E3 ubiquitin ligase siah-1 nuclear accumulation is critical for homocysteine-induced impairment of C6 astrogliaoma cells

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Abstract. Elevated plasma homocysteine (Hcy), known as hyperhomocysteinemia (HHcy), is an independent risk factor for neurodegenerative diseases. Hcy, even at a low concentration, can promote free radical formation and increase oxidative stress, leading to neuronal death, which may be an important mechanism underlying the pathogenesis of neurodegenerative diseases. Although several reports have indicated that the nuclear translocation of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) may be involved in Hcy-induced apoptosis, the exact mechanism remains to be fully elucidated. The siah E3 ubiquitin protein ligase 1 (siah-1) gene was found to be critical for the translocation of GAPDH from the cytoplasm to the nucleus. In the present study, the role of siah-1 was investigated in the nuclear translocation of GAPDH in rat C6 astrogliaoma cells treated with Hcy. C6 cells were treated with various concentrations of Hcy for 48 h and the expression level of siah-1 was examined using reverse transcription-quantitative polymerase chain reaction and western blotting analysis. In addition, the subcellular localization of siah-1 and GAPDH and the interaction between these two factors were investigated by immunofluorescence staining and co‑immunoprecipitation assay, respectively. The results showed that Hcy at a high concentration increased the expression of siah-1 and induced nuclear translocation of siah-1 and GAPDH. In addition, siah-1 knockdown by siah-1 small interfering RNA significantly decreased the Hcy-induced nuclear accumulation of GAPDH and inhibited the impairment of C6 cells. These findings suggest that siah-1 is involved in Hcy-induced cell damage by promoting the nuclear translocation of GAPDH.

Introduction

Several neurodegenerative conditions, including Alzheimer's disease (AD) and Parkinson's disease, are associated with homocysteine (Hcy) (1-3). However, the mechanism involved remains to be fully elucidated. Hyperhomocysteinemia (HHcy) is a condition characterized by an elevation in the concentration of L-Hcy in plasma above its physiological level, which varies between 5 and 15 µM (mean 10 µM). Even moderate-mild HHcy is considered to be a risk factor for several neurodegenerative diseases. The mechanisms underlying its toxicity have been reported, including NMDA receptor- and group I metabotropic glutamate receptor-mediated neurotoxicity. In addition, Hcy can induce caspase-dependent apoptosis in human dopaminergic cells, and in rat hippocampal and mouse cortical neurons (4).

Although the neurotoxic effects of Hcy are well documented, few studies have investigated the effect of Hcy in astrocytes (4-6). Maler et al reported that Hcy exhibited a dose-dependent cytotoxic effect at doses ≤2 mM in cortical astrocytes (7). In order to determine the influence of Hcy on the viability of the cells, a previous study cultured glioblastoma cells with Hcy (0.5, 2, and 5 mM) for 72 h; the results indicated that the extent of cell death increased with the concentration of Hcy in the culture medium (8). Astrocytes are an important cell type in the central nervous system and are critical in the glial-vascular interface as part of the blood-brain barrier. Astrocytes have been identified as the support and housekeeping cells of the nervous system and exert structural, metabolic and functional effects on neurons, which are either neurotoxic or neuroprotective (9). It has been shown that non-neural cells, mainly astrocytes, are crucial in the occurrence and development of degenerative diseases (10).

A previous study has shown that Hcy exerts an excitotoxic effect on cells by promoting free radical formation and inducing oxidative stress (11). It has been reported that, under oxidative stress, GAPDH translocates to the nucleus and induces p53-dependent apoptosis (12); Hcy-induced cell apoptosis is also involved in the nuclear translocation of GAPDH (13). GAPDH, as an oxidant stress sensor, contributes to the early stage of apoptosis, during which cellular signals initiate the translocation of GAPDH into the nucleus.

