Caffeic Acid Inhibits Acute Hyperhomocysteinemia-Induced Leukocyte Rolling and Adhesion in Mouse Cerebral Venules

HAI-PING ZHAO,*†,‡ JUAN FENG,*† KAI SUN,‡ YU-YING LIU,‡ XIAO-HONG WEI,‡ JING-YU FAN,‡ PING HUANG,‡ XIAO-WEI MAO,† ZHOU ZHOU,* CHUAN-SHE WANG,‡ XIAN WANG,* AND JING-YAN HAN†,*

*Department of Physiology and Pathophysiology, School of Basic Medical Sciences, Peking University, Beijing, China; ‡Tasly Microcirculation Research Center, Peking University, Beijing, China

Address for correspondence: Jing-Yan Han, M.D., Ph.D., Department of Integration of Chinese and Western Medicine, School of Basic Medical Sciences, Peking University, Beijing, China. E-mail: hanjingyan@bjmu.edu.cn and Xian Wang, M.D., Ph.D., Department of Physiology and Pathophysiology, School of Basic Medical Sciences, Peking University, Beijing 100191, China. E-mail: xwang@bjmu.edu.cn

1Co-first author

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ABSTRACT

Objective: To investigate the effects and possible mechanisms of CA on acute HHcy-induced leukocyte rolling and adhesion in mouse cerebral venules.

Methods: Male C57 BL/6J mice were injected with DL-Hcy (50 mg/kg) and CA (10 mg/kg). The effect of CA on HHcy-induced leukocyte rolling and adhesion in cerebral vessels was assessed using intravital microscopy. Plasma cytokines and chemokines were evaluated by cytometric bead array. ROS production in HUVECs and adhesion molecule expression on leukocytes were determined by flow cytometry. E-selectin and ICAM-1 expression in cerebrovascular endothelium was detected by immunohistochemistry. CD18 phosphorylation and the Src/PI3K/Akt pathway in leukocytes were determined by confocal microscopy and Western blot.

Results: CA inhibited HHcy-elicited leukocyte rolling and adhesion, decreased ROS production in HUVECs, and reduced plasma KC, MIP-2, and MCP-1 levels. CA reduced the E-selectin and ICAM-1 expression on cerebrovascular endothelium and CD11b/CD18 on leukocytes caused by HHcy. Of notice, CA depressed CD18 phosphorylation and the Src/PI3K/Akt pathway in leukocytes.

Conclusions: CA inhibited HHcy-provoked leukocyte rolling and adhesion in cerebral venules, ameliorating adhesion molecule expression and activation, which is related to the suppression of the Src/PI3K/Akt pathway in leukocytes.

Key words: leukocyte adhesion molecule, chemokine, Src, PI3K, Akt

Abbreviations: Akt, protein kinase B; CA, caffeic acid; DCF, dichlorofluorescein; ELISA, enzyme-linked immunosorbent assay; Hcy, homocysteine; HHcy, hyperhomocysteinemia; HUVECs, human umbilical vein endothelial cells; ICAM-1, intercellular adhesion molecule-1; IL-6, interleukin-6; KC, keratinocyte-derived chemokine; MCP-1, monocyte chemoattractant protein-1; MFI, mean fluorescence intensity; MIP-1α, macrophage inflammatory protein-1α; MIP-2, macrophage inflammatory protein-2; PI3K, phosphatidylinositol 3-kinase; PMSF, phenylmethanesulfonyl fluoride; PSGL-1, P-selectin glycoprotein ligand-1; ROS, reactive oxygen species; SABC, streptavidin–biotin complex; Src, proto-oncogene tyrosine-protein kinase; TNF-α, tumor necrosis factor-alpha.


INTRODUCTION

Cerebrovascular disease is a leading cause of death and disability in the world [40]. Increasingly findings suggest that systemic inflammation is the central feature in cerebrovascular disease [12,36]. The cerebral inflammation is initiated by leukocyte recruitment to the endothelium in cerebral venules, with cytokines, chemokines, and adhesion molecules as key mediators.

HHcy is an independent risk factor for atherosclerosis, cardiovascular, and cerebrovascular disease [2,3,15,17,34,37]. Folate/vitamin B6/vitamin B12 are currently used to lower Hcy level of HHcy patients in clinical practice. However, its role in the inhibition of inflammation is controversial.
[5,13,33], thus appealing for novel option to improve the outcome of patients with HHcy.

