Homocysteine induces cytotoxicity and proliferation inhibition in neural stem cells via DNA methylation in vitro

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Abstract

Mild to moderate hyperhomocysteinemia has been implicated in neurodevelopmental disorders and neurodegenerative diseases in human studies. Although the molecular mechanisms underlying the effects of homocysteine (Hcy) neurotoxicity on the nervous system are not yet fully understood, inhibition of neural stem cell (NSC) proliferation and alterations in DNA methylation may be involved. The aim of the present study was to characterize the effects of Hcy on DNA methylation in NSCs, and to explore how Hcy-induced changes in DNA methylation patterns affect NSC proliferation. We found that L-Hcy (30–1000 μM) but not L-cysteine inhibited cell proliferation and reduced levels of global DNA methylation in NSCs from neonatal rat hippocampus and increased cell injury. High levels of Hcy also induced an increase in S-adenosylhomocysteine (SAH), a decrease in the ratio of S-adenosylmethionine (SAM) to SAH, and a reduction in protein expression of the DNA methyltransferases DNMT1, DNMT3a and DNMT3b and their enzymatic activity. Moreover, the DNMT inhibitor zebularine reduced the global DNA methylation level and inhibited NSC proliferation. Our results suggest that alterations in DNA methylation may be an important mechanism by which high levels of Hcy inhibit NSC viability in vitro. Hcy-induced DNA hypomethylation may be caused by a reduction in the DNMT activity which is regulated by the cellular concentrations of SAM and SAH, or their protein expression levels. Our results also suggest that Hcy may play a role in the pathogenesis of certain nervous system diseases via a molecular mechanism that involves negative regulation of NSC proliferation and alterations in DNA methylation.

Introduction

Epidemiological studies show a positive, dose-dependent relationship between mild to moderate increases in plasma total homocysteine (Hcy) concentrations and the risk of neurodegenerative diseases such as Alzheimer’s disease, vascular dementia, cognitive impairment and stroke [1,2]. Increased maternal Hcy is also associated with neural tube defects. Several placebo-controlled trials have shown that therapies that lower Hcy levels reduce the frequency of neural tube defects in both women who have a history of such pregnancies and women who do not [3]. Furthermore, a cross-sectional MRI study showed that elevated Hcy levels are associated with more hippocampal and cortical atrophy in healthy elderly individuals [4]. Hcy may increase the permeability of the blood-brain barrier suggesting that functional disturbances of the blood–brain barrier may lead to increased exposure of the brain to Hcy and the neurotoxic effects of hyperhomocysteinemia [5]. How-
ever, the exact mechanism for direct neurotoxic effects of Hcy has yet to be elucidated.

Self-renewing neural stem cells (NSCs) that are capable of differentiating into neurons, astrocytes and oligodendrocytes play a crucial role in the developing brain as well as the adult brain. NSCs have been the subject of intense study in recent years with regard to their therapeutic potential in treating neurodegenerative diseases and traumatic injuries of the nervous system. Most studies investigating the consequences of Hcy toxicity have mainly focused on injury to mature neurons. Elevated levels of Hcy exert a variety of adverse effects on neural cells, including oxidative stress, activation of caspases, mitochondrial dysfunction, and an increase in cytosolic calcium [6, 7]. As NSCs are necessary for brain development and neural repair, elevated Hcy may cause brain injury by inhibiting neurogenesis through its effects on NSCs. Only few experimental data are available on Hcy neurotoxicity which may be involved in neuronal progenitor cell proliferation. Rabaneda et al. [8] found evidence in vitro and in vivo suggesting that homocysteine inhibits the proliferation of neuronal precursors in the sub-ventricular zone by impairing basic fibroblast growth factor-induced proliferation. A recent study showed that Hcy decreases Extracellular signal-regulated kinase 1/2 (ERK1/2) protein phosphorylation and NSC proliferation in fetal rat brain in vitro [9]. However, evidence for the direct effect of Hcy on NSC viability has been lacking. Here, we examined the effects of elevated Hcy on proliferation and viability of NSCs from neonatal rat hippocampus, and explored the molecular mechanisms of neural cell toxicity induced by Hcy.

