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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-

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taining NAD(P)H (100 μM), Cytochrome *c* (75 μM), with or without SOD (1000 U/ml) at 37 °C. After 30 min of incubation, the absorbance at 550 nm was measured. There was no detectable activity in the absence of NAD(P)H. The activity of NAD(P)H oxidase was calculated as SOD-inhibitable Cytochrome *c* reduction and expressed as superoxide in nmol g^{-1} min^{-1}.

2.7. Quantitative real-time PCR analysis

Total RNA from cells was isolated using a total RNA extract kit (Omega Bio-tek Inc., Doraville, GA). Random-primed cDNAs were generated by reverse transcription of total RNA samples with SuperScript II (Invitrogen, Carlsbad, CA). A real-time-PCR analysis was performed with the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) using SYBR® Premix-Ex Tag™ (Takara, Dalian, China). Beta-actin was used as an internal control. The primers used for PCR were listed in Table S1.

2.8. Western blotting

Cells were lysed on ice for 30 min in lysis buffer (containing 0.15 M NaCl, 30 mM Tris, 1 mM phenylmethanesulfonyl fluoride, 1% Triton X-100, 1 mM EDTA, 10 μg/ml leupetin, 2 μg/ml pepstatin, 2 μg/ml aprotinin and 2 mM Na_3VO_4). The proteins of cell lysates were separated on a 10% SDS polyacrylamide gel, and then transferred to PVDF membranes. Primary antibodies were: anti-actin antibodies (Sigma, St. Louis, MO), anti-phospho Akt (Ser^{473}) antibodies (ABCAM, Cambridge, UK), anti-CD11b antibodies (Biolegend, San Diego, CA), anti-phospho extracellular signal-regulated kinase (ERK)1/2, anti-phospho c-Jun NH_2-terminal kinase (JNK), anti-p38 mitogen-activated protein kinase (MAPK), anti-phospho-p38 MAPK, and anti-p47phox (Santa Cruz Biotech, Santa Cruz, CA), anti-phospho-p47phox antibodies (Ser370) (Abzoom, Dallas, TX). The secondary antibodies Included peroxidase-coupled anti-mouse IgG, anti-rabbit IgG (Amersham Biosciences, Piscataway, NJ), and anti-rat IgG (Boster Biol Tech, Wuhan, China). The bands were visualized using an ECL detection system (Amersham Biosciences). Each blot was stripped with a stripping solution (0.1 M glycine, pH2.9) for 1 h and re-probed with other antibodies.

2.9. Immunohistochemistry

Brains were postfixed in 4% paraformaldehyde for 2 h and placed in 30% sucrose–PBS solution for 2 days. Sections of 20 μm were cut with a cryostat and mounted on Super Frost Plus slides (Fisher Scientific, Pittsburgh, PA). After washes with TTBS, the sections were blocked by 3% BSA–PBS solution at room temperature for 1 h. And then the sections were stained with anti-CD11b (microglia/macrophage; 1:100, Biolegend) at 4 °C overnight. All incubations were performed under humidified conditions. The brain sections were washed with PBS three times for 10 min at room temperature and incubated with AP-conjugated goat anti-rat IgG (Boster Biol Tech) for 60 min at room temperature. Finally, AP-substrate solution BCIP/NBT (Boster Biol Tech) was added until the desired intensity of color developed.

2.10. Immunofluorescent staining

Cells were fixed with methanol at 4 °C for 5 min. The non-specific protein binding was blocked in blocking buffer (1% BSA in TTBS) for 1 h. The cells were incubated at 4 °C for 24 h with anti-CD11b antibodies (1:150 dilution, Biolegend). After washing in TTBS, the cells were incubated for 1 h with anti-rat IgG conjugated with FITC (Boster Biol Tech). Images were captured using a D-eclipse C1 confocal microscope (Nikon, Tokyo, Japan).

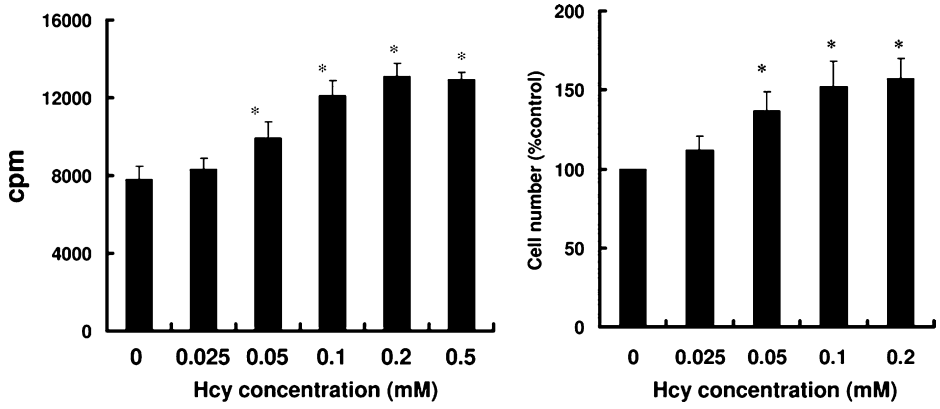

Fig. 1. Homocysteine induces cell proliferation in primary microglia. (A) Primary microglia were incubated with varying concentrations of homocysteine for 24 h. The cell proliferation was assessed by [^{3}H]-thymidine incorporation into DNA. (B) Primary microglia were incubated with varying concentrations of homocysteine (Hcy) for 48 h. Cells were counted in triplicate in a hemocytometer. These results are means ± SD of five experiments. *$P < 0.05$ *versus* control (without homocysteine).

