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γ -Aminobutyric Acid A Receptor Mitigates Homocysteine-Induced Endothelial Cell Permeability

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Abstract

Many cerebrovascular disorders are accompanied by an increased homocysteine (Hcy) levels. We have previously shown that acute hyperhomocysteinemia (HHcy) leads to an increased microvascular permeability in the mouse brain. Hcy competitively binds to γ -aminobutyric acid (GABA) receptors and may increase vascular permeability by acting as an excitatory neurotransmitter. However, the role of GABA-A (GABA_A) receptor in Hcy-induced endothelial cell (EC) permeability remains unclear. In the present study we attempted to determine the role of GABA_A receptor and the possible mechanisms involved in Hcy-induced EC layer permeability. Mouse aortic and brain ECs were grown in Transwells and treated with 50 μ M Hcy in the presence or absence of GABA_A-specific agonist muscimol. Role of matrix metalloproteinase-9 (MMP-9) was determined using its activity inhibitor GM-6001. Involvement of extracellular signal-regulated kinase (ERK) signaling was assessed using its kinase activity inhibitors PD98059 or U0126. EC permeability to the known content of bovine serum albumin (BSA)-conjugated with Alexa Flour-488 was assessed by measuring fluorescence intensity of the solutes in the Transwell's lower chambers. It was found that Hcy induced the formation of filamentous actin (F-actin). Hcy-induced EC permeability to BSA was significantly decreased by GABA and muscimol treatments. Presence of MMP-9 or ERK kinase activity inhibitors restored the Hcy-induced EC permeability to its baseline level. The mediation BSA leakage through the ECs was further confirmed in the experiments where Hcy-induced alterations in transendothelial electrical resistance of confluent ECs were assessed. The data suggest that Hcy increases EC layer permeability through inhibition of GABA_A receptor and F-actin formation, in part, by transducing ERK and MMP-9 activation.

Keywords

Albumin Leakage; Endothelial Gap Formation; ERK; F-Actin; Muscimol

Homocysteine (Hcy) is a homologue of the naturally occurring amino acid, cysteine. Normal concentration of Hcy in plasma is in the range of 5 to 10 μ M. Increased blood level of Hcy is known as hyperhomocysteinemia (HHcy). There are three ranges of HHcy: moderate (10 to 30 μ M), intermediate (31 to 100 μ M), and severe (>100 μ M). Increased blood levels of Hcy

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have been linked to increased risk of premature coronary artery disease, stroke, and thromboembolism (Lentz 2005; Lee et al. 2006). Elevation of blood Hcy content coincides with an increased blood concentration of well-accepted inflammatory markers such as C-reactive protein (Hackam and Anand 2003), interleukin-6 (Araki et al. 2005), and fibrinogen (Zoccali et al. 2002). Because of this association, it was postulated that an increase in Hcy content may promote vascular inflammation (Lentz 2005). In addition, it was shown that increased level of Hcy may cause interleukin-6 accumulation in the monocytes (van Aken et al. 2000), suggesting an association of HHcy and an enhanced formation of interleukin-6, the latter being involved in synthesis of another inflammatory agent fibrinogen (Vasse et al. 1996). However, according to the recent Glasgow Myocardial Infarction Study, correlation of Hcy with the inflammatory markers such as fibrinogen, C-reactive protein, and interleukin-6 was not significant (Woodward et al. 2006), suggesting that, within the population range, increasing Hcy contents have no effect on the systemic inflammatory response. Therefore, it can be stated that Hcy is an independent risk factor for cardiovascular diseases and stroke (Seshadri et al. 2002; Tyagi 1999).

Many cardiovascular diseases, e.g., hypertension and diabetes, are considered inflammatory diseases. Increased microvascular permeability, also typical to these diseases, is one of the manifestations of inflammation. A net loss of blood plasma components into the interstitium causes edema. Albumin, the most abundant plasma protein, helps maintain the oncotic pressure and protects endothelial barrier integrity by its interaction with glycocalyx (Huxley and Curry 1985, 1987). Thus, changes in albumin transport through the endothelial cell (EC) layer may cause significant damage to tissue and induce inflammatory responses (Mehta and Malik 2006).