HHcy has been reported to evoke leukocyte rolling and adhesion to the microvascular wall, with increasing expression of adhesion molecules such as CD11b/CD18 on leukocytes and ICAM-1 on the endothelium [15,34]. Thus, the inhibition of adhesion molecule expression could reduce leukocyte–endothelium interaction so as to alleviate inflammation, which might be important for the treatment of cerebrovascular disease related to HHcy. However, the study of the mechanism underlying the action of Hcy on leukocyte CD11b/CD18 expression is limited, which hinders the development of new management for HHcy. Moreover, in addition to the upregulation of integrins CD11b/CD18 on leukocytes, the phosphorylation of CD18 is also known to be implicated in integrin activation [44]. However, no study has been published regarding the role of Hcy in CD18 phosphorylation and the related mechanism. In view of the fact that the Src/PI3K/Akt pathway has been reported to be related to TNF-α-induced CD11b/CD18 upregulation in neutrophil [28], we investigated the effect of HHcy on leukocyte integrin activation and the possible involvement of this pathway. CA (3,4-dihydroxyxannamic acid), one of the active constituents of Radix Salviae Miltiorrhizae, exhibits antioxidant and anti-inflammatory activities [9,31,35,38]. An in vitro study has shown that CA inhibits TNF-α-induced monocyte adhesion to HUVECs by decreasing the expression of E-selectin and ICAM-1 on HUVECs [29]. However, the effect of CA on the expression of leukocyte adhesion molecules such as CD11b/CD18 and potential mechanism remains unclear.

In this study, we explored the role of CA in acute HHcy-provoked leukocyte rolling and adhesion in mouse cerebral venules and the possible mechanism. Our results showed that CA inhibited Hcy-induced leukocyte rolling and adhesion in mouse cerebral venules, decreased the release of chemokines, and suppressed the expression of adhesion molecules on leukocytes and cerebrovascular endothelium. The inhibitory effect of CA on the expression of CD11b/CD18 and phosphorylation of CD18 on leukocytes was associated with the depression of the Src/PI3K/Akt pathway. Our work provides new information for the treatment of cerebrovascular disease related to HHcy.

**MATERIALS AND METHODS**

**Animals**

Male C57 BL/6J mice weighing 20–24 g, eight weeks old, were purchased from the Animal Center of Peking University Health Science Center. The certificate code of these animals was SCXK 2006-0008. This study was carried out in strict accordance with the Guide for the Care and Use of Laboratory Animals of the Health Science Center of Peking University. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Health Science Center of Peking University (Permit Number: 16559). All surgeries were performed under sodium pentobarbital (50 mg/kg, i.p.) anesthesia, and all efforts were made to minimize suffering of animals.

**Animal Preparation and Grouping**

The mice were anesthetized with sodium pentobarbital and the femoral vein was cannulated for the administration of various reagents. Acute HHcy was produced by intravenous injection of high concentration of DL-Hcy (Sigma, St. Louis, MO, USA), as described [15,34], and CA (Fengshanjian Medical Research, Kunming, China) was slowly injected in one minute. The mice were divided into three groups: control group (saline 0.4 mL), Hcy group (Hcy 50 mg/kg dissolved in 0.4 mL saline), and Hcy + CA group (Hcy 50 mg/kg dissolved in 0.2 mL saline + CA 10 mg/kg dissolved in 0.2 mL saline). The number of animals used in different experiment groups and for various parameters is detailed in Table 1.

**Table 1. Number of animals for different experiment groups and various parameters**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Plasma Hcy levels (5, 30, 90 minutes)</th>
<th>Leukocyte rolling and adhesion</th>
<th>Levels of plasma cytokines and chemokines (30, 90 minutes)</th>
<th>E-selecting and ICAM-1 expression (90 minutes)</th>
<th>L-selectin, PSGL-1, CD11b, CD18 expression (45, 90 minutes)</th>
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<tr>
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*For the multiple time-point experiment, the figures represent the animal number per time point.
†The animals were selected from those for leukocyte rolling and adhesion after observation.
**Plasma Hcy Level**

Anti-coagulated (50 U/mL heparin) blood samples of 50 µL were taken from the mouse femoral vein after 5, 30, and 90 minutes of Hcy administration, and the plasma was submitted to Peking University Third Hospital Clinic Laboratory for the determination of total Hcy concentration by ELISA.