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2.11. Plasmid construction for the short hairpin RNAs

To induce stable RNAi-mediated knockdown of the p47phox, pSilencer2.1-U6 plasmids (a gift from Dr. Jianguo Wu, Wuhan University, China) were constructed to express short hairpin RNAs (shRNA). Phosphorylated and annealed 64-mer double-strand DNA oligonucleotides incorporating BamHI and HindIII restriction sites were ligated into the respective sites in the pSilencer2.1-U6 vector. Five separate hairpin sequences were analyzed for reduction of p47phox gene expression (data not shown). The RNAi sequence, which was effective for p47phox knockdown as measured by real-time PCR, was shRNA 1.1: 5′-TGTGATGTCAGCTACGTTA-3′.

2.12. Transfection

Transfection was performed using Lipofectamine 2000® Reagent in OPTI-MEM medium (Invitrogen) in BV-2 microglial cells. The BV-2 immortalized microglial cell line has been extensively characterized and has many of the features of primary microglia (Murphy et al., 1998). In parallel experiments, the expression vectors were replaced with empty vectors. 48 h following transfection, the medium was replaced with fresh DMEM containing 10% FBS. Stable cell lines of BV-2 cells that express p38 dominant negative mutant or shRNA for p47phox gene, were produced by transfection of pcDNA3-p38DN plasmid (a gift from Dr. Jiahuai Han, Xianmen University, China) or pSilencer2.1-U6-shRNA followed by positive colony selection using G418 (Sigma) at a concentration of 500 μg/ml.

2.13. Statistical analysis

Data from experiments were expressed as mean ± SD. Statistical difference between the groups was analyzed using one-way ANOVA, followed by a Newman–Keuls test. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Homocysteine-induced proliferation in primary microglia

In this study, primary mouse microglia were cultured to investigate the effect of homocysteine on DNA synthesis. [^{3}H]-thymidine incorporation was then determined to assess the rate of DNA synthesis after incubation with homocysteine for 24 h. As shown in Fig. 1, homocys-

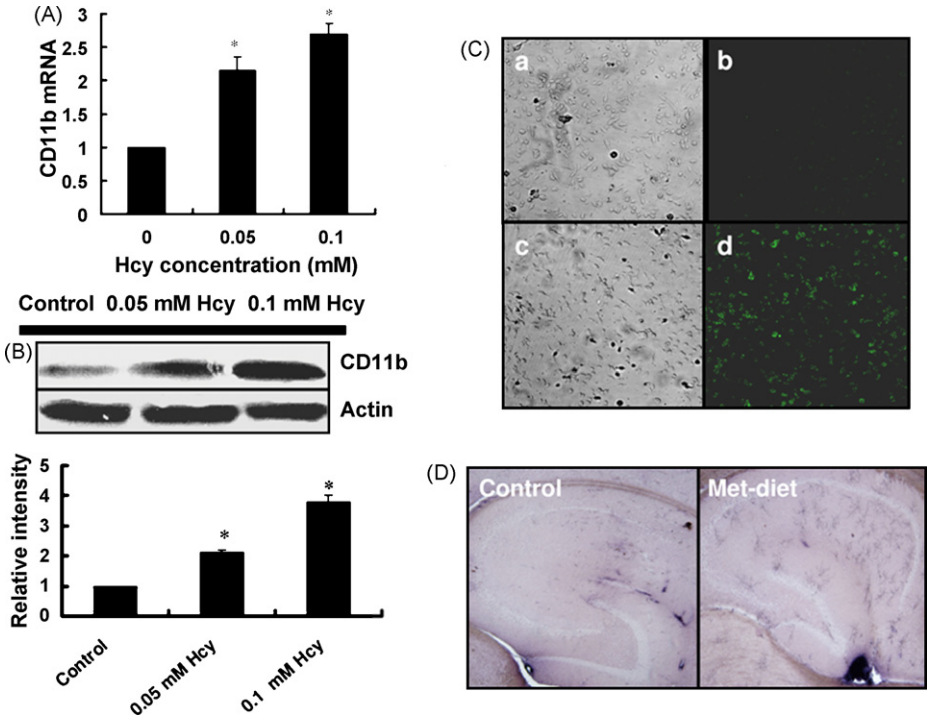

Fig. 2. Homocysteine promotes microglial activation. Primary microglia were incubated with 0.05 or 0.1 mM homocysteine (Hcy) for 8 h. (A) The mRNA levels were detected by real-time PCR. All results are standardized to the levels of actin and are the means ± SD of five experiments. *$P < 0.05$ *versus* control (without homocysteine). (B) The protein levels were detected by Western blotting. The blot is typical of five experiments. *$P < 0.05$ *versus* control (without homocysteine). (C) After cells were fixed with methanol, CD11b was detected in microglia by immunofluorescent staining using anti-CD11b antibodies. Cellular fluorescence intensity was visualized by using a confocal microscopy. (a and b) Control (without homocysteine), (c and d) Homocysteine (0.1 mM) treatment for 8 h. Photographs are representative of three studies with similar results. Original magnification ×100. (D) Hyperhomocysteinemia in mice was induced by feeding a diet enriched in methionine. Sections of brain were stained as described in "Section 2". Representative photomicrographs are shown. Original magnification ×40.

