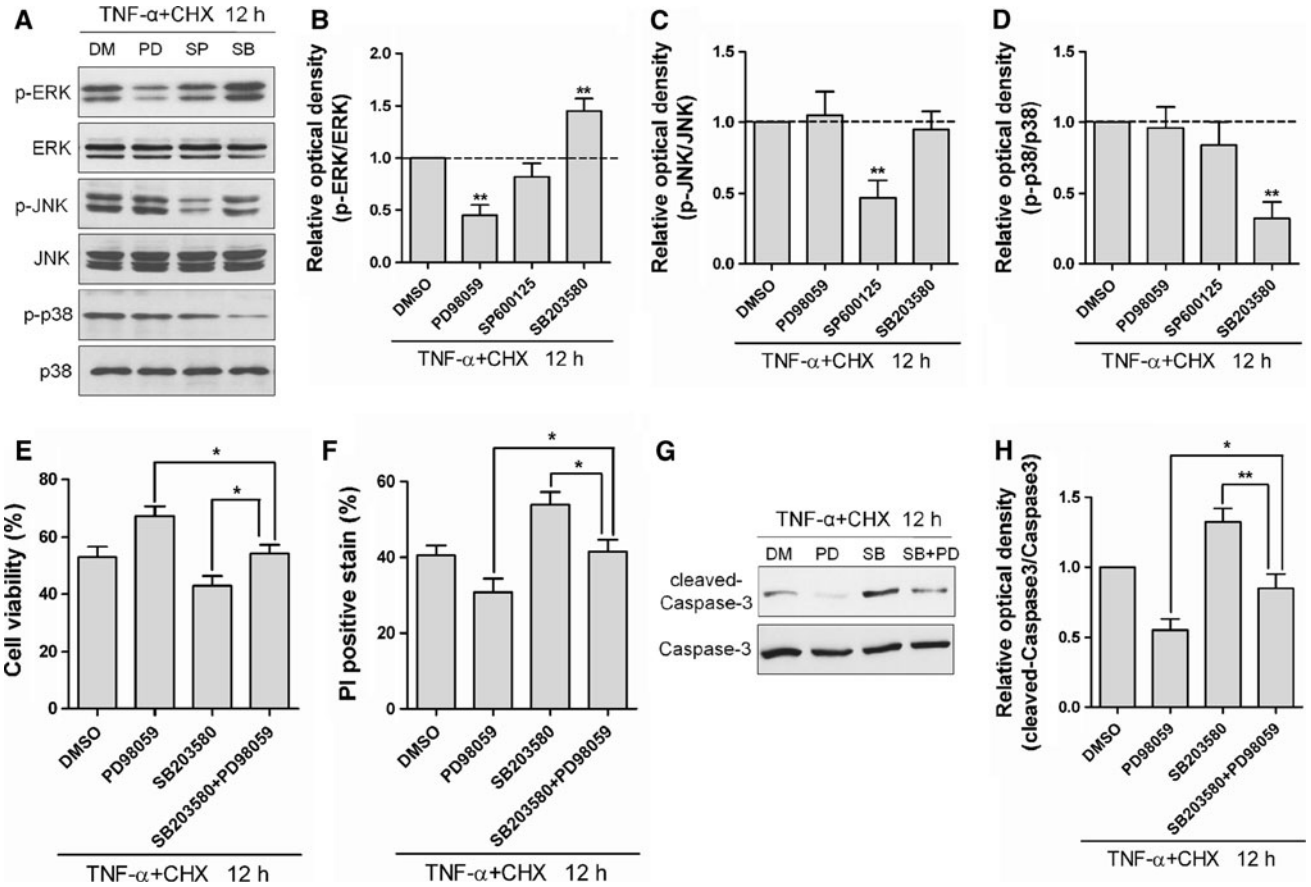

Fig. 7 Involvement of interaction between MAPK pathways in regulating apoptotic cell death. PC12 cells were respectively incubated with PD98059 (PD, 40 μM), SP600125 (SP, 20 μM), and SB203580 (SB, 20 μM) at 1 h before TNF-α/CHX treatment. DMSO (DM, 0.1 % V/V) was used as a vehicle control. **a** Western blot analysis for p-ERK, p-JNK, and p-p38 after TNF-α/CHX treatment for time indicated. ERK, JNK, and p38 were used as the loading control respectively. **b–d** The quantitative analysis for **a** were shown in the *bar graphs*. PC12 cells were incubated with PD98059 (PD, 40 μM), SB203580 (SB, 20 μM), and PD98059/SB203580 respectively at 1 h before TNF-α/CHX treatment. DMSO (DM, 0.1 % V/V) was used as a vehicle control. **e**, **f** After treatment with TNF-α/CHX for time indicated, cell viability and cytotoxicity were measured by WST and PI staining in PC12 cells. **g** Western blot analysis for cleaved-caspase-3 and caspase-3. **h** The quantitative analysis for **g** was shown in the *bar graphs*. The *bars* are the means and SE of five independent experiments. * $p < 0.05$ and ** $p < 0.01$ by using Bonferroni's test after ANOVA

death by regulating Homer 1a. In support of this idea, we found that down-regulation of Homer 1a reduced the protective effect of ERK1/2 inhibitor against TNF-α/CHX-induced apoptotic cell death. But, this protective effect was just