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Homocysteine promotes proliferation and activation of microglia

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Abstract

Epidemiological and experimental studies have correlated hyperhomocysteinemia to a range of neurodegenerative conditions, including Alzheimer's disease, stroke, and Parkinson's disease. Although homocysteine-induced apoptosis in neurons has been extensively studied, little information is available regarding the effect of homocysteine on microglia. In this report, we demonstrated that homocysteine promoted proliferation and up-regulated the expression of CD11b (a marker of microglial activation). Consistent with our *in vitro* results, a significant increase in the number of CD11b-positive microglia was also observed in brain sections of mice with hyperhomocysteinemia. Homocysteine promoted the activity of NAD(P)H oxidases, resulting in the generation of reactive oxygen species. Up-regulation of NAD(P)H oxidase activity by homocysteine appears to be due to its ability to induce the phosphorylation of p47phox through the p38 MAPK pathway. Furthermore, inhibition of reactive oxygen species significantly blocked cellular proliferation and activation in microglia. Since microglial proliferation and activation play an important role in the development of several neurodegenerative disorders, our results reveal a novel role of homocysteine in the pathogenesis of neurodegenerative diseases.

Keywords: Homocysteine; Microglia; Proliferation; Activation; NAD(P)H oxidases; Reactive oxygen species; p47phox; p38 MAPK

1. Introduction

Homocysteine is formed as an intermediate in sulfur amino acid metabolism. Elevated levels of circulating homocysteine, a condition known as hyperhomocysteinemia, are regarded as an independent risk factor for cardiovascular diseases (Zou and Banerjee, 2005). In addition to its association with cardiovascular diseases, homocysteine also plays an important role in several neurological and psychological disorders (Mattson and Shea, 2003). Epidemiological and experimental studies correlate hyperhomocysteinemia to a range of neurodegenerative conditions, including Alzheimer's disease, stroke, and Parkinson's disease (Mattson and Shea, 2003; Zou and Banerjee, 2005). The mechanism underlying homocysteine mediated-pathogenesis of neurodegenerative disorders is not yet fully understood. *In vitro* experiments have demonstrated that homocysteine can cause apoptosis in cultured neurons (Kruman et al., 2000; Kruman et al., 2004). Additionally, mice with hyperhomocysteinemia show a substantial increase of apoptotic neurons and astrocytes in the CA1 hippocampal layer of rats (Blaise et al., 2007). Homocysteine-induced neuronal death is often associated with increased levels of cytosolic calcium and reactive oxygen species (ROS) formation (Lipton et al., 1997; Ho et al., 2002; Tjiattas et al., 2004).

Microglia are resident brain macrophages, which function as immune effectors (Cabral and Marciano-Cabral, 2005). Microglia become activated and proliferate following brain injury or stimulation by various proinflammational factors. It has been suggested that microglial activation plays an important role in the initiation and development of several

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neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease (Nelson et al., 2002; Liu and Hong, 2003; Guillemin and Brew, 2004). The activated microglia may secret a diverse range of proinflammatory and neurotoxic factors, such as nitric oxide, arachidonic acid, and cytokines which contribute to their neurotoxicity (Vegeto et al., 2001; Liu and Hong, 2003).

Homocysteine has been shown to elicit cellular proliferation in vascular smooth muscle cells (VSMCs) (Tsai et al., 1994), splenic B lymphocytes (Zhang et al., 2001) and mesangial cells (Yang and Zou, 2003). The promitogenic effects of homocysteine are associated with the generation of ROS (Zhang et al., 2001; Yang and Zou, 2003). Homocysteine can stimulate ROS formation in a number of different cell types, such as splenic B lymphocytes, mesangial cells, monocytes, and VSMCs (Zhang et al., 2001; Yang and Zou, 2003; Zeng et al., 2003; Liu et al., 2008). To better understand the role of hyperhomocysteinemia in the pathogenesis of neurodegenerative disorders, we investigated the effect of homocysteine on the proliferation and activation of microglia. We found that homocysteine stimulated DNA synthesis and the activation of microglia. The action of homocysteine is due to enhancing ROS formation by activation of NAD(P)H oxidase.