**Leukocyte Rolling and Adhesion in Mouse Cerebral Venules**

For observation of cerebral microcirculation, a noninvasive method was applied. Briefly, the skull over the left parieto-occipital cortex was ground down with a cranial drill, and local microcirculation was recorded in real time through the thinnest part of the skull. Immersion oil (Olympus, Tokyo, Japan) was dropped on the skull to make it more transparent for observation of the cerebral venules under a microscope. Leukocyte rolling and adhesion in pial cerebral venules were assessed in each group before reagent administration (time zero) as baseline, and at 15, 30, 45, 60, and 90 minutes after reagent administration, respectively, by an upright fluorescence microscope (DMLFS; Leica, Mannheim, Germany) mounted with super-sensitive CCD camera (USS-301; UNIQ Vision, Santa Clara, CA, USA). Single unbranched venules without an obvious bend and with diameters ranging from 30 to 50 µm and lengths of about 200 µm were selected. The fluorescent marker rhodamine 6G (Sigma) of 0.2 mg/kg was injected to label leukocytes. The image was projected onto a monitor (J2118A; TCL, Seoul, Korea) and recorded with a DVD recorder (DVR-R25; Malata, Shenzhen, China). To examine leukocyte rolling and adhesion in venules, the dynamic behavior of leukocytes was examined by replaying the recorded video. Leukocytes moving slower than erythrocytes in the same vessel were defined as rolling. Adherent leukocytes were defined as cells that attached to the same site for more than 30 seconds, and the number of adherent leukocytes was counted along venules (200 µm in length) [41].

**Plasma Levels of Cytokines and Chemokines**

At 30 and 90 minutes after reagent administration, 100 µL anti-coagulated blood samples were collected from the mouse femoral vein to determine plasma levels of TNF-α, IL-6, KC, MIP-2, MIP-1α, and MCP-1 by cytometric bead array. For this purpose, 50 µL plasma or standard substance was incubated with 50 µL capture beads for one hour at room temperature and then mixed with 50 µL PE-labeled TNF-α, IL-6, KC, MIP-2, MIP-1α, or MCP-1 antibodies, incubated for two hours at room temperature. MFI was detected using flow cytometry (FACS Calibur, BD, San Jose, CA, USA) after the addition of 1 mL washing buffer (BD Pharmingen, San Diego, CA, USA). The data were analyzed by BD Cytometric Bead Array analysis software.

**Oxidative Stress in HUVECs**

Primary HUVECs were obtained from Clonetics (San Diego, CA, USA). Cells between passages 3 and 5 were used in the experiments. ROS formation was evaluated by intracellular DCF fluorescence. Confluent cells were exposed to Hcy 1 mM, Hcy 1 mM + CA 10 µM, Hcy 1 mM + CA 100 µM, or Hcy 1 mM + CA 400 µM for two hours, rinsed with PBS, and loaded with 5 µM H2DCF-DA (Molecular Probes, Leiden, The Netherlands) at 37°C in the dark for 20 minutes. The blank control cells were subjected to the same incubation but without DCF. Cells were trypsinized, resuspended in PBS at 1 × 10⁶/mL, and analyzed on a Coulter EPICS 753 flow cytometer (Beckman, Cincinnati, OH, USA) for DCF fluorescence (excitation 488 nm, emission 530 nm).

**E-selectin and ICAM-1 Expression in Cerebrovascular Endothelium**

At the end of microcirculation observation, the mice were perfused with 4% formaldehyde, then the brain was removed and cut into blocks, embedded in paraffin, and sectioned at 6 µm. Immunohistochemistry was employed to document the expression of E-selectin and ICAM-1 on cerebrovascular endothelium. Following overnight incubation with goat antimouse polyclonal primary antibodies targeting E-selectin and ICAM-1 (Santa Cruz, Santa Cruz, CA, USA) (1:50, 4°C), slides were treated for one hour with horseradish peroxidase-conjugated secondary antibodies (1:200). The expression of E-selectin and ICAM-1 was detected by using the SABC method [26]. Images were collected via light microscopy (20×). A total of 18 images, 6 per animal for 3 animals, were analyzed in each group. The positive vessels (30–50 µm in diameter) for E-selectin and ICAM-1 were counted in each image and averaged for each group.