partially inhibited by down-regulation of Homer 1a. Compared to pretreatment with DMSO, ERK1/2 inhibitor did not affected the induction of Homer 1a (Fig. 8f, g), but improved cell viability and attenuated the cytotoxicity and activation of caspase-3 in PC12 cells transfected with si-H1a (Fig. 8h–k). These results suggested that mediation of Homer 1a was one of mechanisms underlying the regulation of ERK1/2 pathway on apoptotic cell death.

Although Our results has showed that MAPK pathways might be the upstream of Homer 1a, it is still unclear whether Homer 1a reversely regulates the activation of MAPK pathways. In present study, we found that up-regulation of Homer 1a by transfection with LV-H1a reduced the phosphorylation of ERK1/2 after TNF-α/CHX treatment, whereas down-regulation of Homer 1a by transfection with si-H1a enhanced the phosphorylation of ERK1/2 after TNF-α/CHX treatment. On the contrary, up-regulation of Homer 1a nor down-regulation of Homer 1a significantly affect the phosphorylation of JNK and p38 (Fig. 9). These results showed that there was a reciprocal interaction between Homer 1a and ERK1/2 pathway.

Discussion

In this report, we showed that endogenous Homer 1a was induced by signal transduction pathways responsible for TNF-α/CHX-induced apoptotic cell death. In previous studies, the alteration of Homer 1 protein is stimulus-dependent [23, 36–40]. We observed that the expression of

