Neurochemistry International 58 (2011) 833–838

Contents lists available at ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/neuint

Homocysteine levels impact directly on epigenetic reprogramming in astrocytes

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ARTICLE INFO

Article history:
Received 29 October 2010
Received in revised form 8 March 2011
Accepted 9 March 2011
Available online 16 March 2011

Keywords:
Astrocytes
Epigenetics
DNA methylation
Homocysteine

ABSTRACT

Although the neurotoxic effects of homocysteine have been well elucidated, the effects of homocysteine in astrocytes have received little attention until recently. Previously we have demonstrated that elevated levels of homocysteine caused significant metabolic changes and altered mitochondrial function in primary cultures of astrocytes. However, the mechanisms behind such alterations remain unclear. As homocysteine is a key metabolite in one-carbon metabolism the present study examined if the effects of homocysteine on astrocyte function are mediated through an epigenetic mechanism. Following exposure to homocysteine for 72 h, global DNA methylation and H3K9 acetylation were examined using flow cytometric analysis. Total DNA methyltransferase activity and protein levels of DNA methyltransferase 3B were measured. Exposure to homocysteine resulted in global DNA hypomethylation ($p < 0.05$) and histone hyperacetylation ($p < 0.05$). Total DNA methyltransferase activity significantly decreased following exposure to homocysteine (from 11.5 ± 3.9 to 6.0 ± 1.7 OD/h/mg protein, $p < 0.01$) which was accompanied by a significant reduction in protein levels of DNA methyltransferase 3B ($p < 0.05$). Treatment of astrocytes with the DNA methyltransferase inhibitor, 5-aza-2′-deoxycytidine, mimicked the functional changes induced by homocysteine. In conclusion, the results demonstrate significant epigenetic modifications following exposure to homocysteine in astrocytes and these changes seem to mediate functional alterations.

1. Introduction

Homocysteine is a non-essential sulfur-containing amino acid that has been linked with neurodegenerative diseases and aging (Isobe et al., 2005; Oulhaj et al., 2009). Previous studies have shown that an increased plasma homocysteine level is an independent risk factor for the development of dementia, Alzheimer's disease (AD) and Parkinson's disease (PD) (Prins et al., 2002; Ravaglia et al., 2005; Seshadri et al., 2002). More recently, evidence exists for the role of homocysteine in the pathogenesis of Alzheimer's disease (Isobe et al., 2009) however, the underlying molecular mechanisms are unknown.

Studies examining the effects of homocysteine on neuronal cells have clearly demonstrated its neurotoxic effects. A variety of mechanisms of action have been proposed including but not limited to apoptosis, oxidative stress, over activation of glutamate receptors, mitochondrial dysfunctions and activation of caspases (Ho et al., 2003; Kruman et al., 2000; Robert et al., 2005). Additionally, it has been shown that homocysteine induces activation of both N-methyl-D-aspartate (NMDA) and group I metabotropic glutamate receptors (mGluR), which may play a role in homocysteine mediated excitoxicity (Ho et al., 2002; Zieminska et al., 2003). Cytoplasmic calcium influx, as a consequence of both excitoxicity and oxidative stress has also been suggested to play a crucial role in homocysteine induced neurotoxicity (Loureiro et al., 2008; Robert et al., 2005). Several studies have proposed that homocysteine induces neuronal apoptosis by damaging DNA: neuronal DNA strand breaks have been demonstrated in rat hippocampal neurons in response to elevated homocysteine (Ho et al., 2002; Kruman et al., 2000, 2002; Liu et al., 2009).

Epigenetic modifications such as DNA methylation play a significant role in transcriptional regulation. In recent years, the importance of epigenetic modifications in disease pathogenesis have begun to emerge (Mattson, 2003; Santos-Reboucas and Pimentel, 2006; Wilson et al., 2007). Hyperhomocysteinemia results in increased S-adenosylhomocysteine (SAH) levels which intriguingly is a potent inhibitor of S-adenosylmethionine-dependent DNA methyltransferases (DNMTs). However, high levels of SAH do not always translate to lack of DNA methylation

Abbreviations: SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; Hcys, homocysteine; DNMT, DNA methyltransferase; NMDA, N-methyl-D-aspartate; mGluR, metabotropic glutamate receptors.