**L-Selectin, PSGL-1, CD11b, and CD18 Expression on Leukocytes**

At 45 and 90 minutes after reagent administration, 200 µL anti-coagulated blood samples were collected from the mouse femoral vein and incubated with FITC-labeled antibodies against L-selectin or CD11b, or PE-labeled antibodies against PSGL-1 or CD18 (BD Pharmingen) for 20 minutes at room temperature in the dark. Erythrocytes were lysed with 2 mL of 1× FACS Lysing Solution (BD Pharmingen). Leukocytes were washed twice with PBS. Five thousand leukocytes were acquired for each sample, and the MFI was evaluated by flow cytometry [41].

**Leukocyte Isolation and Culturing**

For the in vitro studies below, leukocytes were isolated from mouse peripheral blood and cultured. Briefly, blood was harvested from normal mice, mixed with 5× sample volume of RBC lysis buffer (150 mM NH₄Cl, 10 mM
NaHCO₃, EDTA 1 mM), and centrifuged for three minutes, then the precipitated cells were washed with PBS. The procedure of lysis and wash was repeated once. The isolated leukocytes were plated at 2 × 10⁶ cells/well in 48-well round-bottom plates and cultured with RPMI-1640 medium containing 10% fetal bovine serum. The cell viability was determined to be >95% by trypan blue exclusion.

Confocal Microscopy of CD18 Phosphorylation in Leukocyte
To assess the role of CA on CD18 phosphorylation in leukocyte upon Hcy stimulation, the cells were divided into three groups: control, Hcy 1 mM, and Hcy 1 mM + CA 400 μM. Cells were incubated at 37°C for 60 minutes, then washed with PBS, dropped on slide, dried at 37°C for five minutes, fixed in prechilled acetone (−20°C) for 10 minutes, and finally permeabilized in PBST (0.3% Triton X-100-PBS) for 15 minutes. After blocked with 5% BSA-PBST for 10 minutes, cells were incubated with antibody against p-CD18 S745 (1:300; Abcam, Cambridge, UK) at 4°C overnight. The slides were washed and incubated with DyLightTM 488-labeled goat anti-rabbit IgG (H + L) (KPL, Gaithersburg, MD, USA) at a dilution of 1:100 for one hour. The nuclei were counterstained with Hoechst 33342 (Invitrogen, Carlsbad, CA, USA) at a dilution of 1:1000 for one hour. The slides were washed and mounted by confocal fluorescence microscopy (Bio-Rad, Hertfordshire, UK).

Immunoblot of CD18 Phosphorylation in Leukocytes
To evaluate the effect of CA on Hcy-induced CD18 phosphorylation in leukocytes by immunoblot, the cells were divided into five groups: control, Hcy 1 mM, Hcy 1 mM + CA 10 μM, Hcy 1 mM + CA 100 μM, and Hcy 1 mM + CA 400 μM. Cells were incubated at 37°C for 60 minutes, then lysed with cell lysis buffer (Applygen, Beijing, China) plus 1 mM PMSF. Total protein was quantified by the bicinchoninic acid assay (Pierce, Rockford, IL, USA). Proteins were separated by 10% SDS–PAGE, and electrophoretically transferred onto nitrocellulose membranes. Immunoblot was carried out with antibody against p-CD18 S745 diluted at 1:1000. Primary antibody was visualized with HRP-conjugated secondary antibody against rabbit IgG (Santa Cruz, Santa Cruz, CA, USA), using a chemiluminescent detection system (Applygen).

Assessment of the Src/PI3K/Akt Pathway in Integrin Activation on Leukocytes
To assess the role of the Src/PI3K/Akt pathway in Hcy-enhanced expression of CD11b/CD18 in leukocytes, the cells were treated as follows: control, Hcy 1 mM, Hcy 1 mM + CA 10 μM, Hcy 1 mM + CA 100 μM, Hcy 1 mM + CA 400 μM, Hcy 1 mM + Su6656 20 μM, and Hcy 1 mM + PP2 20 μM, and Hcy 1 mM + LY294002 50 μM. For inhibitor-blocking groups, the cells were preincubated with the Src inhibitors Su6656 and PP2 or the PI3K inhibitor LY294002 (Sigma) for 10 minutes before 90 minutes challenge with Hcy. The expression of CD11b/CD18 in leukocytes was evaluated by flow cytometry as described above.