Siah-1 proteins are a conserved family of E3 ubiquitin ligases that have been implicated in a variety of cellular...
processes, including mitosis, DNA damage, tumor suppression and apoptotic cell death (14-17). Siah-1 consists of an N-terminal RING domain that can bind to E2 proteins, two novel zinc finger motifs that are involved in protein–protein interactions, and a C-terminal sequence that can regulate oligomerization and bind to target proteins (18-20). GAPDH lacks a nuclear location signal (NLS), whereas siah-1 carries an NLS motif allowing its translocation into the nucleus (21). As a binding partner of GAPDH, siah-1 may translocate GAPDH from the cytosol to the nucleus, contributing to cell death (22).

Glial cells are the most numerous cellular constituent of the brain parenchyma. They serve a major role in sustaining the physiological function of this tissue. Therefore, the present study was undertaken to evaluate the viability of rat C6 cells exposed to Hcy, mimicking HHcy in vitro. The involvement of GAPDH/siah-1 nuclear translocation in Hcy-induced cell damage was investigated in rat C6 cells. The model of Hcy-induced impairment of C6 cells showed that siah-1 was significantly upregulated by Hcy. In addition, siah-1 knockdown using siah-1 small interfering RNA (siRNA) significantly decreased the Hcy-induced nuclear accumulation of GAPDH/siah-1. These lines of evidence show that siah-1 is important in the Hcy-induced inhibition of C6 cell survival.

Materials and methods

Reagents. The following antibodies were used in the present study: Goat anti-siah-1 antibody (cat. no. sc-5505), mouse anti- lamin B antibody (cat. no. sc-374015; both Santa Cruz Biotechnology, Inc., Dallas, TX, USA), mouse anti-GAPDH antibody (cat. no. ab6285), mouse anti-β-actin antibody (cat. no. ab6276; both Abcam, Cambridge, MA, USA), rabbit anti-cleaved caspase 3 antibody (cat. no. 9661), rabbit anti-phospho (p)-p53 antibody (cat. no. 9284), mouse anti-p53 (cat. no. 2524; all Cell Signaling Technology, Inc., Danvers, MA, USA), IRDye® 800CW goat anti-mouse immunoglobulin G (IGG) heavy and light chain (H+L; cat. no. P/N 925-32210), IRDye® 800CW goat anti-rabbit IgG (H+L; cat. no. P/N 925-32211), IRDye® 800CW donkey anti-goat IgG (H+L; cat. no. P/N 925-32214; all LI-COR Biosciences, Lincoln, NE, USA), Alexa Fluor 488-AffiniPure goat anti-mouse IgG (H+L; cat. no. 115-545-003) and Cy3 AffiniPure rabbit anti-goat IgG (H+L; cat. no. 305-165-003; both Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). Other materials used included D,L-homocysteine (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), Cell Counting kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan), and TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was isolated from the cells in each group using TRIzol reagent according to the manufacturer's protocol. The RNA (2 µg) was reverse transcribed into cDNA with the PrimeScript RT reagent kit (Takara Biotechnology Co., Ltd., Dalian, China). The qPCR protocol was used in conjunction with SYBR FAST qPCR Master mix (KAPA; Roche Diagnostics, Basel, Switzerland). Reaction mix included: 1 µl cDNA, 10 µl SYBR FAST qPCR master mix, 0.4 µl ROX High, 0.4 µl primer forward (10 µM) and 0.4 µl primer reverse (10 µM) in a total volume of 20 µl. The primers were as follows: Siah-1 forward, 5'-CAAAAGTGTCACACTCCCAGAG-3' and reverse, 5'-GGTGGCAATACATATCGAAGCAG-3'; GAPDH forward, 5'-TTTCTCAACCACCATGTGTGGCG-3' and reverse, 5'-CATGAGGTTCACACCTGGTCT-3'; β-actin forward, 5'-TCTCATGTTGGCCTAAGCTCAG-3' and reverse, 5'-GGTGGCATAGAAGCTTCTTACG-3'. Quantitation of mRNA expression was performed on an ABI Prism 7900 Sequence Detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The reaction conditions were as follows: 95°C for 3 min, 95°C for 3 sec, and 60°C for 30 sec, for a total of 40 cycles; following melting curve analysis, the reaction conditions were as follows: 95°C for 15 sec, 60°C for 15 sec, and 95°C for 15 sec. All mRNA expression was normalized to that of β-actin, and the detection was performed in triplicate. Data normalization was performed using β-actin, and the 2^ΔΔCq method was used to calculate the relative gene expression level (23).