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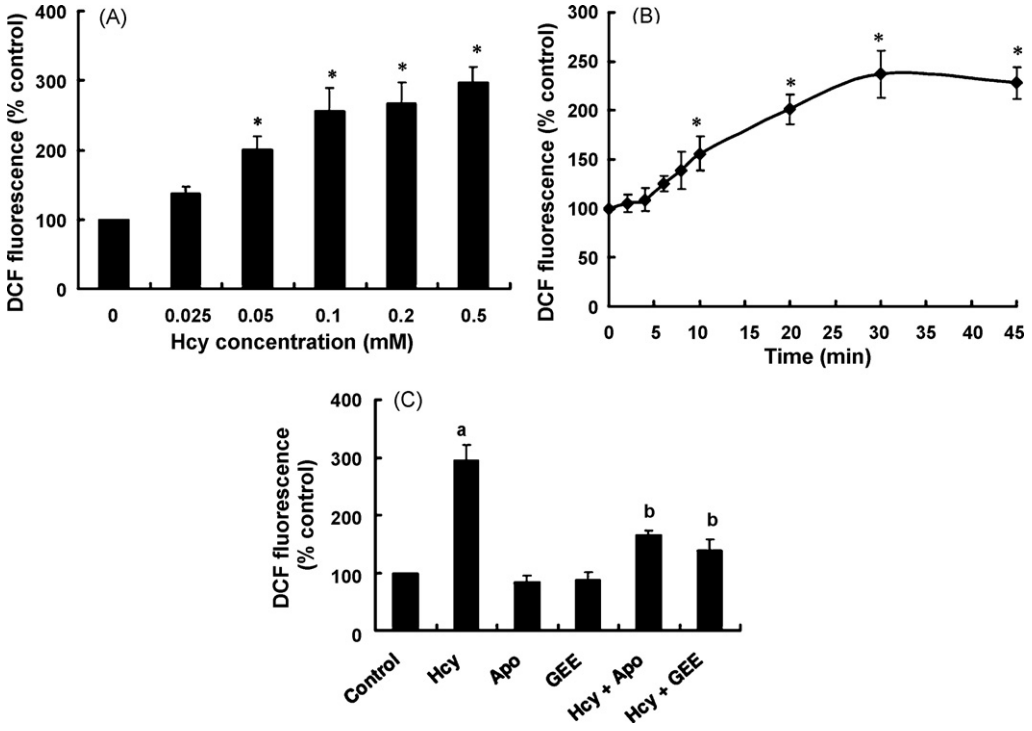

Fig. 3. Homocysteine promotes ROS formation in primary microglia. (A) Cells were loaded with H_2DCFDA (10 μM) for 30 min before incubation with homocysteine (Hcy) for 30 min. Formation of ROS in cells at the indicated concentrations of homocysteine was determined by fluorescent plate reader. *$P < 0.05$ *versus* control (without homocysteine). (B) Cells were loaded with H_2DCFDA (10 μM) for 30 min before incubation with 0.1 mM homocysteine (Hcy). Formation of ROS in cells was determined at the indicated times. (C) Cells were preincubated with glutathione ethyl ester (5 mM) (GEE) or apocynin (100 μM) (Apo) for 30 min before incubated with 0.1 mM homocysteine. The ROS levels were evaluated by performing DCF measurement at 30 min. Data are presented as relative increase compared with control (100%) ($n = 5$). [a]$P < 0.05$ *versus* control (without homocysteine); [b]$P < 0.05$ *versus* homocysteine treatment;

teine significantly promoted [^{3}H]-thymidine incorporation. The effect of homocysteine on [^{3}H]-thymidine incorporation was found to be dose-dependent, and 0.2 mM was sufficient to achieve a maximal effect. Furthermore, homocysteine-induced cellular proliferation as also indicated by cell numbers. By 48 h, homocysteine at 0.025, 0.05, 0.1 and 0.2 mM increased cell number by 12%, 37%, 52% and 57%, respectively (Fig. 1B). We found that cysteine (0.05–1 mM), another thiol-containing amino acid, did not significantly promote proliferation (data not shown), indicating this effect was selective for homocysteine.

A recent study has reported that homocysteine (100 μM) in combination with S-adenosylhomocysteine (0.5–4.0 μM), but not homocysteine alone, significantly decreased the cell viability at an incubation time of 72 h (Lin et al., 2007). We found that no obvious lactate dehydrogenase leakage was observed after microglia were incubated with homocysteine up to a concentration of 500 μM and an incubation time of 72 h (data not shown).

3.2. Homocysteine induced the activation of primary microglia

Since increased expression of CD11b is one typical feature of microglial activation, the effect of homocysteine on the expression of CD11b was examined. As shown in Fig. 2A and B, both the mRNA and protein levels of CD11b were evaluated after homocysteine treatment for 8 h. The effect of homocysteine on the expression of CD11b in microglial cells was further examined by immunofluorescence analysis. Compared with control cells, the intensity of CD11b immunofluorescence staining was markedly enhanced in primary microglia exposed to homocysteine for 8 h (Fig. 2C). Microglia activation is often accompanied by increased NO production (Dheen et al., 2005). We thus determined the effect of homocysteine on NO production in primary microglia. As shown in Fig. S1, the concentrations of 0.05, 0.1, and 0.2 mM homocysteine resulted in 65%, 126%, and 143% increase in the amount of NO. As a positive control, lipopolysaccharide (100 ng/ml) elicited 221% increment in primary microglia.