Previous studies have demonstrated an essential role for epigenetics in NSC development, and that inhibiting DNA methylation prevents NSC differentiation [10, 11]. Hcy in the body is solely of metabolic origin, ultimately derived from metabolic cycling of methionine. Methionine is converted through S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) to Hcy. SAM and SAH are metabolites of Hcy. SAM is the substrate and SAH is the product of methyltransferase reactions. SAM and SAH levels are important metabolic indicators of cellular methylation status. Thus, Hcy may influence global and gene promoter-specific DNA methylation. To date, no studies have examined the relationship between Hcy and total DNA methylation in NSCs. In the present study, the levels of the methylation intermediates SAM and SAH, DNA methyltransferase activity, and global DNA methylation in NSCs were explored to test the hypothesis that Hcy-induced inhibition of NSC proliferation is caused by modifications in DNA methylation.

Results

Effects of Hcy on neurosphere growth and NSC proliferation

NSCs derived from the neonatal rat hippocampus began to form suspended neurospheres after 2 days in vitro. As shown in Fig. 1, undifferentiated NSCs in neurospheres were characteristically spherical [12]. Most floating spheres increased in size over time, and reached a diameter of ~ 150 μm after 5–6 days in vitro. The neurosphere size reflects the proliferative capacity of cells. Dramatic reductions in the mean diameters of the neurospheres were observed after exposure to all three concentrations of Hcy (P < 0.05). Furthermore, the effect appeared to be dose-dependent; the diameter was gradually reduced with increasing concentration (30 > 300 > 1000 μM). The mean diameter

![Fig. 1. Inhibition of NSC neurosphere formation. (A) Representative images of live floating neurospheres prepared from neonatal rat hippocampus under various Hcy treatments (C, control; L, Hcy-L; M, Hcy-M; H, Hcy-H). Scale bar = 100 μm. (B) The diameter of neurospheres was measured using cellSens Dimension software. Eight bright-field images were randomly captured under a phase-contrast microscope. *P < 0.05 versus control; **P < 0.05 versus Hcy-L; ***P < 0.05 versus Hcy-M.](image-url)
of the neurospheres after treatment with 1000 μM Hcy was significantly lower than those seen after treatment with 30 or 300 μM Hcy (P < 0.05) (Fig. 1).

SRY (sex-determining region Y)-box 2 (Sox 2) is essential to maintain self-renewal of undifferentiated stem cells, and is widely used to identify multipotent neural stem/progenitor cells in vitro. We performed double labeling with 5′-bromo-2′-deoxyuridine (BrdU) and Sox2 to assess the proliferative potential in NSCs. Almost all the cells in the neurospheres expressed Sox2 protein, which appeared as green cytoplasmic staining (Fig. 2A). The percentage of BrdU-positive/Sox2-positive cells was significantly lower in the Hcy-exposed groups compared to the control (Fig. 2B). These results suggest that the smaller size of the neurospheres formed in the presence of Hcy may be a consequence of the decreased proliferative capacity of the NSCs.

To rule out a possible contribution to NSC proliferation of redox action of the thiol group of Hcy, 300 μM l-cysteine, a structurally related sulfur amino acid, was used to treat NSCs instead. As shown in Fig. 2B, no significant difference in NSC proliferation was detected between l-cysteine-treated and untreated cells over the 6-day cysteine incubation period (P > 0.05).

The toxic effects of Hcy on NSCs

Neurotoxicity was assessed by measuring the amount of lactate dehydrogenase (LDH) released from the cells following Hcy treatment. The results show that Hcy increased LDH release into the cell culture medium. Comparisons between Hcy treatment groups showed that the LDH levels after treatment with 300 and 1000 μM Hcy were significantly higher than that after treatment with 30 μM Hcy (P < 0.05, Fig. 2C).

SAM, SAH and global DNA methylation levels in Hcy-treated NSCs

Previous studies have reported changes in SAM, SAH and DNA methylation during NSC proliferation and differentiation [10]. Our results showed a significant decrease in global DNA methylation in Hcy-treated NSCs.
groups compared with the control group (Fig. 3A). Moreover, no significant difference in the global DNA methylation level in NSCs was observed between cells treated with 300 µM cysteine and control cells ($P > 0.05$) (Fig. 3A).

To understand the mechanism underlying the change in global DNA methylation caused by Hcy treatment, the cellular concentrations of Hcy-related metabolites (SAM and SAH) were measured by HPLC. We verified that treatment with Hcy did not significantly alter the concentration of SAM in NSCs (Fig. 3B). However, SAH and Hcy concentrations increased in tandem (Fig. 3C). Consequently the SAM : SAH ratio was decreased by Hcy in a concentration-dependent manner (Fig. 3D).