Cell culture. The rat C6 astroglioma cells were purchased from the Cell Bank of Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (both Gibco; Thermo Fisher Scientific, Inc.) at 37°C and 5% CO2 in a humidified atmosphere. The medium was refreshed once every 2-3 days, and cells were passaged when the cell confluence reached 100%.

Cell proliferation assay. The proliferation of the C6 cells was detected using a CCK-8 assay. The cells were seeded into 96-well plates (3,000 cells/well). After 24 h, the medium was refreshed and the cells were treated with Hcy at various concentrations (0, 2, 4, 6, 8 and 10 mM) for 48 h. Following rinsing twice with PBS, 10 µl of CCK-8 solution was added to each well (100 µl). The absorbance was measured at 450 nm using a microplate reader (Synergy 2; BioTek Instruments, Inc., Winooski, VT, USA) after 2 h incubation at 37°C.

Western blotting. The cells were placed into radioimmuno-precipitation assay buffer and total protein was extracted. The nuclearic fractions were prepared using Nuclear and Cytoplasmic Protein Extraction kit (Beyotime Institute of Biotechnology, Haimen, China). The bovine serum albumin (BSA; Gibco; Thermo Fisher Scientific, Inc.) was diluted to make a standard solution. Subsequently, the protein samples, the standard solution and the bicinchoninic acid assay kit (Beyotime Institute of Biotechnology) were separately added to the 96-well plate. The samples were incubated at 37°C for 30 min in the dark. The optical density at 562 nm was measured using an ELISA kit (ELX-800; BioTek Instruments, Inc.) and the protein concentration was calculated. A total of 50 µg protein was loaded per lane. Proteins were separated by 10% SDS-PAGE. Following electrophoretic separation for 2 h at 80 V, the gel was subsequently transferred onto a
PVDF membrane at 20 mA for 1 h. The membranes were blocked in 5% BSA (Gibco; Thermo Fisher Scientific, Inc.) at room temperature for 1 h and subsequently incubated overnight at 4°C with primary antibodies (anti-β-actin, 1:5,000; anti-lamin B, 1:400; anti-GAPDH, 1:1,000; anti-siah-1, 1:200; anti-p-p53, 1:1,000; anti-p53, 1:1,000; and anti-cleaved caspase 3, 1:1,000). The free antibodies were washed away with 0.1 M Tris-buffered saline with 0.1% Tween-20 buffer. The membrane was then incubated with the appropriate secondary antibody (peroxidase-conjugated goat antibody anti-rabbit or mouse immunoglobulin G and peroxidase-conjugated donkey antibody anti-goat immunoglobulin G; each secondary antibody was diluted 1:2,000) for 1 h at room temperature. The IRDye-conjugated antibody was scanned with the Odyssey imaging system (LI-COR Biosciences), analyzed with the ImageJ software (version 1.8.0; National Institutes of Health, Bethesda, MD, USA). Lamin B served as the nuclear fractionation control, β-actin was used to determine total protein concentration.