To test whether homocysteine influence the expression of CD11b *in vivo* in the central neuronal system, we induced hyperhomocysteinemia in mice fed a high-methionine diet. After 3 months, plasma levels of homocysteine were approximately five-fold higher in mice on the diet than in control mice (16.7 ± 2.3 vs 3.1 ± 0.2 μM, respectively). Immunohistochemical analysis revealed that an increase in the number of CD11b-positive microglia was observed in brain sections of mice with hyperhomocysteinemia compared with those of normal mice (Fig. 2D).

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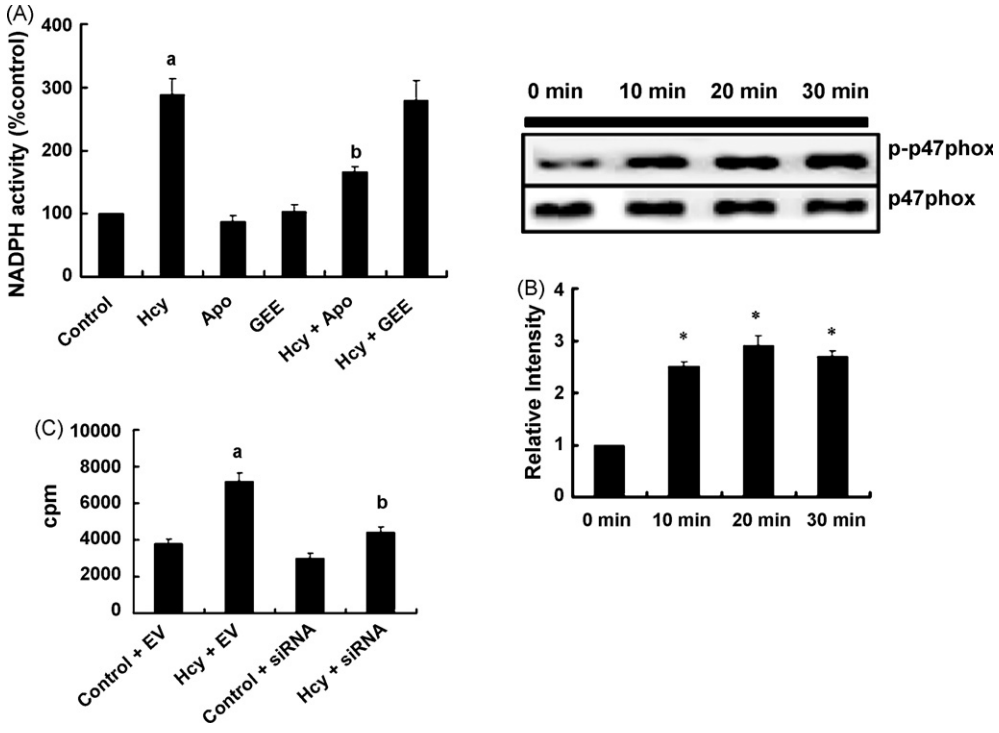

Fig. 4. Homocysteine activates NAD(P)H oxidases by promoting phosphorylation of p47phox. (A) Primary microglia were preincubated with apocynin (100 μM) (Apo) or glutathione ethyl ester (5 mM) (GEE) for 30 min. After cells were incubated with 0.1 mM homocysteine for 30 min, NAD(P)H oxidase activity was determined in cells as described in "Section 2" ($n = 5$). [a] $P < 0.05$ *versus* control (without homocysteine); [b] $P < 0.05$ *versus* homocysteine treatment; (B) After primary microglia were incubated with 0.1 mM homocysteine at the indicated times, the protein levels were detected by Western blotting. The blot is typical of three experiments. * $P < 0.05$ *versus* control (without homocysteine). (C) Stable cell lines of BV-2 microglia that express shRNA for p47phox gene, were produced by transfection of pSilencer2.1-U6-shRNA followed by positive colony selection using G418. The cell proliferation was assessed by [^{3}H]-thymidine incorporation into DNA. These results are means ± SD of five experiments. EV, empty vector, [a] $P < 0.05$ *versus* control (without homocysteine); [b] $P < 0.05$ *versus* homocysteine treatment alone.

3.3. Homocysteine elicits ROS production via NAD(P)H oxidase

Previous studies have demonstrated that homocysteine induces generation of ROS in a variety of cell types (Yang and Zou, 2003; Wang et al., 2005; Koh et al., 2006; Papatheodorou and Weiss, 2007). We then examined the effect of homocysteine on formation of ROS using a fluorescent probe, H_2DCFDA in primary microglia. As shown in Fig. 3A, homocysteine at 0.025, 0.05, 0.1, 0.2, 0.5 mM increased intracellular ROS levels in 30 min of incubation by 138%, 201%, 253%, 267%, 298%, respectively. As shown in Fig. 3B, homocysteine also induced formation of ROS in a time-dependent manner. DCF fluorescence started increasing in 10 min and reached a maximal level at 30 min. Glutathione ethyl ester (5 mM) (a cell permeable scavenger of H_2O_2) significantly suppressed homocysteine-induced ROS formation (Fig. 3C). However, glutathione ethyl ester by itself did not markedly alter the levels of DCF fluorescence in the absence of homocysteine.

To study the role of NAD(P)H oxidase in formation of ROS induced by homocysteine, primary microglia were treated with a specific inhibitor of NAD(P)H oxidases, apocynin. Apocynin can block the assembly of the multisubunit enzyme complex (Stolk et al., 1994). As shown in Fig. 3C, apocynin (100 μM) significantly inhibited ROS formation after homocysteine treatment. However, apocynin by itself did not markedly alter the levels of DCF fluorescence in the absence of homocysteine. Similar results were obtained using diphenyleneiodonium (10 μM), another inhibitor of NAD(P)H oxidase (data not shown).