**DNMT expression and activity in Hcy-treated NSCs**

A family of DNMTs, including DNMT3a, DNMT3b and the maintenance methyltransferase DNMT1, mediates the methylation reaction, in which a methyl group ($-\text{CH}_3$) is added to the 5' position of the pyrimidine ring of cytosine residues, primarily at CpG dinucleotides. Our results showed that protein expression of all DNMT isoforms decreased in NSCs with increasing Hcy concentration (Fig. 4A). The total activity of all DNMT enzymes was also significantly decreased after Hcy treatment ($P < 0.05$) (Fig. 4B).

Thus, the Hcy treatment-induced changes in total DNMT activity may be associated with changes in DNMT1, DNMT3a and DNMT3b protein expression.

**Effects of zebularine on NSC proliferation and global DNA methylation level**

Zebularine, a DNA methylation inhibitor that acts by forming a covalent complex with DNMTs [13], was used to determine whether the decrease in DNMT activity resulted in reduced cell proliferation or not. Our results show that zebularine decreased the global DNA methylation in NSCs to lower levels than in the control group ($P < 0.05$) (Fig. 2A). Inhibition of DNMT activity also significantly decreased the percentage of proliferative cells in the zebularine treatment group compared with the control group ($P < 0.05$). A zebularine concentration of 150 µM, which inhibited DNMT activity by 50% (data not shown), was chosen to ensure minimal cytotoxicity *in vitro*, and had a maximal inhibitory effect on NSC proliferation.

**Discussion**

NSCs are crucial for both brain development and adult neurogenesis. Because of their capacity to undergo self-renewal and to differentiate into multiple cell types, stem cells have the potential to serve as...
valuable sources of neural cells during attempts to induce regeneration and tissue repair in response to the devastating effects of neurodegenerative disorders. In this study, Hcy decreased NSC proliferation and increased cell injury in vitro. These results suggest that the inhibitory effects of Hcy on NSC proliferation may further hinder neural regeneration and repair following neurodegeneration. This finding may help to explain why hyperhomocysteinemia often coincides with some neurological diseases.

DNA methylation is important in cellular processes during embryonic development and stem cell differentiation. Recently, DNA methylation has been shown to be closely associated with fate specification in NSCs [10]. On the other hand, Hcy disrupts the DNA methylation cycle inducing DNA damage, cell death, and alterations in gene expression. Perturbations in methyl group metabolism and Hcy balance have emerged over the past few decades as playing defining roles in a number of pathological conditions [2]. The present results showed that Hcy decreases global DNA methylation in NSCs. Moreover, our findings suggest that these changes in DNA methylation may be at least partially responsible for the inhibitory effects of Hcy on NSC proliferation.

DNA methylation, which adds a methyl group to a cytosine base at CpG dinucleotide residues, is catalyzed by DNMTs. The degree of DNA methylation regulates gene expression patterns by altering chromatin structures. Three active DNMTs have been identified in mammals: DNMT1, DNMT3a, and DNMT3b. DNMT1 is essential for maintaining methylation patterns during DNA replication, whereas DNMT3a and DNMT3b are primarily responsible for de novo methylation in embryonic and postnatal tissues [14,15]. In the developing mouse brain, deletion of DNMT1 in progenitor cells impairs neuronal maturation and survival, and causes precocious astroglial differentiation [16]. DNMT3a is expressed in postnatal NSCs and is required for neurogenesis. In the postnatal forebrain, DNMT3a is expressed in the subventricular zone and the hippocampal dentate gyrus. DNMT3a mediates methylation at non-promoter regions in genes related to nervous system development and neurogenesis, but methylates the proximal promoter regions of non-neuronal genes such as glial fibrillary acidic protein (GFAP) [17]. During neurogenesis, DNMT3b is predominantly expressed in proliferating neural precursor cells within the cerebral cortex [18,19]. DNMT3b may play an important role in the initial steps of progenitor cell differentiation, although this function remains to be demonstrated. Thus, it appears that the DNA methylation status during new neural cell formation is regulated by these DNMTs. The present study showed...
that inhibition of DNMT activity by Zebularine treatment decreased the total DNA methylation level and the percentage of proliferative NSCs. This suggests that, by regulating these DNMTs, varying concentrations of Hcy may alter global DNA methylation in proliferating NSCs, further affecting neurogenesis in the developing and mature central nervous system.

SAH is produced as a by-product of SAM-dependent methyltransferase reactions. Most cellular methyltransferases are inhibited by accumulation of intracellular and cellular SAH. High-affinity binding of SAH to the active site of cellular methyltransferases results in product inhibition of the enzyme activity [20]. The DNMT enzyme activity may be also affected by SAM/SAH levels in the cell. The SAM : SAH ratio is frequently used as an indicator of cellular methylation potential. A decrease in the SAM : SAH ratio is predictive of a reduced methylation capacity. The present results show that elevated Hcy levels are associated with an increase in SAH and a decrease in the SAM : SAH ratio in NSCs. We conclude that Hcy may decrease DNMT activity by altering the concentration of SAH and the SAM/SAH ratio. In addition, our observation that Hcy decreased the protein expression of DNMT1, DNMT3a and DNMT3b suggests that the reduction in enzymatic DNMT activity may also be due in part to reduced protein expression.