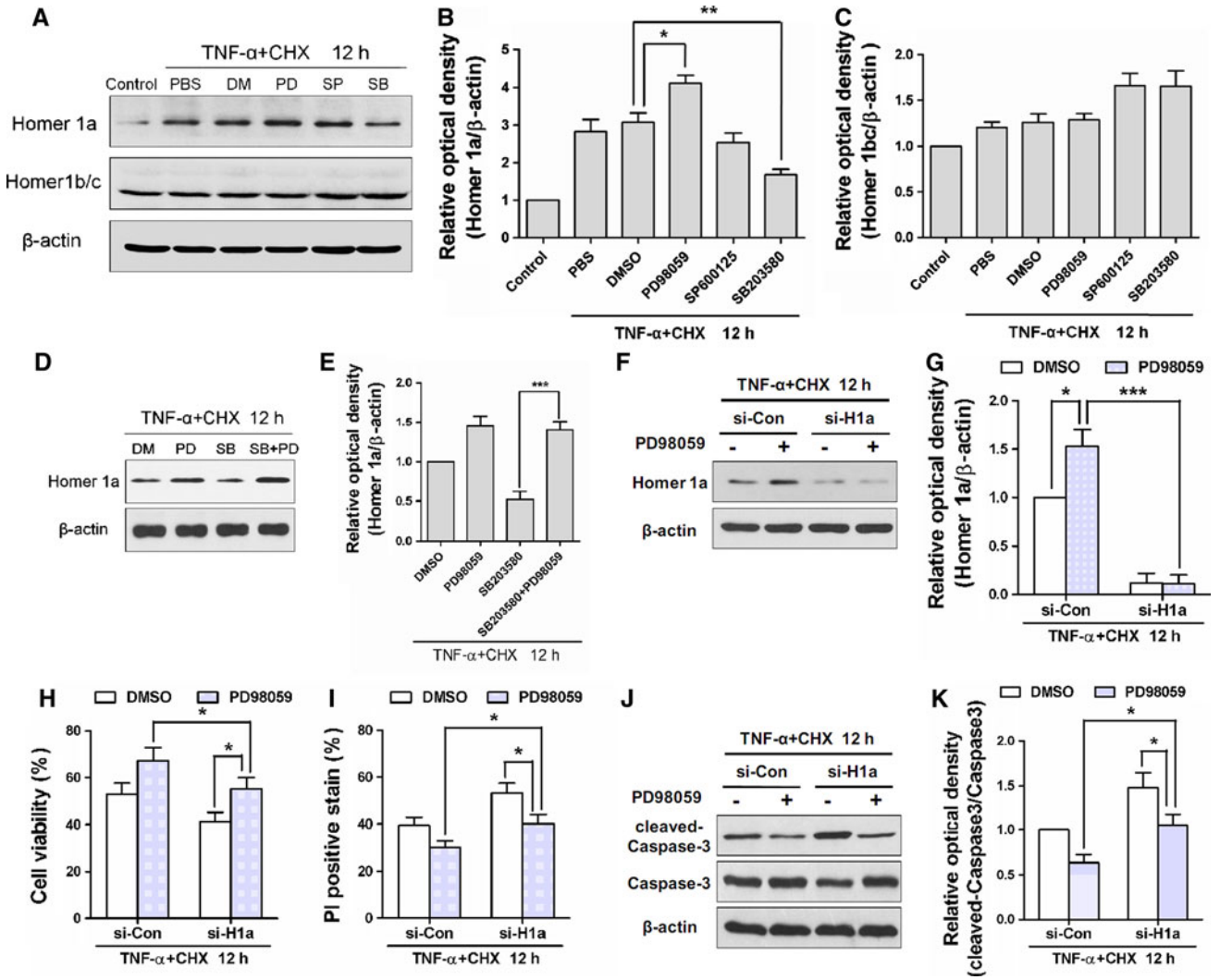

Fig. 8 Induction of Homer 1a is mediated by MAPK pathways. PC12 cells were respectively incubated with PD98059 (PD, 40 μM), SP600125 (SP, 20 μM), and SB203580 (SB, 20 μM) at 1 h before TNF-α/CHX treatment. DMSO (DM, 0.1 % V/V) was used as a vehicle control and PBS was used as a sham control. **a** Western blot analysis for Homer 1a and Homer 1b/c. **b**, **c** The quantitative analysis for **a** was shown in the *bar graphs*. PC12 cells were incubated with PD98059 (PD, 40 μM), SB203580 (SB, 20 μM), and PD98059/SB203580 respectively at 1 h before TNF-α/CHX treatment. DMSO (DM, 0.1 % V/V) was used as a vehicle control. **d** Western blot analysis for Homer 1a. **e** The quantitative analysis for **d** was shown in the *bar graphs*. PC12 cells were transfected with si-H1a or si-Con and then incubated with PD98059 (PD, 40 μM) at 1 h before TNF-α/CHX treatment. **f** Western blot analysis for Homer 1a. **g** The quantitative analysis for **f** was shown in the *bar graphs*. **h**, **i** after treatment with TNF-α/CHX for time indicated, cell viability and cytotoxicity were measured by WST and PI staining in PC12 cells. **j** Western blot analysis for cleaved-caspase-3 and caspase-3. **k** The quantitative analysis for **j** was shown in the *bar graphs*. The *bars* are the means and SE of five independent experiments. * $p < 0.05$ and ** $p < 0.01$ by using Bonferroni's test after ANOVA