Activation of the Src/PI3K/Akt Pathway in Leukocytes
To detect the effect of CA on Hcy-induced activation of the Src/PI3K/Akt pathway in leukocytes, the leukocytes were distributed into six groups: control, Hcy 1 mM, Hcy 1 mM + CA 100 μM, Hcy 1 mM + CA 400 μM, Hcy 1 mM + Su6656 20 μM, and Hcy 1 mM + LY294002 50 μM. Cells were incubated at 37°C for 10 minutes, and the total protein was collected for detecting Src and Akt phosphorylation, whereas the cytoplasmic and membrane proteins were extracted using the Nucl–Cyto–Mem preparation kit (Applygen) and used for detecting p85 translocation. Antibodies used were those against p-Src (Y416), t-Src, p-Akt (S473), t-Akt, p85a, and β-actin (Cell Signaling Technology, Danvers, MA, USA), which were all diluted at 1:1000. After immunoblotting with p-Src and p-Akt, the blots were stripped and reprobed with total Src and Akt.

Statistical Analysis
Data were expressed as means ± SEM. Data analysis involved one-way ANOVA F-test and t-test for comparison of data. Statistical significance was accepted for p < 0.05.

RESULTS
CA Does Not Influence the Plasma Concentration of Hcy
To show the time course of plasma concentration of Hcy after its administration and the influence of CA on it, we detected it after 5, 30, and 90 minutes of Hcy administration. The results show that the plasma level of Hcy was constant over the observation. The plasma level of Hcy in the Hcy group increased to 1010.2 ± 50.4 μM five minutes after administration, and then decreased to 350.5 ± 20.4
CA inhibits Hcy-induced leukocyte rolling and adhesion in mouse cerebral venules

Leukocyte recruitment to the venular wall is a multistep process in the inflammatory response. We explored the role of CA on Hcy-provoked leukocyte rolling and adhesion in mouse cerebral venules using a noninvasive method (Figure 2). At time zero, there was no significant difference in the number of leukocyte rolling and adhesion to the venules among the three groups, and in the control group, there was only a slight increase in cellular rolling and adhesion by the end of the observation. In contrast, values of rolling in the Hcy group showed an impressive linear increase over time, from 5.8 ± 0.9 cells/30 seconds at 45 minutes after Hcy injection, to 7.8 ± 1.2 cells/30 seconds at 90 minutes, representing an increase of about sevenfold of the baseline. The values of leukocyte adhesion in the Hcy group also showed a linear increase, from 2.1 ± 0.4 cells/200 μm at 60 minutes after Hcy injection, to 3.4 ± 0.5 cells/200 μm at 90 minutes. The representative images of leukocyte adhesion to cerebral venular wall at 90 minutes are illustrated in Figure 2 (upper panel). Notably, treatment with CA resulted in a significant attenuation of Hcy-induced rolling and adhesion of leukocytes to the venular wall.

CA decreases Hcy-induced increment in plasma KC, MIP-2, and MCP-1 levels

In view of the importance of cytokines and chemokines in the initiation and progression of inflammation, we determined the plasma levels of TNF-α, IL-6, KC, MIP-2, MIP-1α, and MCP-1 at 30 and 90 minutes after Hcy and CA injection. As shown in Figure 3, the concentration of all the cytokines and chemokines tested was elevated dramatically by Hcy stimulation in comparison with the control group, suggesting the involvement of TNF-α, IL-6, KC, MIP-2, MIP-1α, and MCP-1 in the Hcy-induced inflammatory reaction. Among the chemokines tested, KC and MIP-2 induce neutrophil chemotaxis, whereas MIP-1α and MCP-1 induce monocyte chemotaxis. Interestingly, KC, MIP-2, and MCP-1 levels were attenuated by CA treatment, which indicates a decreased recruitment of neutrophils and monocytes after CA treatment. The result also showed that KC and MIP-2 were increased by Hcy more significantly than MIP-1α and MCP-1, suggesting that neutrophil chemotaxis possibly occurs prior to monocyte chemotaxis in acute HHcy.