Numerous studies in recent years have investigated the role of Hcy as a cause of brain damage. It was found that Hcy itself can cause disturbed redox potentials, thus promoting calcium influx, accumulation of amyloid and tau proteins, apoptosis and neuronal death [21]. Thus, it is possible that Hcy, as a sulfur-containing amino acid, may affect NSC viability and proliferation via an alteration in the redox status of Hcy. In this study, L-cysteine was used to detect the effect of the redox actions of the thiol group on NSC proliferation. A 300 μM concentration of L-cysteine was used to reflect the moderate homocysteine concentration used here. Recently, L-cysteine at lower doses (0.5–5 μM) had been shown to improve the cell viability of NSCs, and 1 μM L-cysteine had the optimal effect on cell viability [22]. On the other hand, cysteine has been found to be toxic to cultured neurons at relatively high concentrations. It was reported that 1 mM L-cysteine decreased neuronal survival to 30.5%, whereas we found that L-cysteine had no cytotoxic effects on proliferative NSCs when it was used at a concentration of 300 μM [23]. The results suggest that redox actions of the thiol group of Hcy may be not the major cause of Hcy-induced cell proliferation inhibition, at least at a concentration of 300 μM.

In summary, our study confirmed that Hcy at high concentrations inhibited NSC proliferation and exerted a cytotoxic effect. This may explain why high Hcy levels result in neural tube defects and some neurodegenerative diseases. Moreover, the DNMT inhibitor zebularine reduced cell proliferation and the levels of global DNA methylation in NSCs. Hcy affects DNA methylation in NSCs, which may be mediated by decreased DNMT enzyme activity. The inhibition of DNMT activation caused by Hcy treatment may be associated with a high concentration of cellular SAH, a decreased SAM : SAH ratio, and reduced DNMT protein expression.

Experimental procedures

Materials

Dulbecco’s modified Eagle’s medium, basic fibroblast growth factor and epidermal growth factor were obtained from Gibco (Carlsbad, CA, USA). D,L-homocysteine, L-cysteine, BrdU, SAM and SAH were purchased from Sigma (St Louis, MO, USA). Zebularine was purchased from Merck Chemicals (Darmstadt, Germany). The DNA methyltransferase activity/inhibition assay kit was purchased from Active Motif (Carlsbad, CA, USA). Mouse monoclonal antibodies against BrdU, β-actin, DNMT1 and DNMT3a were obtained from Cell Signaling Technology Inc. (Boston, MA, USA). Rabbit monoclonal antibodies against DNMT3b and Sox2 were obtained from Abcam (Cambridge, UK). All secondary antibodies were obtained from Zhongshan Goldbridge Biotechnology (Beijing, China).

NSC culture

The Tianjin Medical University Animal Ethics Committee approved the experimental protocols in this study (study number TMUaMEC 2012016). Neonatal Sprague Dawley rats were purchased from Beijing Medical Laboratory Animal Co. Ltd. (Beijing, China). NSCs were isolated and maintained as previously described [24]. The cultured cells were assigned to seven treatment groups: control (Hcy-free), Hcy-L (low Hcy, 30 μM), Hcy-M (moderate Hcy, 300 μM), Hcy-H (high Hcy, 1000 μM), Cys (300 μM L-cysteine), Zeb (150 μM zebularine) and Zeb + Hcy-M (150 μM zebularine + 300 μM Hcy). The duration of the Hcy treatment was 6 days. After treatment, NSCs were harvested for the following immunocytochemistry and western blot analysis.

Determination of neurosphere size

To determine the size of the neurospheres after 6 days of treatment with varying concentrations of Hcy, eight bright-
field images for each group were randomly captured using an inverted microscope and digital camera (Olympus, Tokyo, Japan). The diameters of the neurospheres were measured using cellSens Dimension software (Olympus).