Immunofluorescence staining. The cells were treated with Hcy for 48 h. Following fixation in 4% paraformaldehyde for 30 min at room temperature, the cells were washed three times with PBS, blocked with 1% BSA and treated with 0.1% Triton X-100 for 30 min at room temperature. Then, cells were incubated overnight at 4°C with mouse anti-GAPDH (1:500) and anti-siah-1 (1:200) prior to the treatment with secondary antibody (Dylight 488 AffiniPure Goat anti-mouse IgG, 1:200; Cy3 AffiniPure rabbit anti-goat IgG, 1:200) and DAPI (0.2 μg/ml) for 30 min at room temperature. The fluorescence intensity was measured under a ZEISS LSM710 confocal microscope (Zeiss GmbH, Jena, Germany; magnification, x100).

Co-immunoprecipitation assay. The cells were lysed in immunoprecipitation buffer (protease inhibitor and phosphatase inhibitor mixture) and equal quantities of protein were obtained from each sample (800 μg). The Agarose Protein A+G beads were mixed and divided into two groups, one for removing non-specific binding and one for binding to antibodies. The sample was pretreated with Agarose A+G, shaken slowly for 2 h at 4°C, and the pretreated sample was added to a new EP tube, following which the protein A+G beads were removed. The anti-siah-1 antibody (3 μg) was added to the total protein to react with the target protein, with slow agitation of the antigen-antibody mixture overnight at 4°C. Following washing with pre-cooled PBS, the immune complexes were detected by western blotting. GAPDH was used as a positive control, eliminating false positive interference.

siRNA transfection. The cells were transfected with 40 nM siRNA targeting rat siah-1 (GenePharma, Shanghai, China) in the presence of Lipofectamine™ 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer’s protocol. The two RNAi oligos targeting siah-1 were 1#-siRNA siah-1: 5’-GCAACAGCCAUAUG AAUATT-3’ and 2#-siRNA siah-1: 5’-UAUUCAGUAGG CUGUUGCTT-3’. The medium was refreshed 6 h later and the cells were maintained for another 24 h. The transfection efficiency was detected by RT-qPCR and western blot analyses.

Statistical analysis. The data were analyzed with either one-way or two-way analysis of variance, followed by the Newman-Keuls post hoc test for pairwise comparisons, using SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Hcy affects the viability of C6 cells. The viability of C6 cells was determined following incubation with Hcy at various concentrations for 48 h. The viability of the Hcy-treated cells was normalized to that of cells without Hcy treatment. As shown in Fig. 1, Hcy treatment reduced cell viability in a dose-dependent manner as assessed using a Cell Counting kit-8 assay. *P<0.05 vs. control group. Hcy, homocysteine.

Hcy increases the expression of siah-1. Siah-1 is expressed in several tissues, including the brain (24). A previous study showed that apoptosis-inducing stimuli may markedly elevate cellular expression of siah-1 (15). Therefore, the expression of siah-1 was also measured following Hcy treatment. The cells were treated with Hcy at various concentrations for 48 h. The results showed that Hcy significantly increased the mRNA expression of siah-1 within 48 h compared with the untreated cells (Fig. 2A). In addition, a significant increase in the protein expression of siah-1 was observed following Hcy treatment, as shown by the western blotting (Fig. 2B). The increases in the mRNA and protein expression levels of siah-1 were dependent on the concentrations of Hcy.

Hcy induces the nuclear accumulation of siah-1 and GAPDH in C6 cells. To confirm that Hcy induces the nuclear accumulation of siah-1 and GAPDH, western blotting was used to determine the expression levels of siah-1 and GAPDH in the nucleus following treatment with Hcy at 8 mM for 48 h. As shown in Fig. 2C, the nuclear protein expression levels of siah-1 and GAPDH were significantly increased in a dose-dependent manner following Hcy treatment. The
localization of siah-1 and GAPDH expressed in the C6 cells was also determined by immunofluorescence staining. As shown in Fig. 2D, compared with the control cells, a high proportion of cells showed nuclear expression of siah-1 and GAPDH following Hcy treatment. Therefore, it was hypothesized that Hcy promoted the translocation of siah-1 and GAPDH into the nucleus.