CA decreases Hcy-induced e-selectin and ICAM-1 expression on mouse cerebrovascular endothelium

Adhesion molecules on the vascular endothelium play an important role in leukocyte recruitment, wherein E-selectin mediates leukocyte capture and rolling, and ICAM-1 mediates stable adhesion. Consistent with others [34,45], we observed a significant increase in the expression of E-selectin (Figure 5A2,C) and ICAM-1 (Figure 5B2,D) on cerebrovascular endothelium in the Hcy group at the end of observation of cerebral microcirculation. In contrast, this Hcy increased the expression of E-selectin, and ICAM-1 was suppressed to a half level by CA treatment, as denoted by the number of positive vessels of E-selectin (Figure 5A3,C) or ICAM-1(Figure 5B3,D).

CA decreases Hcy-induced CD11b/CD18 upregulation on leukocytes in vivo

Leukocyte adhesion molecules also play an important role in leukocyte recruitment, with L-selectin and PSGL-1 mediating leukocyte capture and rolling, and integrin CD11b/CD18 mediating stable adhesion. As leukocyte rolling was increased at 45 minutes after Hcy administration, we detected the effect of CA on the expression of leukocyte adhesion molecules induced by Hcy at 45 and 90 minutes. As shown in Figure 6, Hcy significantly downregulated the expression of L-selectin and PSGL-1 at 45 and 90 minutes after administration, whereas upregulated CD11b expression at 90 minutes and CD18 expression on leukocytes at 45 and 90 minutes after administration. CA treatment...
significantly diminished the Hcy-unregulated expression of CD11b and CD18 on leukocytes without influencing the downregulation of L-selectin and PSGL-1.

CA Decreases Hcy-Induced CD11b/CD18 Expression and CD18 Phosphorylation in Leukocytes In Vitro

Several lines of evidence suggest that increased surface expression of integrins does not contribute to adhesion unless activated [7,18,19]. During integrin activation, the intracellular C-terminal tail of CD18 chain becomes phosphorylated on serine and threonine residues. A pilot experiment revealed that Hcy induced significant phosphorylation of CD18 at Ser745 from 10 to 60 minutes, and then declined at 90 minutes (data not shown). We chose 60 minutes as the time point to detect the effect of CA on Hcy-induced CD18 phosphorylation in leukocyte. Immunofluorescence confocal microscopy revealed that CA decreased the phosphorylation of CD18 in neutrophils (Figure 7B). Western blot further showed that CA at 10, 100, and 400 μM dose-dependently suppressed Hcy-induced CD18 phosphorylation (Figure 7C).

Src/PI3K/Akt Pathway is Involved in Hcy-Induced CD11b/CD18 Upregulation and CD18 Phosphorylation in Leukocytes

As the Src/PI3K/Akt pathway has been reported to be related with TNF-α-induced CD11b/CD18 upregulation in

Figure 2. Effect of CA on Hcy-induced leukocyte rolling and adhesion in mouse cerebral venules within 90 minutes. Representative images are for leukocyte adhesion in mouse cerebral venules of control group (A), Hcy group (B), and Hcy + CA group (C) at baseline (1) and 90 minutes (2) after intravenous injection of DL-Hcy (50 mg/kg) and CA (10 mg/kg). White arrows indicate the adherent leukocytes. Displayed at the bottom are the statistical results of the number of leukocytes rolling and adherent in cerebral venules over time. The abscissa represents time after Hcy injection. Data are expressed as mean ± SEM from five mice. *p < 0.05 vs control group; #p < 0.05 vs Hcy group.
neutrophil [28], we detected the relationship between this signaling pathway and Hcy-induced integrins expression. Flow cytometry analysis showed that the upregulated CD11b⁄CD18 expression on leukocytes was significantly abrogated by preincubation with the Src kinase inhibitor Su6656, PP2, and the PI3K inhibitor LY294002 (Figure 7A). Western blot showed that Su6656, PP2, and LY294002 evidently suppressed Hcy-induced CD18 phosphorylation (Figure 7D). Taken together, the Src/PI3K/Akt pathway is involved in Hcy-induced CD11b⁄CD18 upregulation and CD18 phosphorylation in leukocytes.

