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Research article

Homocysteine inhibits neural stem cells survival by inducing DNA interstrand cross-links *via* oxidative stress

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HIGHLIGHTS

- Hcy inhibits neural stem cells survival.
- Hcy causes cell interstrand cross-links by oxidative stress and activates FA pathway.
- Inhibiting Hcy-envoked reactive oxidative species generation could ameliorate DNA damage and cell survival.

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ABSTRACT

Elevated plasma levels of homocysteine have been implicated in neurodevelopmental and neurodegenerative disorders in human studies. Although the molecular mechanisms underlying the effects of homocysteine (Hcy) cytotoxicity on the nervous system are not yet fully unknown, induction of DNA interstrand cross-links and inhibition of neural stem cells (NSCs) survival may be involved. The objective of our study was to investigate the effects of Hcy on DNA interstrand cross-links in NSCs, and to explore its possible mechanisms. We also found that Hcy induced cell DNA damage on a dose-dependent manner and evoked reactive oxidative species (ROS) production, leading to elevated apoptosis in NSCs. Moreover, Hcy exposure activated the Fanconi anemia (FA) pathway, which was characterized by increases in monoubiquitination of Fanci and Fancd2 and enhancement of the interaction between above two proteins. On contrary, *N*-Acety-L-Cysteine (NAC) decreased Hcy-evoked ROS production and significantly ameliorated DNA damage and improved cell survival. These data suggest that Hcy may play a role in the pathogenesis of neurological diseases *via* a molecular mechanism that induces DNA interstrand cross-links *via* oxidative stress and involves in negative regulation of NSCs survival.

1. Introduction

Homocysteine (Hcy) plays a role in developmental and adult neurological disorders [1,2]. Elevated maternal Hcy is associated with neural tube defects (NTDs) of fetus [1,3]. Epidemiological study also shows a positive relationship between plasma Hcy level and the risk of neurodegenerative diseases such as cognitive impairment, Alzheimer's disease and stroke [2]. Hcy is toxic to human and murine neuronal cells *in vitro* and in mouse brain [4]. Neural stem cells (NSCs) that are capable to differentiate into neurons, oligodendrocytes and astrocytes play a crucial role in the embryonic neurogenesis as well as the injury repair of adult brain [5]. NSCs are sensitive to extracellular Hcy which decreases the numbers of NSCs and impairs their proliferation and differentiation capacities [5,6]. However, the underlying mechanism for direct neurotoxic effects of Hcy on NSCs has remained obscure.

Hcy is a metabolite of the essential amino acid methionine. Methionine involves in the generation of methyl groups required for the DNA synthesis. Patients with hyperhomocysteinemia exhibit increased DNA damage in white blood cells [7]. DNA damage, which has been proved in Alzheimer's disease and aging patients [8] and NTDs experimental models [9], is the main reason for neuron apoptosis. The extensive neuron loss induced by apoptosis could be contributed to the initiation and progression of abnormal neurogenesis and degenerative diseases [10].

NSCs are particularly sensitive to DNA damage, such as DNA strand breaks, telomere damages and DNA interstrand cross-links

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(ICLs) [11]. ICLs are extremely toxic since they prevent DNA replication and transcription, generate DNA strand breaks at collapsed replication forks, and lead to cell death. Fanconi anemia (FA) pathway is responsible for removing ICLs. Eight FA proteins (A–M) are assembled into a nuclear FA core complex at the sites containing ICLs. The FA core complex is a multisubunit E3 ubiquitin ligase that subsequently monoubiquitinates two additional FA proteins (FANCI and FANCD2) [12]. The monoubiquitinated FANCI (ub-FANCI) and FANCD2 (ub-FANCD2) then recruit endonucleases to excise ICLs. Thus, FANCI and FANCD2 play an important role in repairing ICLs. Dysfunction of FANCI or FANCD2 would induce DNA damage and extensive apoptosis in NSCs [13].

However, whether Hcy induces NSCs apoptosis by ICLs is remain unknown. In this study, we characterize the effects of Hcy on apoptosis and DNA damage in NSCs, and to explore its possible mechanisms.

2. Material and methods

2.1. Material

Dullbecco's modified Eagle's medium was obtained form Gibco (Carlsbad, CA, USA). D,L-homocysteine, *N*-Acetyl-cysteine (NAC) and protease inhibitor cocktail were purchased from Sigma (St Louis, MO, USA). Lipofectamine 2000 for cell transfection was obtained from Invitrogen (Grand Island, NY, USA). The Fanci and Fancd2 open reading frame plasmids were purchased from Origene (Fredrick, MD, USA). The Comet assay kit, FITC Annexin V apoptosis detection kit, Caspase-3 activity assay kit and Cell Counting Kit (CCK8) were obtained from Trevigen (Gaithersburg, MD, USA), B.D. Biosciences Pharmingen (Franklin Lakes, NJ, USA), Cell Signaling Technology (Danvers, MA, USA) and Dojindo Laboratories (Mashikimachi, Kumamoto, Japan), respectively. Anti-H2ax (1:1000) and anti-γ-H2ax (1:1000) antibodies were purchased from Cell Signaling Technology (Danvers,MA, USA); anti-Fanci (1:2500) and anti-Fancd2 (1:200) antibodies were purchased from Abcam (Cambridge, UK) and Santa Cruz Biotechnology (DBA, Milano, Italy), respectively. All secondary antibodies were obtained from SouthenBiotech (Birminghan, AL, USA).