**Hcy increases the interaction between siah-1 and GAPDH.** To determine whether Hcy induces the formation of siah-1-GAPDH complexes, co-immunoprecipitation was performed using equal quantities of nuclear fractions from cells following treatment with 8 mM Hcy for 48 h. The results showed that siah-1 and GAPDH formed complexes in response to Hcy treatment. As shown in Fig. 3, Hcy significantly induced the formation of GAPDH/siah-1 complexes compared with the control group.

**Siah-1 mediates the nuclear accumulation of GAPDH.** To assess whether a decrease in siah-1 is involved in the nuclear accumulation of GAPDH, the expression of siah-1 was silenced by siRNA-mediated knockdown in C6 cells, which were then treated with Hcy at 8 mM for 48 h. Pull-down assays were performed, as described above. The knockdown efficiency and
other quality control aspects of the siRNA experiments are shown in Fig. 4A. The results demonstrated that siah-1 siRNA significantly reduced the mRNA and protein levels of siah-1 by >80% in the C6 cells.

The nuclear interaction between GAPDH/siah-1 following treatment with Hcy was inhibited in C6 cells following siah-1 silencing (Fig. 4B). In addition, nuclear aggregation and nuclear translocation of siah-1 and GAPDH was decreased in cells transfected with siah-1 siRNA compared with the group treated with Hcy (Fig. 4C).

Siah-1 silencing suppresses Hcy-induced cell death. p53 is one of the most important tumor suppressor factors in cells, and it has a key role in cell cycle regulation, DNA repair and apoptosis. p53 can be phosphorylated by multiple protein kinases at multiple sites, and Serine 15 is the most common phosphorylation site of p53, leading to its transcriptional activity during cell death. To determine whether siah-1 is critical for Hcy-induced cell death, the expression levels of phosphorylated and total p53 protein under different treatment conditions were measured and normalized with actin, and p-p53 was normalized to total p53. As shown in Fig. 5A, Hcy increased the expression levels of p-p53 (ser15) and total p53 compared with levels in the control cells. Following siRNA interference to knock down the expression of siah-1, the increases in the expression of p-p53 and total p53 caused by Hcy were significantly inhibited. The knockdown of siah-1 also inhibited the phosphorylation of p53 at serine 15. For further confirmation, the protein levels of cleaved caspase 3 were also measured. The results showed that 8 mmol/l Hcy significantly upregulated the protein expression of cleaved caspase 3, whereas siah-1 silencing with target siRNA significantly reduced the protein expression of cleaved caspase 3 in the Hcy-treated C6 cells (Fig. 5B).

Discussion

An important pathological feature of neurodegenerative diseases is the apoptosis and necrosis of cells. The abnormal apoptosis of nerve cells is the ultimate cause of neurodegenerative diseases. In order to further investigate the pathogenesis of neurodegenerative diseases, the present study established a variety of cell and animal models, through which it has been confirmed that Hcy can exert its neurotoxic effect in various nerve cells. The detrimental effects of Hcy on neurons is well documented in the literature, however, few studies have probed the effects of Hcy in astrocytes. It is widely recognized that Hcy can induce cell death, however, the specific molecular mechanism remains to be fully elucidated. Studies have shown that Hcy-induced cell death involves DNA damage (25), promotes the expression level of poly-ADP-ribosomederase activation and mitochondrial dysfunction following the activation of caspase-3 (26,27). A previous study showed that the interaction between GAPDH and siah-1 was associated with cell death in neuronal and non-neuronal cell types (28). Therefore, it was hypothesized that the cytotoxicity of Hcy may be associated with its interaction with GAPDH/siah-1. In the present study, the nuclear accumulation of GAPDH/siah-1 was detected in Hcy-treated C6 cells.