Next, we examined the effect of homocysteine on the activity of NAD(P)H oxidases. As shown in Fig. 4A, homocysteine significantly promoted the activity of NAD(P)H oxidases in primary microglia after 30 min incubation. Apocynin markedly abolished the stimulatory effect of homocysteine on the activity of NAD(P)H oxidases. However, apocynin by itself did not significantly alter the levels of NAD(P)H oxidases in the absence of homocysteine. Similar results were obtained using diphenyleneiodonium (10 μM) (data not shown).

Since ROS production was derived from NAD(P)H oxidase, we examined the effect of homocysteine on the expression of subunits of NAD(P)H oxidase. We found that there was no change in the mRNA levels of p40phox, p47phox, p67phox, p22phox and gp91phox (data not shown). However, we found that homocysteine treatment resulted in a significant increase in the phosphorylation of p47phox

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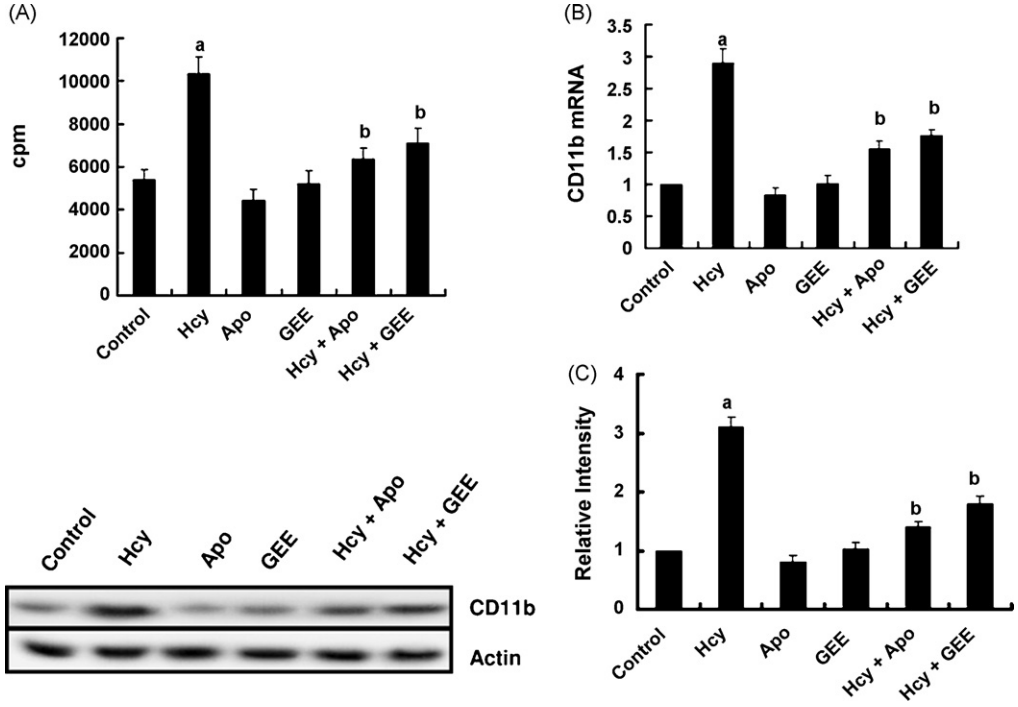

Fig. 5. Inhibition of ROS formation suppresses homocysteine-induced cellular proliferation and activation. Primary microglia were preincubated with apocynin (100 μM) (Apo) or glutathione ethyl ester (5 mM) (GEE) for 30 min. (A) After cells were incubated with 0.1 mM homocysteine (Hcy) for 24 h, cellular proliferation was assessed by [^{3}H]-thymidine incorporation into DNA. These results are means ± SD of five experiments. [a] $P < 0.05$ *versus* control (without homocysteine); [b] $P < 0.05$ *versus* homocysteine treatment alone. (B) After cells were incubated with 0.1 mM homocysteine for 8 h, the mRNA levels were detected by real-time PCR. All results are standardized to the levels of actin and are the means ± SD of five experiments. [a] $P < 0.05$ *versus* control (without homocysteine); [b] $P < 0.05$ *versus* homocysteine treatment. (C) After cells were incubated with 0.1 mM homocysteine for 8 h, the protein levels were detected by Western blotting. The blot is typical of three experiments. [a] $P < 0.05$ *versus* control (without homocysteine); [b] $P < 0.05$ *versus* homocysteine treatment.

in primary microglia after 10 min incubation (Fig. 4B). Apocynin (100 μM) and glutathione ethyl ester (5 mM) failed to abolish homocysteine-induced phosphorylation of p47phox (data not shown). To address the role of p47phox in homocysteine-induced proliferation, endogenous p47phox expression was ablated by shRNA in BV-2 microglial cells (Fig. S2). Homocysteine-induced proliferation was significantly inhibited in BV-2 microglia transfected with shRNA against p47phox mRNA (Fig. 4C).

3.4. Inhibition of ROS production blocks microglial proliferation and activation

In this study, we examined whether inhibition of ROS formation could block microglial proliferation and activation induced by homocysteine. As shown in Fig. 5A, pretreatment of primary microglia with glutathione ethyl ester (5 mM) or apocynin (100 μM) markedly inhibited [^{3}H]-thymidine incorporation stimulated by homocysteine. However, both compounds did not significantly alter [^{3}H]-thymidine incorporation.