Homer 1b/c was not affected by treatment with CHX, TNF-α, or TNF-α/CHX. Compared with Homer 1b/c, Homer 1a expression increased rapidly after the administration of TNF-α/CHX, but the administration of TNF-α or CHX alone did not significantly alter its expression. As a protein synthesis inhibitor, CHX did not directly trigger apoptotic cell death, but it suppressed the synthesis of the short-lived anti-apoptotic proteins, such as cellular FLICE-like inhibitory protein (c-FLIP), which serves as an endogenous caspase-8 inhibitor [28, 30]. However, our results demonstrated that concurrent treatment with TNF-α and CHX significantly induced the expression of Homer 1a. Similarly, previous study also reported that Bak protein was significantly increased under CHX and lexatumumab co-treatment-induced apoptosis [41]. Thus, CHX might not completely block some proteins synthesis after induction of apoptotic cell death.

After induction of apoptotic cell death, both pro-apoptotic and anti-apoptotic signaling pathways are activated. Here, we found that up-regulation of exogenous Homer 1a

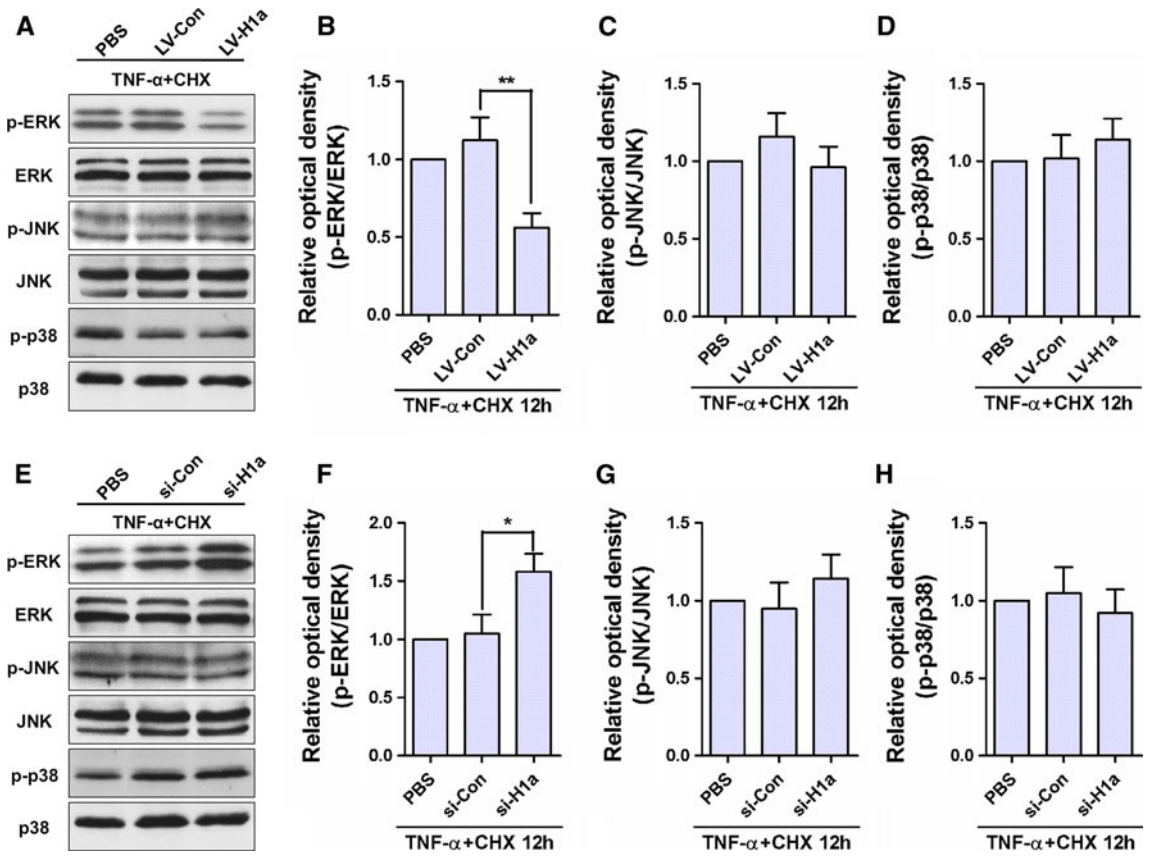

Fig. 9 Homer 1a reversely affects the activation of MAPK pathways. PC12 cells were infected with LV-H1a vectors expressed Homer 1a. LV-Con was used as a vehicle control. **a** Western blot analysis for p-ERK, p-JNK, and p-p38 after TNF-α/CHX treatment for time indicated. ERK, JNK, and p38 were used as the loading control respectively. **b–d** The quantitative analysis for **a** was shown in the *bar graphs*. PC12 cells were infected with si-H1a. si-Con was used as a vehicle control. **e** Western blot analysis for p-ERK, p-JNK, and p-p38 after TNF-α/CHX treatment for time indicated. ERK, JNK, and p38 were used as the loading control respectively. **f–h** The quantitative analysis for **e** were shown in the *bar graphs*. The *bars* are the means and SE of five independent experiments. * $p < 0.05$ and ** $p < 0.01$ by using Bonferroni's test after ANOVA