A previous study showed that Hcy concentrations >2 mM were required to induce cell death (7), however, the mode of action remained unknown. In the present study, C6 cells were treated with various concentrations of Hcy to mimic the pathological changes in Hcy. The results showed that treatment of cells with Hcy >2 mM significantly reduced their proliferation capacity, and the higher the concentration of Hcy, the lower the proliferation capacity, showing a dose-dependent effect.

As a key enzyme in the glycolysis pathway, GAPDH is also involved in DNA repair, cell membrane fusion and transport, transcriptional regulation and apoptosis caused by oxidative stress. In cells, GAPDH acts as an oxidant-stress sensor. It has been reported that, under oxidative stress, GAPDH translocates to the nucleus, leading to cell death (29). GAPDH possesses a region homologous to a nuclear localization sequence motif (NLS), and its nuclear transport can occur via this NLS (30). A previous study identified siah-1 as a potential carrier/shuttle protein; GAPDH binds the NLS-bearing siah-1, forming a complex and subsequently promoting the translocation of GAPDH from the cytosol to the nucleus (22). The present study demonstrated that the NLS on siah-1 can promote nuclear movement of this complex. The results showed that Hcy inhibited the survival of C6 cells, which was at least partially dependent on the nuclear interaction between GAPDH and siah-1.

Siah-1 is an E3-ubiquitin-ligase involved in the ubiquitination and proteasome-mediated degradation of proteins, and it has been shown to be involved in the regulation of a variety of substrate proteins that are involved in different signal transduction pathways, including cell...
Figure 4. Siah-1 mediates the nuclear accumulation of GAPDH. (A) Expression of siah-1 was significantly decreased by siah-1 siRNA. (B) Nuclear interaction between GAPDH/siah-1 following Hcy treatment was inhibited in C6 cells following siah-1 silencing. (C) Nuclear translocation of siah-1 and GAPDH following Hcy treatment in C6 cells with siah-1 silencing. Images were acquired with a Zeiss LSM710 confocal microscope (magnification, x100). *P<0.05 vs. NC group. Hcy, homocysteine; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; siRNA, small interfering RNA; NC, negative control.
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cycle, cell differentiation, apoptosis and neurodegenerative diseases (31). It has been suggested that siah-1 is a potential metastatic protease of GAPDH from the cytoplasm to the nucleus. In the present study, it was found that Hcy not only induced a high expression of GAPDH in C6 cells, but also induced the nuclear transposition and nuclear aggregation of GAPDH. To verify the molecular mechanism underlying the nuclear transposition of GAPDH, the effects of Hcy on the expression and localization of siah-1 were also observed. The experimental results showed that when Hcy caused the overexpression of GAPDH, it also induced the overexpression of siah-1. Therefore, it was concluded that the nuclear transposition of GAPDH and siah-1 is involved in the cytotoxic processes.

As a tumor suppressor, siah-1 encodes nuclear protein and promotes apoptosis (32). It has been demonstrated that siah-1 can bind to GAPDH and then translocate from the cytoplasm to the nucleus, inducing the accumulation of GAPDH and siah-1 in the nucleus. The nuclear GAPDH/siah-1 complex may stabilize siah-1 and enhance its E3 ligase activity, thereby causing cell death (21). To investigate whether siah-1 is involved in the nuclear translocation of GAPDH following Hcy treatment, the expression of siah-1 was silenced with a specific siRNA. The results showed that silencing siah-1 inhibited the nuclear accumulation of GAPDH, and inhibited the Hcy-induced impairment of C6 cells. This suggests that siah-1 is a key factor in Hcy-induced cell damage.
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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions
YT made substantial contributions to the conception and design of the study. XZ conducted and supervised the experiments. XT and LG conducted the experiments and wrote the manuscript. AJ and YW prepared the figures, analyzed and interpreted the data, and drafted the manuscript. All authors critically revised the manuscript, and read and approved the final version of the manuscript.

Ethics approval and consent to participate
Not applicable.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interest.

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