Next, we tested the effect of glutathione ethyl ester or apocynin on microglial activation. Although both compounds did not significantly alter the expression of CD11b as well as the production of NO in the absence of homocysteine, they markedly suppressed homocysteine-induced-expression of CD11b and NO production in primary microglia (Figs. 5B and C and S3).

3.5. p38 MAPK is involved in homocysteine-induced proliferation and activation of microglia

To clarify the signaling mechanism underlying homocysteine-induced cellular proliferation and activation, primary microglia were preincubated with the inhibitors of MEK1/2 (PD98059, 10 μM), p38 MAPK (SB 202190, 20 μM), JNK (curcumin, 20 μM), PI3K (wortmannin, 5 μM) for 60 min. These inhibitors did not significantly alter the phosphorylation of p47phox and the formation of ROS in the absence of homocysteine. Of these tested inhibitors, only SB 202190 significantly blocked the phosphorylation of p47phox and the formation of ROS (Fig. 6). Western blotting revealed that homocysteine induced the phosphorylation of p38 MAPK as early as 5 min (Fig. 7A). In contrast, homocysteine did not modify the phosphorylation of ERK1/2, JNK and Akt with in 30 min (not shown).

To further confirm the role of p38 MAPK, the effects of SB 202190 on the proliferation and activation of primary microglia were investigated. SB 202190 by itself did not significantly alter [^{3}H]-thymidine incorporation, the expression of CD11b, and NO production in the absence of

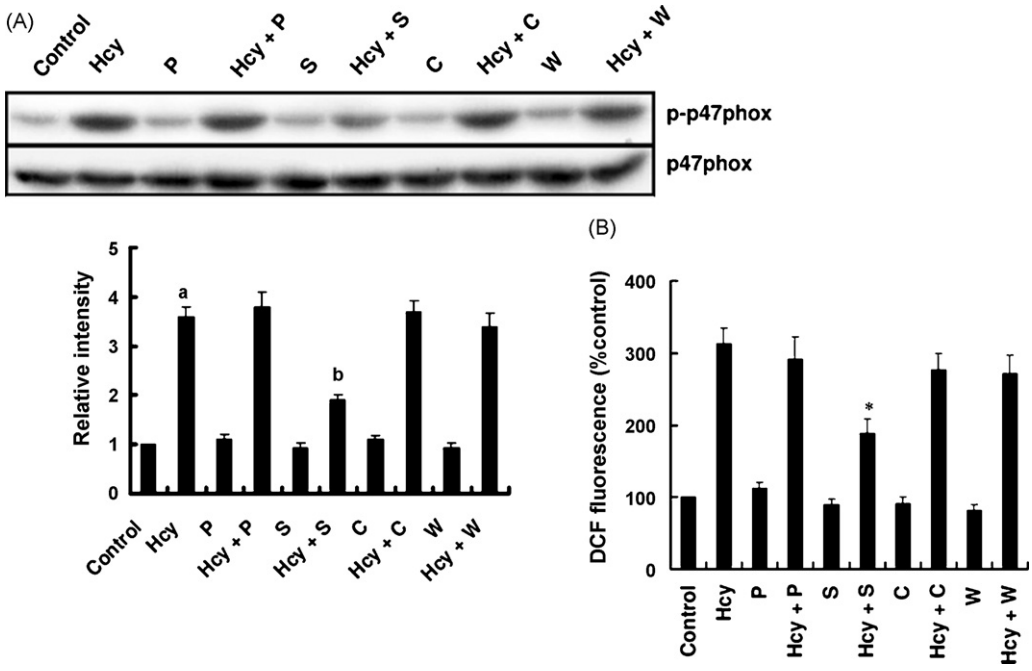

Fig. 6. Homocysteine induces the phosphorylation of p47phox and ROS formation through the p38 MAPK pathway. Primary microglia were preincubated with PD98059 (10 μM) (P), SB202190 (20 μM) (S), curcumin (20 μM) (C), wortmannin (5 μM) (W) for 30 min. (A) After cells were incubated with 0.1 mM homocysteine (Hcy) for 30 min. The protein levels were detected by Western blotting. The blot is typical of three experiments. [a] $P < 0.05$ *versus* control (without homocysteine); [b] $P < 0.05$ *versus* homocysteine treatment. (B) After cells were incubated with 0.1 mM homocysteine for 30 min, the ROS levels were evaluated by performing DCF measurement. Data are presented as relative increase compared with control (100%) ($n = 5$). * $P < 0.05$ *versus* homocysteine treatment.