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doi:10.1016/j.neuint.2011.03.012

(Barić et al., 2004; Buist et al., 2006; Devlin et al., 2005). Moreover, recent studies have highlighted the importance of tissue-specificity for DNA-methylation and the importance of multiple factors governing the DNA methylation pattern (Dayal and Lentz, 2008; Devlin et al., 2005; Pogribny et al., 2008).

Although the neurotoxic effects of homocysteine have been well elucidated, the effects of homocysteine in astrocytes have been under studied until recently (Jin and Brennan, 2008; Loureiro et al., 2010; Maler et al., 2003). Astrocytes constitute a major class of cells in central nervous system (CNS) and play a crucial role in neuronal survival. Therefore, any alteration in astrocytic function could potentially have deleterious effects on neurons. Previous experiments carried out in our laboratory have demonstrated that an elevated level of homocysteine resulted in significant metabolic changes and altered mitochondrial function in primary cultures of astrocytes. More recently, Lourerio and colleagues showed that cytoskeleton remodeling of cortical astrocytes was induced by an increased levels of homocysteine (Loureiro et al., 2010). Considering the emerging important role of astrocytes in neurodegenerative diseases, elucidating the mechanisms by which homocysteine causes functional alterations in astrocytes is warranted. The objective of the present study was to examine the effects of homocysteine on the epigenetic signature in astrocytes.

2. Experimental procedures

2.1. Reagents

All chemicals were obtained from Sigma–Aldrich Chemical Company (Poole, Dorset, UK). Culture media and fetal bovine serum were obtained from Gibco (Glasgow, UK). For flow cytometry, anti-acetylated histone 3 lysine 9 (AcH3K9) and anti-5′Methylcytidine (5′MeC) antibodies were purchased from Abcam (Cambridge, UK). IgG negative control (mouse IgG1) and fluorescein isothiocyanate (FITC)-polyclonal rabbit anti mouse secondary antibodies were from Dako, Denmark. Nuclear extraction and enzyme activity assay kits were purchased from Epigentek (Brooklyn, NY, USA).

2.2. Cell culture and treatment

Rat astrocytes were prepared from 2 to 3 days old Wistar rats as previously described (Jin and Brennan, 2008; Richter-Landsberg and Besser, 1994). All procedures were performed in accordance with Irish Government ethical guidelines. The cerebral hemispheres were isolated and the meninges was carefully removed. The cerebral hemispheres were then mechanically disrupted. Cells were grown in DMEM containing 10% (v/v) FBS for 7–10 days in T175 flasks (Greiner). The non-astrocytic cells were removed by vigorous shaking and the astrocytes were sub-cultured for 20–23 days during which time the media was changed twice a week. Cells were seeded at a density of 1×10^6 cells/for 6 well plates and 0.4×10^5 cells/well for 96 well plates. Cells were maintained in a Forma Scientific incubator at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. For all the experiments, cells were pre-incubated for a period of 72 h in the presence or absence of 400 μM homocysteine. As a D,L-mixture of homocysteine was used the effective concentration used in the present studies was 200 μM. A previous study showed that homocysteine concentrations above 2 mM were needed to induce cell death (Maler et al., 2003). Previous work from our laboratory has shown that this concentration (200 μM) is well below this toxic concentration and is within the range used in other in vitro studies using brain cells (20 μM to 25 mM) (Jin and Brennan, 2008). In parallel experiments, 10 μM 5-aza-2′-deoxycytidine (5-azadC), a DNMT inhibitor, was used as a positive control. Cells were incubated for 72 h with 10 μM 5-azadC and the media replaced every 24 h with fresh media containing 5-azadC.