It has been shown that HHcy induced by folate depletion caused increased arterial permeability and stiffness in rats (Symons et al. 2002, 2006). The results of our previous study suggest that an acute increase in blood Hcy content enhances leakage of pial microvessels in mice (Lominadze et al. 2006). Although the role of Hcy-induced matrix metalloproteinase-9 (MMP-9) activation in vascular remodeling is well known, the mechanism of Hcy-induced increased microvascular permeability was not clear. Along with extracellular matrix (ECM) degradation, tissue plasminogen activator causes proteolytic shedding of γ -aminobutyric acid (GABA) receptors (Mataga et al. 2002; Frey et al. 1996). GABA receptors play a significant role in brain microvascular permeability, which if inhibited, alters the neuronal environment and leads to ECM degradation and edema (Lee et al. 1995; Limmroth et al. 1996; Fruscella et al. 2001; Lazzarini et al. 2001). Hcy competes with GABA receptors and acts as an excitatory neurotransmitter. Therefore, an increase in blood Hcy level may attenuate the function of GABA receptor, leading to the disruption of EC layer integrity. In the present study, we tested the hypothesis that high levels of Hcy may increase EC leakage to albumin through extracellular signal regulated kinase (ERK) signaling and filamentous actin (F-actin) formation. The role of GABA-A (GABA_A) receptor in Hcy-induced EC permeability is shown.

METHODS

Materials and Reagents

Bovine serum albumin (BSA) 1-palmitoyl-sn-glycero-3-phosphocholine (LPC), Hcy, fibronectin, and muscimol (GABA_A receptor-specific agonist) were purchased from Sigma (St. Louis, MO). Specific inhibitors of the mitogen-activated protein kinase known as extracellular signal-regulated kinase (ERK), kinase, PD98059 (2'-amino-3'-methoxyflavone), and U0126 (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene), and GM-6001 (MMP activity blocker) were purchased from Calbiochem (La Jolla, CA). Monoclonal antibody to phosphorylated ERK1/2 (p44/42) was purchased from Cell Signaling Technology (Beverly, MA) and the antibody to ERK2 was purchased from Santa Cruz Biotechnology

(Santa Cruz, CA). Horseradish peroxidase (HRP)-conjugated antibody was obtained from Cappel Laboratories (Durham, NC). BSA conjugated with Alexa Flour-488 dye (BSA-488), Alexa Flour-594 Phalloidin (300 U), and a secondary antibody conjugated with Texas Red dye were purchased from Molecular Probes (Eugene, OR).

Endothelial Cell Culture

Mouse aortic endothelial cells (MAECs) were a generous gift from Kathleen Bove, Stratton VA Medical Center, Albany, NY. The endothelial nature of the cells was verified by uptake of acylated low-density lipoprotein and positive staining for CD-31 (Lincoln et al. 2003). Mouse brain endothelial cells (MBECs) were purchased from American Type Culture Collection (ATCC, Manassas, VA) at 21th passage. The endothelial nature of these cells was confirmed by the observed expression of von Willebrand factor and uptake of fluorescently labeled low-density lipoprotein (Montesano et al. 1990). The MAECs were grown in Dulbecco's modified Eagle's medium/Ham's F-12 50/50 mix (DMEM/F12–50/50; Cellgrow, Mediatech, Herndon, VA), whereas MBEC were grown in DMEM (ATCC). Both culture media were supplemented with 10% fetal bovine serum (HyClone, Logan, UT) 1% L-glutamine and 1% penicillin-streptomycin (Gibco, Grand Island, NY). Cells were grown at 37°C at 5% CO₂ in a humidified environment. For the experimentation, MAECs and MBECs were used at the 10th and 22nd passages, respectively.

Endothelial Cell Permeability Assays

Albumin leakage through endothelial cell monolayer was assessed according to the method described earlier (Tyagi et al. 2007b). Transwell permeable supports (Corning, Corning, NJ) with polycarbonate membranes (Nuclepore Track-Etch; 6.5 mm in diameter, 0.4- μ m pore size, and pore density of 10⁸/cm²) were coated with fibronectin for 1 h. The membranes were seeded with MAECs or MBECs and the cells were grown to confluence. To confirm the cell confluence and the presence of an intact monolayer on the membranes, cells in a separate test well (not membrane) were monitored by a light microscope. Cells in the test membranes were labeled with fluorescence (2',7'-bis(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester [BCECF, AM]; Molecular Probes, Eugene, OR) and observed under a microscope (Carl Zeiss Axiovert-100, objective 10 $\times$) with a fluorescence filter (488-nm excitation and 516-nm emission) for absence of visible gaps in the cell monolayer. The permeability assays were done after confirming that the cells in the test wells and Transwell membranes were fully confluent and formed an intact monolayer.