CA Suppresses Hcy-Caused Activation of the Src/PI3K/Akt Pathway in Leukocytes

We next assessed the influence of CA on the activation of the Src/PI3K/Akt pathway in leukocytes. We first determined Src phosphorylation in mouse leukocytes exposed to Hcy for different time periods, and found that Hcy-induced phosphorylation of Src took place within five minutes, peaked at 10 minutes, and then declined till 40 minutes (Figure 8A). Therefore, we assessed the effect of CA on the Src/PI3K/Akt pathway in leukocytes by treating the cells with Hcy + CA for 10 minutes. Obviously, addition of CA reduced the phosphorylations of Src and Akt (Figure 8B,D) and the translocation of p85 to membranes (Figure 8C) induced by Hcy, an effect similar to that of the corresponding specific inhibitor. The levels of total Src and Akt in leukocytes remained constant and were not affected by the treatment with Hcy or CA.

DISCUSSION

The current study shows an inhibitory effect of CA on HHcy-evoked leukocyte rolling and adhesion in mouse cerebral venules, which may be related to its depression on Hcy-induced release of chemokines and upregulation of adhesion molecules E-selectin and ICAM-1 on cerebrovascular endothelium and CD11b/CD18 on leukocytes in vivo. Our in vitro study revealed that CA significantly reduced Hcy-induced ROS production in HUVECs. CA remarkably attenuated...
Hcy-caused CD11b/CD18 upregulation and CD18 phosphorylation in leukocytes in vitro, which is related to its restoration of the Hcy-activated Src/PI3K/Akt signaling pathway.

Hcy has been documented to enhance the interaction between leukocytes and endothelial cells in some occasions, such as rat mesenteric venules and cultured human cells [5,39]. Here, CA was proved to inhibit Hcy-induced leukocyte adhesion in mouse cerebral venules by a noninvasive technique, a method that virtually does not perturb the microenvironment for observation of pial venules by intravital microscopy, suggesting its potential application in HHcy-related cerebrovascular diseases.
The potential of Hcy to induce oxidative stress has been reported as a mechanism for its pathogenesis [4,43]. However, CA has been proved to be a potent antioxidant [9,31,35,38], able to inhibit ROS production and eliminate active oxygen in macrophages and neutrophils [21,23]. CA derivatives have been reported to ameliorate oxidative stress in endothelial cells and protect endothelial cells from oxidative stress [20,45]. The in vitro experiment in the present study showed that CA inhibited the superoxide release from HUVECs induced by Hcy, implying that the antioxidant properties of CA may contribute to inhibiting Hcy-induced endothelial injury and further leukocyte recruitment.

Leukocyte rolling and adhesion to the microvessels is an initial step of circulating leukocyte recruitment to inflammatory sites [6,25], which are mediated by cytokines, chemokines, and cell adhesion molecules. Hcy has been reported to induce the secretion of chemokines and cytokines, including MCP-1, IL-8, IFN-γ, IL-2, TNF-α, and IL-10 [10,11,42]. Moreover, it was reported that Hcy increased the expression of adhesion molecules ICAM-1 [34] and E-selectin [46] on endothelial cells as well as CD11b/CD18 on all cell types of leukocytes in human blood [18]. In line with the above results, we show here that the administration of Hcy elevated the plasma level of IL-6, TNF-α, KC, MIP-2, MCP-1, and MIP-1α, as well as increased the expression of CD11b/CD18. Moreover, we demonstrated that CA treatment partially reduced the level of KC, MIP-2, and MCP-1, suggesting the involvement of neutrophil and monocyte chemotaxis attenuation in CA action. Nevertheless, more works are required to elucidate the rational for CA reduction of KC, MIP-2, and MCP-1 levels.

Moreover, this study was undertaken with an acute model of HHcy to make the experimental condition as simple as possible. However, the situation may become complicated in chronic HHcy models and in patients with HHcy, due to the concurrence of some conditions, such as metabolism disorders and atherosclerosis, which may inter-
fere in the outcome. To this end, a recent publication reported that under certain circumstances, CA can be pro-inflammatory, particularly during increased glycation [47], implying that the action of CA in patients with HHcy in the clinic might be more complicated, and the applicability of the present findings to a chronic model or patients with HHcy remains to be verified.