2.3. Flow cytometry

Following 72 h treatment in 6 well plates, cells were rinsed in ice-cold PBS and then harvested by trypsinisation. Cells were labeled with monoclonal anti-acetylated histone 3 lysine 9 (AcH3k9, Abcam) and anti-5′Methylcytidine (5′MeC, Abcam) as described previously (Watson et al., 2009). To investigate the histone acetylation modification, cells were washed with PBS containing 1% EDTA and fixed in 70% ice-cold methanol at 4 °C for 2 h. Following fixation, cells were washed twice in PBS containing 1% Tween 20. Cells were then collected by centrifugation, and the cell pellet was resuspended in 200 μl of PBS containing 1% bovine serum albumin and 0.5% of anti-AcH3k9 antibody and incubated at 4 °C for overnight. For the global DNA methylation study, cells were washed with PBS containing 1% EDTA and fixed with ice-cold Carnoy's fixative (methanol:acetic acid = 3:1) at 4 °C for 2 h. Subsequent to fixation, cells were washed twice with PBS containing 1% Tween 20. To investigate global DNA methylation, cells were pre-incubated with 1 M HCl at 37 °C for 1 h. Cells were then washed twice in PBS containing 1% Tween 20, collected by centrifugation and resuspended in 200 μl of PBS containing 1% bovine serum albumin and 0.2% of anti-5′MeC antibody and incubated overnight at 4 °C. IgG negative controls were used at the same concentration as the primary antibody in experiments.

Following overnight incubation with the primary antibody, cells were washed twice in PBS containing 1% Tween 20 and resuspended in 200 μl of PBS containing 1% bovine serum albumin and 0.1% of FITC – polyclonal rabbit anti mouse secondary antibody (DaKo, Denmark) at room temperature under light exclusion for 1 h. Analysis was performed on a CYAN flow cytometry and results assessed using SUMMIT software (Dako, Denmark). Median channel fluorescence (MCF) values were calculated with each cell population following the subtraction of IgG negative control (MCF) values.

2.4. Immunostaining

Following sub-culture for 20–23 days, cells were seeded in poly-D-lysine-coated glass coverslips and treated for 72 h as described above. To assess histone acetylation modification, cells were rinsed in PBS and fixed in 70% ice-cold methanol at 4 °C for 2 h. After blocking with PBS containing 1% BSA, cells were incubated with the primary mouse monoclonal anti-AcH3k9 antibody overnight at 4 °C followed by incubation with an Alexa Fluor 488-conjugated goat anti-mouse secondary antibody (Invitrogen) for 1 h. To investigate global DNA methylation, cells were fixed with ice-cold Carnoy's fixative (methanol:acetic acid = 3:1) at 4 °C

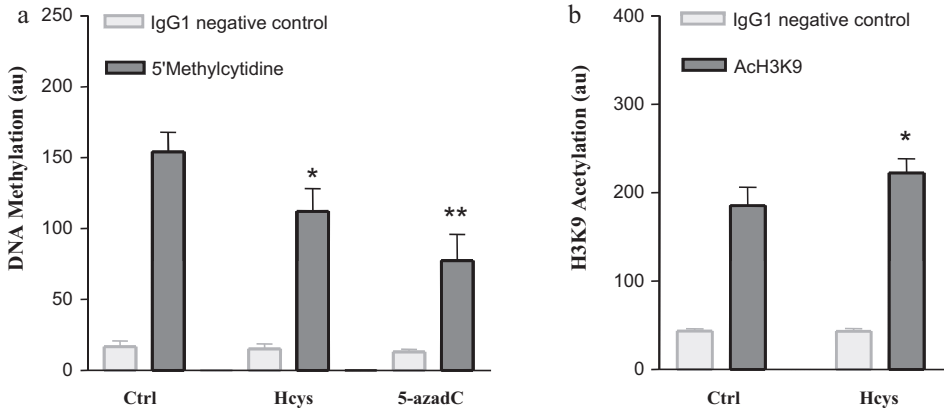

Fig. 1. Effects of homocysteine (Hcys) exposure on global epigenetic patterns of DNA methylation and histone acetylation. Primary astrocytes were cultured in the presence or absence of 400 μM homocysteine for 72 h and stained with (a) anti-5′Methylcytidine (5′MeC) and (b) anti-acetylated histone 3 lysine 9 (AcH3K9), subsequently incubated with a FITC tagged secondary antibody and assessed quantitatively using flow cytometry. Appropriate IgG1 negative control samples were also applied. The data represents the median channel fluorescence (arbitrary units (au)) ± S.D. $n = 5$. (a) Cells treated with homocysteine exhibited a significant global DNA hypomethylation compared to the control group ($^*p < 0.05$). 5-azadC, used as a positive control, exhibited DNA hypomethylation compared to the control group ($^{**}p < 0.001$). (b) Cells treated with homocysteine induced a significant hyperacetylation compared to control group ($^*p < 0.05$).