**BrdU incorporation and immunocytochemistry**

NSC proliferation was determined by in vitro labeling with the thymidine analog BrdU. On the 6th day of culture, the cells were incubated with BrdU (10 μg·mL⁻¹) for 24 h. The cultures were then processed for double-labeled immunofluorescence. The cells were fixed in 4% paraformaldehyde for 30 min at room temperature, permeabilized using 0.1% Triton X-100 in NaCl/Pi for 5 min, and incubated with blocking buffer for 1 h with 1% fetal bovine serum. The cells were incubated overnight at 4 °C in a mixture of rabbit monoclonal anti-Sox2 antibody (1 : 100) and mouse monoclonal anti-BrdU antibody (1 : 100). Subsequently, cells were incubated for 2 h at room temperature with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG and tetramethylrhodamine isothiocyanate-conjugated goat anti-mouse IgG. Immunostained cells were visualized by indirect fluorescence under a fluorescent microscope (Olympus).

**Measurement of LDH activity**

LDH, an indicator of cell injury, was detected by colorimetric (440 nm) assay with the LDH cytotoxicity detection Kit (Nanjing Jiancheng Biochemical Reagent Co., Nanjing, China), according to the manufacturer’s instructions. The assay is based on measurement of LDH released into the culture medium from damaged cells. The released LDH can be quantified by a coupled enzymatic reaction. First, LDH catalyzes the conversion of lactate to pyruvate via reduction of NAD⁺ to NADH. Second, diaphorase uses NADH to reduce a tetrazolium salt to a red formazan product. Briefly, the cells were cultured in the presence of Hcy at various concentrations (0, 30, 300 and 1000 μM) for 48 h. The cell medium (50 μL) was then gently aspirated and used for LDH determination. After the reaction, the absorbance for each sample was read at wavelength of 440 nm.

**Global DNA methylation detection**

Global methylation levels were measured using a Methylamp Global DNA Methylation Quantification Ultra kit (Epigentek Group Inc., New York, NY, USA) as previously described [25]. According to the manufacturer’s instructions, DNA is immobilized to the strip well specifically coated with a DNA affinity substance. The methylated fraction of DNA can then be recognized by a 5-methylcytosine antibody and quantified through an ELISA-like reaction. The amount of methylated DNA is proportional to the absorbance at 450 nm.

**HPLC analysis**

Cell cultures were rinsed twice with NaCl/Pi, and frozen at −80 °C. After thawing, cells were scraped into 1 mL deionized water and sonicated for 15 s on ice. The macromolecules were precipitated using 1.5 mM perchloric acid solution at 4 °C for 1 h, adjusting the pH to 4–5 with KOH, and then centrifuged for 15 min at 9000 g. The supernatant was freeze-dried and later subjected to HPLC analysis [26]. HPLC was performed on a Waters HPLC system (Waters Corp., Milford, MA, USA) using a Venusil MP C18 column (particle size 5 μm, diameter 4.6 mm, length 250 mm) at 30 °C. The mobile phase consisted of 50 mM NaH₂PO₄ (pH 4.38), 10 mM C₇H₁₅NaO₃S and 20% methanol. The flow rate was 1 mL·min⁻¹, and detection was performed at 254 nm.

**Western blotting**

Protein expression of DNMT isoforms in cultured cells was assessed by western blot analysis. The cells were washed with ice-cold phosphate-buffered saline, and lysed with TNE/NP-40 buffer (50 mM Tris-HCl pH 7.4, 100 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40). Proteins were separated by 12% SDS/PAGE, and transferred to polyvinylidene difluoride membranes. The membranes were blocked using 5% non-fat milk, and incubated with primary antibodies anti-DNMT1, anti-DNMT3a and anti-DNMT3b (all at a 1 : 1000 dilution) overnight at 4 °C, followed by appropriate secondary antibodies for 1 h at room temperature. Proteins were detected by chemiluminescence assay. Quantification of proteins was performed by densitometric analysis using IMAGEJ software (version 1.61, National Institute of Mental Health, Bethesda, MD, USA). The intensity of each protein band was normalized to that of the respective β-actin band (detected using a 1 : 5000 dilution of antibody).

**DNMT activity**

Global DNMT activity was evaluated in nuclear fractions using an Active Motif DNA methyltransferase activity/inhibition colorimetric assay kit, according to the manufacturer’s instructions. The same quantity of cells from each sample was analyzed. A lot-specific standard curve was generated using the DNMT1 enzyme provided in the kit. Absorbance was measured on a microplate reader at 450 nm, and DNMT activity was calculated according to the following formula: DNMT activity (absorbance/hour of incubation time) × 1000.
Statistical analysis

The data are expressed as means ± SD, and were analyzed using spss13.0 statistical software (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls test for multiple comparisons were used to determine significant differences among the experimental groups. The criterion for statistical significance was \( P < 0.05 \).

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References