CA was also found to decrease the expression of E-selectin and ICAM-1 on cerebrovascular endothelium and CD11b/CD18 on leukocytes, consistent with the well-recognized anti-inflammatory potential of CA [9,31,35,38], implying that CA diminished Hcy-induced leukocyte recruitment through interfering in the expression of adhesion molecules on leukocytes and endothelial cells. Obviously, the downregulation of E-selectin and ICAM-1 on endothelial cells by CA is related to inhibiting NF-κB nuclear translocation [29]. However, the underlying mechanism responsible for CA downregulation of integrin CD11b/CD18 on leukocytes remains unclear.

Several lines of evidence suggest that solely increased surface expression of integrins does not trigger adhesion unless activated. For example, as a necessary step for adhesion, the intracellular C-terminal tail of the β2 integrin subunit CD18 has to become phosphorylated on serine and threonine residues [7,18,19]. Likewise, our work showed that Hcy not only enhanced the expression of CD11b/CD18 but also induced CD18 phosphorylation at Ser745. Of notice, the Hcy-induced CD18 phosphorylation was found to be attenuated by CA as well, suggesting that CA acts as an inhibitor for Hcy-elicited leukocyte recruitment via depression of both membrane integrin expression and CD18 phosphorylation on leukocytes.

Several signaling pathways, including PI3K, have been reported to be implicated in integrin activation [1,14,22,30,32]. Moreover, Src-family tyrosine kinases plays a critical role in initiating the numerous intracellular signaling pathways in leukocytes [24]. Specifically, Src kinase is known to be involved in Hcy-potentiated collagen-stimu-

Figure 8. Effect of CA on Hcy-induced activation of Src/PI3K/Akt signaling in mouse leukocytes. Leukocytes were treated with DL-Hcy (1 mM) alone or DL-Hcy + CA (100 μM, 400 μM) or SU6656 20 μM or LY294002 50 μM for 10 minutes, then p-Src Y416, total Src, p-Akt S473, and total Akt, or membrane and cytosolic p85 were detected by Western blot. (A) Src phosphorylation in mouse leukocytes exposed to DL-Hcy (1 mM) for different time periods. (B-D) Effect of CA and inhibitors on Hcy-induced activation of Src/PI3K/Akt signaling in leukocytes. The blots were representatives of three independent experiments. *p < 0.05 vs control group; #p < 0.05 vs Hcy group.
lated aggregation of human platelets [27], and activation of PI3K/Akt signaling is reported to be responsible for Hcy-promoted human vascular smooth muscle cell proliferation [8]. In addition, the Src/PI3K/Akt pathway has been reported to be related to TNF-α-induced CD11b/CD18 upregulation in neutrophils [28]. However, no study has been published as yet regarding the correlation of the Src/PI3K/Akt pathway with Hcy-induced integrin activation in leukocytes. In the present study, we first proved that Src/PI3K/Akt signaling plays an important role in Hcy-induced integrin activation in leukocytes, as indicated by the significant increase in the phosphorylation of Src Y416 and Akt S473, and the translocation of p85 to the membrane in leukocytes after Hcy stimulation. Meanwhile, CA was found to inhibit this Hcy-activated signal cascade, acting as the specific inhibitor for the Src/PI3K/Akt pathway, highly suggesting that the Src/PI3K/Akt pathway is at least one of the targets for CA action in the present circumstance.

In summary, this study demonstrated that CA inhibits Hcy-promoted leukocyte rolling and adhesion in mouse cerebral venules. CA exerts this action possibly via reduction of plasma chemokine levels, downregulation of ROS production from endothelial cells, depression of E-selectin and ICAM-1 expression on cerebrovascular endothelium, and decrement of CD11b/CD18 expression and CD18 phosphorylation in leukocytes. Importantly, we provided evidence for the first time that the Src/PI3K/Akt pathway is implicated in the Hcy-elicited CD11b/CD18 expression and activation, and CA is able to inhibit the activation of this pathway. These results open a new prospect to the development of drugs for relief of the patients with HHcy, and provide novel clue for better understanding the anti-inflammatory properties of CA.

**REFERENCES**

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