2.2. Cell culture, treatment and transfection

C17.2 mouse neural stem cells, originally obtained from European Collection of Cell Culture, were maintained in Dulbecco's modified Eagle's medium (5 mM glucose) supplemented with 10% fetal bovine serum, 5% horse serum, 2 mM glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin in a humidified atmosphere with 5% carbon dioxide at 37 °C. Under that culture condition, C17.2 cells could differentiate into neurons and glia cells [14]. Homocysteine was prepared at 0.125 mM, 0.25 mM or 0.5 mM in fresh medium as used previously [4,15]. 5 mM NAC were diluted with deionized water and added into culture 1 h before homocysteine addition. Plasmids were transfected with Lipofectamine 2000 according to the manufacturer's protocol for 48 h.

2.3. Comet assay for DNA damage

The Comet assay kit is a simple method for measuring several kinds of DNA damage including ICLs, DNA strand breaks and adduct [16]. After treatment, harvested C17.2 cells were embedded in 1% low melting point agarose on a microscope slide and then lysed with a chilled lysis solution at 4 °C overnight. DNA was allowed to unwind and denature at room temperature for 20 min, and then electrophoresis was performed for 30 min under alkaline conditions at 300 mA. Following electrophoresis, slides were stained with SYBR green and viewed (excitation 425–500 nm) with a Leica DMI 4000 B epifluorescence microscope (Leica, Wetzlar, Germany). In C17.2 cells with DNA damage, DNA supercoils were relaxed and broken ends were able to migrate toward the anod. In normal cells, DNA without breaks lacks of free ends and is confined mostly to the nucleus. Percent of Comet positive cell and Olive tail movement (OTM), which is the percent DNA in the tail multiplied by the distance between the means of the head and tail distributions, are useful measures for DNA damage [16].

2.4. Measurement of intracellular reactive oxygen species

The production of reactive oxygen species (ROS) was quantified by fluorescent staining with H_2DCF-DA, a non-fluorescent probe, which is oxidized to the highly fluorescent compound DCF aldehyde upon exposure to ROS, as previously described [17]. After treatment, C17.2 cells were incubated with 5 μM H_2DCF-DA (dissolved in DMSO) for 30 min at 37 °C. Then cells were harvested by non-enzymatic cell dissociation solution and resuspended in 500 μl of PBS supplemented with 0.1 M KH_2PO_4 and 0.5% Triton X-100. Cell debris were pelleted by centrifugation at 12,000 rpm for 10 min, and the supernatants were analyzed under fluorescein optics, at an excitation wavelength of 480 nm and an emission wavelength of 540 nm. Cell lysates were analyzed for protein content using the Bradford method, and H_2DCF fluorescence was normalized against total protein content.

2.5. Flow cytometry

Cell apoptosis was assessed by flow cytometric analysis. In brief, C17.2 cells were harvested at the indicated time points and resuspended in 1 × binding buffer (0.01 M HEPES, 0.14 M NaCl, 2.5 mM $CaCl_2$, pH 7.4) at a concentration of 1×10^6 cells/mL. Samples were stained with 5 μl FITC-annexin V and 5 μl propidium iodide (PI) for 15 min at room temperature and protected from light before 400 μl of binding buffer was added. Flow cytometric analysis was carried out on Accurri C6 (B.D. Biosciences Pharmingen, Franklin Lakes, CA, USA) and then analyzed using FlowJo7.6.2 (Tree Star, CA, USA).

2.6. Caspase-3 assay

Harvested C17.2 cells were washed with ice-cold PBS and subsequently cell lysis buffer was added. Cells were scraped off the plate and collected in a tube. Cell lysates were diluted to a concentration of approximately 3 mg/mL. Caspase-3 activity assay kit was used according to the manufacture's protocol. Fluorescence was measured with excitation wavelength at 380 nm and an emission wavelength of 460 nm and expressed in relative fluorescence units.

2.7. Cell proliferation assay

To assess the effect of Hcy on C17.2 cells viability, a CCK8 assay was performed. C17.2 cells were seeded in 96-well plates (the initial cell number is 4×10^3/well) and incubation. At the end of incubation, 10 μl CCK8 was added to each well and cells were incubated for 2 h at 37 °C. Optic density (OD) value at 450 nm was obtained using a microplate reader (BioTek, VT, USA). Growth curve was drawn using the mean value of OD every day for three days.

2.8. Western blotting

Westren blotting was carried out as previously described [12]. To extract protein, cultured C17.2 cells were sonicated in ice-cold lysis buffer (0.5% NP40) containing protease inhibitor cocktail, and the supernatants were collected by centrifugation at 13,000 rpm at 4 °C for 15 min. Cell protein extract was separated by 10%