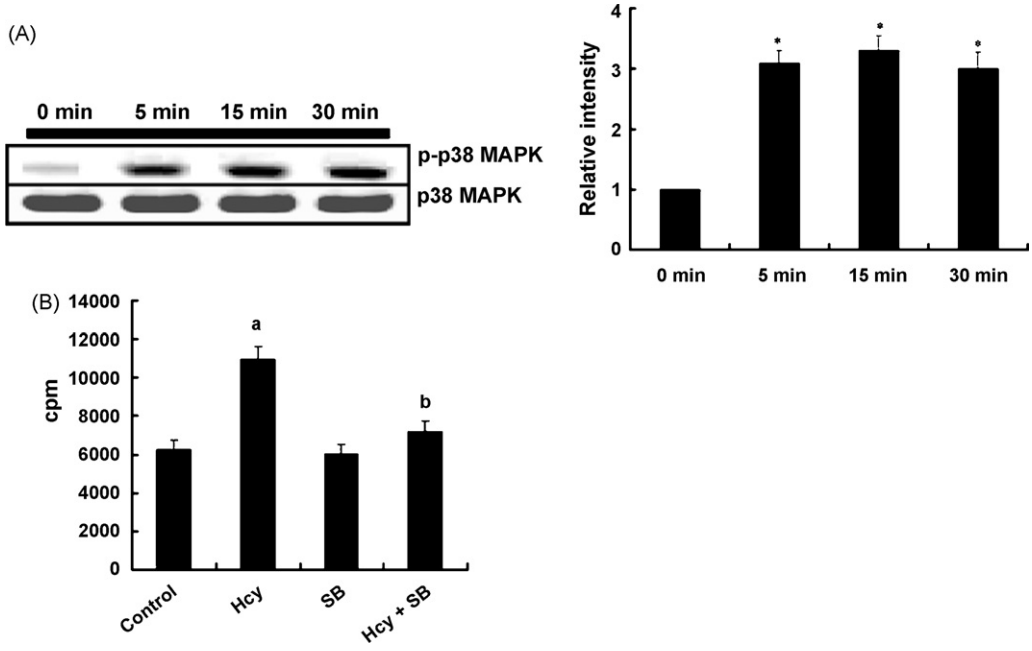

Fig. 7. p38 MAPK is involved in homocysteine-induced proliferation. (A) Primary microglia were incubated with 0.1 mM homocysteine at the indicated times. The protein levels were detected by Western blotting. The blot is representative of three independent experiments. *$P < 0.05$ *versus* control. (B) Primary microglia were preincubated with SB 202190 (20 μM) for 30 min before incubated with 0.1 mM homocysteine for 24 h, proliferation was assessed by [^{3}H]-thymidine incorporation into DNA. These results are means ± SD of five experiments. [a] $P < 0.05$ *versus* control (without homocysteine); [b] $P < 0.05$ *versus* homocysteine treatment.

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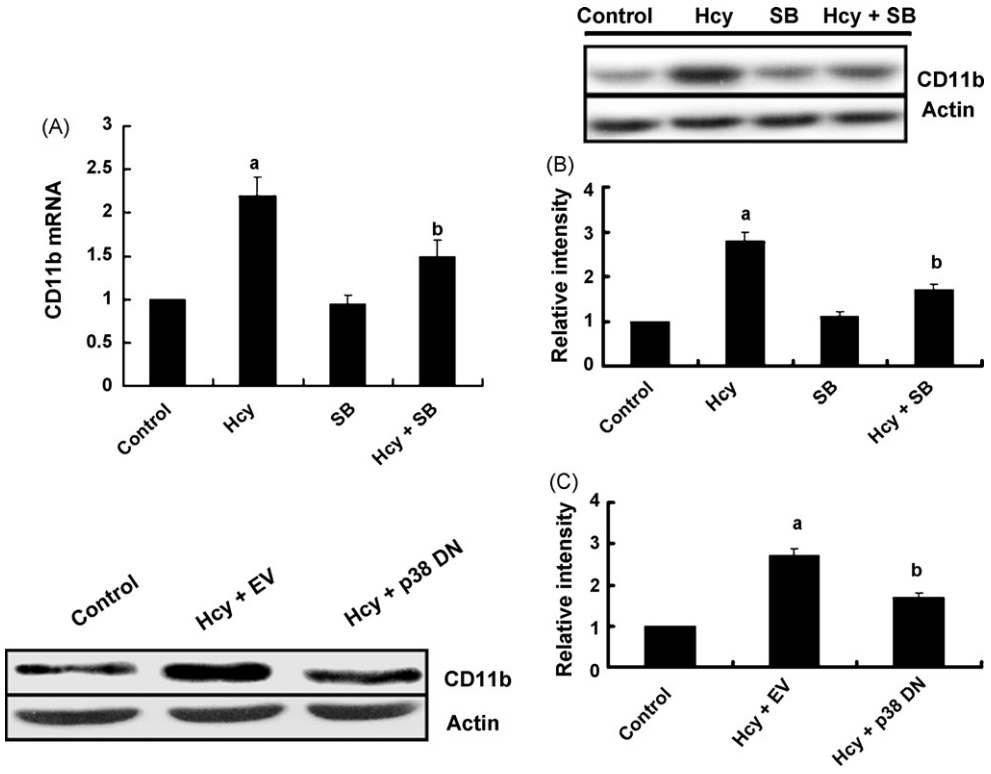

Fig. 8. p38 MAPK is involved in homocysteine-induced activation in microglia. (A) After primary microglia were incubated with 0.1 mM homocysteine for 8 h, the mRNA levels were detected by real-time PCR. All results are standardized to the levels of actin and are the means ± SD of five experiments. $^{a}P < 0.05$ *versus* control (without homocysteine); $^{b}P < 0.05$ *versus* homocysteine treatment. (B) After primary microglia were incubated with 0.1 mM homocysteine for 8 h, the protein levels were detected by Western blotting. The blot is typical of three experiments. $^{a}P < 0.05$ *versus* control (without homocysteine); $^{b}P < 0.05$ *versus* homocysteine treatment. (C) Stable cell lines of BV-2 microglia that express p38 MAPK dominant negative mutant were produced by transfection of pcDNA3-p38DN, followed by positive colony selection using G418. After cells were incubated with 0.1 mM homocysteine for 8 h, the protein levels were detected by Western blotting. The blot is typical of three experiments. EV, empty vector. $^{a}P < 0.05$ *versus* control (without homocysteine); $^{b}P < 0.05$ *versus* homocysteine treatment.

homocysteine. However, SB 202190 significantly inhibited [^{3}H]-thymidine incorporation (Fig. 7B), the expression of CD11b as well as NO production induced by homocysteine in primary microglia (Figs. 8A and B and S4). Furthermore, the expression of CD11b induced by homocysteine in BV-2 microglia were also suppressed by overexpressing dominant-negative mutant of p38α (Fig. 8C).