2. Materials and methods

2.1. Induction of hyperhomocysteinemia

Adult Balb/c mice were obtained from Baiyao Pharmacological Co. (Kunming, China). The animals were fed one of two diets: (i) Control diet (LM-485 chow, Harlan Teklad, Madison, WI); (ii) high-methionine diet (LM-485 chow with drinking water supplemented with 0.5% L-methionine). Mice were sacrificed after 3 months on the diets. The protocol of the experiments was approved by the Animal Care and Use Committee of Yunnan University. Homocysteine levels in plasma of mice were determined by using an ELISA kit (Axis-Shield, UK).

2.2. Isolation of mouse microglia

Mouse primary microglial cells were prepared from mixed glial cultures, as described previously (Park et al., 2002). Briefly, cortices were dissected from newborn BALB/c mice and dissociated by trypsinization and mechanical disruption. Cells were plated on 24-well plates. On days 10–14, microglia were harvested by shaking the cultures (120 rpm) and collecting the floating cells. These cells were seeded into plastic tissue culture flasks. After incubation at 37 °C for 1 h, non-adherent cells were removed by replacing culture medium. The purity of microglia was verified at 96% by CD68 (ABCAM, Cambridge, UK) immunoreactivity. The cells were grown in DMEM (Gibco BRL, Gaithersburg, MD) with 10% fetal bovine serum (FBS) (Gibco) and maintained at 37 °C, 5% CO_2.

The BV-2 immortalized microglial cell line was obtained from The Cell Bank of Chinese Medical Academy of Sciences. The cells were grown in DMEM (Gibco BRL, Gaithersburg, MD) with 10% FBS and maintained at 37 °C, 5% CO_2.

2.3. Determination of cell growth and [^{3}H]-thymidine incorporation assay

Primary microglia were grown in DMEM supplemented 10% FBS and maintained at 37 °C. Before addition of homocysteine, we made cells quiescent by incubating them in medium without FBS for 24 h. Experiments were initiated with fresh DMEM with 1% FBS and containing DL-homocysteine (Sigma, St. Louis, MO). Cells were counted in triplicate in a hemocytometer after 48 h of incubation with homocysteine.

After pretreated with homocysteine for 18 h, the cells were added 50 μl of serum-free medium containing [^{3}H]-thymidine (Atom High-Tech Co., Beijing, China) at 1 μCi/ml to each well. After incubation for an additional 6 h, cells were washed with PBS three times. Then these cells were harvested and transferred onto glass fiber filters. After treated with 5% TCA solution, the cells were then rinsed in 100% ethanol. The filters were dried in air and transferred into scintillation vials including 10 ml scintillation fluid (2 g PPO, 0.05 g POPOP, 333 ml methylbenzene, 167 ml Triton-X-100). After 3 min of shaking, the vials were subjected to radioactivity assay. The radioactivity was measured by a LKB 1214 scintillation counter (Rackbeta, Stockhlom, Sweden).

2.4. Measurement of ROS

Formation of ROS in cells was measured using a probe, 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) (Molecular Probes, Junction City, OR). Cells were pretreated with (10 μM) H_2DCFDA for 30 min and then with homocysteine. DCF fluorescence intensity was measured by using a Cytofluor II fluorescent plate reader (excitation 485/emission 530; Millipore Corp., Bedford, MA).

2.5. Measurement of nitric oxide (NO)

The amount of NO formed in the reaction mixture was measured from the accumulation of the stable NO metabolite nitrite by using the Griess assay according to the protocol of the manufacturer (Beyotime Institute Biotechnology, Jiangmen, China), as absorbance at 540 nm from the ELISA plate reader. The amount of nitrite was normalized to the amount produced by untreated microglia.

2.6. Measurement of NAD(P)H oxidase activity in cells

NAD(P)H oxidase activity was determined in cells as previously described (Shi et al., 2001). After washed with PBS twice, cells were incubated with reaction buffer con-