played a protective role in apoptotic cell death. In contrast, down-regulation of Homer 1a aggravated apoptotic cell death. These results indicated that apoptotic cell death-induced Homer 1a was responsible for a survival pathway and Homer 1a might be a potential endogenous anti-apoptotic agent. In previous studies, BDNF has been shown to induce endogenous Homer 1a expression [35, 42] and used as an activator of endogenous Homer 1a [14]. In this study, we observed that BDNF could induce endogenous Homer 1a expression in a concentration-dependent manner. Since BDNF attenuated the TNF-α/CHX-induced apoptotic cell death and this effect was reduced by down-regulation of Homer 1a, Homer 1a-associated anti-apoptotic effects contributed to the protective effect of BDNF against apoptosis. In addition to BDNF, Pituitary adenylate cyclase activating polypeptide (PACAP) also serves as an activator of endogenous Homer 1a [43, 44]. But, neither BDNF nor PACAP could be used as the specific agonist of Homer 1a, since some other molecules are also induced by BDNF or PACAP treatment.

Either TNF-α alone or TNF-α/CHX leads to the activation of MAPK pathways in cell lines and primary culture cells [29, 31–33]. However, the role of MAPK pathways in apoptosis is still controversial. Previous studies showed that phosphorylation of ERK1/2 improved PC12 cell survival and protected PC12 cells from apoptotic cell death [45–47]. However, we observed that inhibition of the ERK1/2 pathway reduced TNF-α-induced apoptotic cell death. Similar with our results, some other recent studies indicated that the ERK1/2 pathway promoted TNF-α-induced apoptotic cell death in other cell types [48–50]. Although pro-apoptotic and anti-apoptotic effects were reported in the activation of the p38 pathway [51, 52], the protective effect of the p38 pathway was reported in TNF-α/CHX-induced PC12 apoptosis [29], which is consistent with our observation in present study. Here, we reported that inhibition of the JNK pathway did not affect TNF-α/CHX-induced apoptosis in PC12 cells. A similar study indicated that TNF-α induced apoptosis through a JNK/Bax-dependent pathway in differentiated, but not naive,

PC12 cells [53]. Moreover, the cross-talk of MAPK pathways might be involved in the regulation of apoptotic cell death, since the activation of the ERK1/2 pathway was affected by JNK and p38 pathways [54, 55]. Present study supported that the effect of the p38 pathway on the apoptotic cell death was partially dependent on the ERK1/2 pathway. Therefore, the role of MAPK in apoptotic cell death may vary based on different insults and cell types.