4. Discussion

Hyperhomocysteinemia is associated with a range of neurodegenerative conditions, including Alzheimer's disease, stroke, and Parkinson's disease (Mattson and Shea, 2003; Zou and Banerjee, 2005). One mechanism underlying homocysteine mediated-pathogenesis of neurodegenerative disorders is due to its ability to induce neuronal apoptosis by increasing levels of cytosolic calcium and ROS formation in neurons (Lipton et al., 1997; Kruman et al., 2000; Ho et al., 2002; Kruman et al., 2004; Tjiattas et al., 2004). Although homocysteine-induced neurodegenerative disorders have been extensively studied, little information is available regarding the effect of homocysteine on microglia. In this report, we demonstrated that homocysteine promoted the proliferation and activation of microglia. It has been shown that the proliferation and activation of microglia are involved in a variety of neuropathological processes such as Alzheimer's disease, Parkinson's disease (Vegeto et al., 2001; Nelson et al., 2002; Stoll and Jander, 1999; Liu and Hong, 2003; Guillemin and Brew, 2004). Thus, our current results provide a novel mechanistic explanation for the development and progression of neurodegenerative diseases in hyperhomocysteinemia.

Homocysteine has been shown to promote cellular proliferation in a variety of cells (Tsai et al., 1994; Zhang et al., 2001; Yang and Zou, 2003). Moreover, homocysteine can stimulate ROS formation in a variety of cell types (Yang and Zou, 2003; Wang et al., 2005; Koh et al., 2006; Papatheodorou and Weiss, 2007). In this study, our data also revealed that homocysteine markedly stimulated ROS formation in microglia (Fig. 3A). Treatment with glutathione ethyl ester abolished the effects of homocysteine on microglial proliferation and activation (Fig. 4). In addition, the inhibitor of NAD(P)H oxidases, apocynin, significantly antagonized the stimulatory effects of homocysteine on microglial proliferation and activation (Fig. 4). Recently, Mander et al. (2006)

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demonstrated that ROS from NAD(P)H oxidases is involved in microglial proliferation induced by IL-1β or TNF-α. These findings are consistent with the view that promitogenic effects of homocysteine are associated with the generation of ROS (Zhang et al., 2001; Yang and Zou, 2003). ROS not only cause oxidative insult in cells but also function as the molecules of cell signaling. One of mechanisms underlying the mitogenic effects of ROS is probably due to the inhibition of protein tyrosine phosphatases (Hecht and Zick, 1992).

In this study, homocysteine stimulated the activity of NAD(P)H oxidase (Fig. 3A). The NAD(P)H oxidase in microglia consists of at least five components: two membrane proteins, p22phox and gp91phox, three cytoplasmic proteins, p47phox, p67phox, and p40phox (Vilhardt et al., 2002; Li et al., 2005). However, we found that homocysteine treatment did not alter the expression of these five subunits. Instead, homocysteine significantly enhanced the phosphorylation of p47phox (Fig. 3D). Homocysteine has been shown to cause the phosphorylation of p47phox in monocytic cell line THP-1 (Siow et al., 2006). p47phox phosphorylation can lead to its translocation from cytoplasm to membrane and the activation of NAD(P)H oxidase (Siow et al., 2006). In deed, we found that inhibition of the phosphorylation of p47phox by a specific inhibitor of p38 MAPK, SB 202190, markedly suppressed the ROS production (Fig. 6A and B). Thus, these data indicate that increased phosphorylation of p47phox is considered as the major cause of elevated ROS production.

In the present study, exposure to homocysteine promoted a rapid increase in phosphorylation of p38 MAPK (Fig. 7A). Inhibition of p38 MAPK by SB 202190 markedly abolished microglial proliferation and activation (Fig. 7B–D), suggesting the p38 MAPK pathway is predominantly involved in this process. However, how homocysteine acts to initiate phosphorylation of p38 MAPK is unclear. It has been shown that ROS function as an intermediator to activate a signaling pathway involving p38 MAPK, mediating homocysteine-induced chemotaxis in VSMCs (Akasaka et al., 2005) and monocytes (Zeng et al., 2003), respectively. However, we found that either apocynin or glutathione ethyl ester failed to suppress the phosphorylation of p38 MAPK induced by homocysteine (data not shown). In this study, a significant increase in the ROS production was not observed at 5 min after incubation with homocysteine (Fig. 3B). In contrast, homocysteine induced the phosphorylation of p38 MAPK after 5 min of incubation. Thus, these results suggest that the effect of homocysteine on the phosphorylation of p38-MAPK is ROS-independent.

In summary, the present study demonstrates that homocysteine promotes proliferation and activation in microglia. Homocysteine activates p38MAPK, which in turn promotes the phosphorylation of p47phox, resulting in the activation of NAD(P)H oxidases and the generation of ROS. ROS is involved in cellular proliferation and activation in microglia. Our findings reveal a novel role of homocysteine in the pathogenesis of neurodegenerative diseases.

Conflicts of interest

The authors declare that they have no potential conflicts of interest to disclose.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neurobiolaging.2008.11.007.

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