As an potential endogenous protective agent, induction of Homer 1a should be regulated by the intracellular signaling pathways, such as MAPK pathways. In different MAPK pathways, ERK1/2 pathway has been shown to induce the expression of Homer 1a mRNA and protein [16, 34, 35]. Here, we found that inhibition of the ERK1/2 pathway promoted the expression of Homer 1a. Although we also found that inhibition of the p38 pathway attenuated Homer 1a expression, the further study showed that p38 pathway affected the induction of Homer 1a by cross-talking with ERK1/2 pathway, suggesting ERK1/2 pathway is critical for the regulation of Homer 1a. However, the role of ERK1/2 pathway in present study was inconsistent with some previous studies. On the one hand, different cell types or insults could affect the effects of MAPK pathways on induction of Homer 1a. On the other hand, according to the involvement of ubiquitin–proteasome systems in the degradation of Homer 1a expression [56], MAPK pathways might affect the expression of Homer 1a via regulating proteolytic systems. But, further studies are needed to elucidate the mechanism underlying this process.

Furthermore, induction of Homer 1a has been implicated to inhibit the glutamate receptor activation-induced phosphorylation of ERK1/2 pathway in neurons [16], indicating a reciprocal effect between Homer 1a and ERK1/2 pathway. The results in present study suggested that the interaction between Homer 1a and ERK1/2 pathway existed in the TNF-α/CHX-induced apoptosis in PC12 cells. The specific proline-rich sequence including Pro–Pro–x–x–Phe (x is any amino acid) [57], which interacts with the EVH1-like domain of Homer 1a, has not been recognized in ERK1/2 and its upstream kinases. Intracellular Ca^{2+} mobilization in neuronal cells has been implicated to regulate the activation of ERK1/2 pathway. Previous studies had shown that Homer proteins not only affected the Ca^{2+} influx from extracellular space, but also regulates the release of intracellular Ca^{2+} from endoplasmic reticulum (ER) [58]. Our recent study showed that up-regulation of Homer 1a reduced hydrogen peroxide-induced intracellular Ca^{2+} mobilization in PC12 cells, suggesting that Ca^{2+} was an important downstream mediator of Homer 1a [59]. Thus, the effect of Homer 1a on ERK1/2 pathway might be associated with its regulation of intracellular Ca^{2+} mobilization. In addition, further studies on downstream of Homer 1a contribute to elucidation of mechanism underlying interaction between Homer 1a and MAPK pathways.

In conclusion, we have shown that endogenous Homer 1a was induced by signal transduction pathways responsible for TNF-α/CHX-induced apoptotic cell death and that this effect was mediated by the ERK1/2 pathway and cross-talk among MAPK pathways. Homer 1a played a protective role in apoptotic cell death that was related to an interaction between Homer 1a and ERK1/2 pathway (Fig. S5). However, our study also have some limitations. For one thing, although PC12 cells have been widely used as a model system for neuronal function, the observed effects of Homer 1a in PC12 cells could be different in other in vitro systems, such as primary neurons. For another, since environment in animal models are more complicated than in vitro systems, the results from in vivo study might be inconsistent with cell-based assay in this study. Therefore, further studies in other in vitro and in vivo systems would lead to the identification of PSD scaffold proteins during apoptotic cell death and sheds light on a novel endogenous anti-apoptotic mechanism.

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