Unlabeled BSA was added to each well to maintain its concentration similar to a normal plasma concentration of albumin (440 μ M) and maintain its activity coefficient close to that in blood (Rivas et al. 1999). The surface levels of solutions in the luminal (200 μ L) and abluminal (600 μ L) compartments of the Transwells were the same. The experimental set-up is similar to that used by Cooper et al. (1987), but the presence of albumin-mediated osmotic pressure in the present study makes it closer to in vivo conditions (Tyagi et al. 2007b).

For the permeability assay, cells were washed with phosphate-buffered saline (PBS) and incubated with various doses of Hcy (5, 25, or 50 μ M) for 24 h, or with 50 μ M of Hcy for 1, 12, or 24 h. To determine a possible role of GABA_A receptor, MMPs, and the mitogen-activated protein kinase kinase (MEK) signaling in Hcy-induced EC permeability, the following substances were added to the Transwells: Hcy (50 μ M) with muscimol (50 μ M), Hcy (50 μ M) with MEK inhibitors (PD98059 or U0126; 50 μ M each), Hcy (50 μ M) with GABA (50 μ M), or Hcy (50 μ M) with GM6001 (1 μ M).

To determine the effect of GABA, muscimol, GM6001, and MEK inhibitors on EC permeability to albumin, the following substances were added to other Transwells without Hcy:

GABA (50 μ M), muscimol (50 μ M), GM6001 (1 μ M), PD98059 (50 μ M), or U0126 (50 μ M). Cells incubated with medium alone were used as a control group. BSA-488 (3 mg/mL) was added to each of the wells described above and experiments were performed five times in duplicate for each treatment.

The cells were incubated in humidified conditions at 37°C. After incubation, media samples were collected from lower and upper wells. Fluorescence intensity of the samples was measured by a microplate reader (SpectraMax M2; Molecular Devices, Sunnyvale, CA) with excitation at 494 nm and emission at 518 nm. Results are expressed as fluorescence intensity units (FIU) and presented as a percent of the control values.

In additional experiments, permeability of the Nuclepore membranes alone to albumin was compared to albumin leakage through the EC layer in Tranwells. The results showed that albumin leakage through the membrane alone (583 ± 34 FIU) was about nine times greater than that (66 ± 12 FIU) in the presence of the cells.

Transendothelial electrical resistance measurement was performed using an electrical cell-substrate impedance system (ECIS; Applied Biophysics, Troy, NY). ECs were seeded onto evaporated gold microplates of the ECIS. The cells were grown to a confluent monolayer that covered the microelectrodes of the system's 8-well chamber (Tiruppathi et al. 1992). Effect of Hcy (50 μ M) was determined in the presence or absence of muscimol (50 μ M), MEK inhibitors (PD98059 or U0126; 50 μ M each), GABA (50 μ M), or with GM6001 (1 μ M). To determine the effect of GABA, muscimol, GM6001, and MEK inhibitors on transendothelial electrical resistance of the cells, the following substances were added to wells without Hcy: GABA (50 μ M), muscimol (50 μ M), GM6001 (1 μ M), PD98059 (50 μ M), or U0126 (50 μ M). Cells incubated with medium alone were used as a control group. Resistance values from each microelectrode were collected and plotted as relative resistance versus time.

F-Actin Formation Assay

Formation of F-actin in cultured ECs was studied according to the method described earlier (Lominadze et al. 2004, 2006; Tyagi et al. 2007). Briefly, MAECs and MBECs were grown until confluent in 8-well coverglass plates coated with fibronectin. The cells were washed and incubated with or without PD98059 or U0126 (50 μ M each), muscimol (50 μ M), or GABA (50 μ M), followed by Hcy (50 μ M) treatment for 24 h. To determine the effect of GABA, muscimol, PD98059, or U0126 on F-actin formation, the separate groups of cells were incubated with following substances without Hcy: GABA (50 μ M), muscimol (50 μ M), PD98059 (50 μ M), or U0126 (50 μ M). Cells incubated with medium alone were used as a control. After the treatments the cells were washed twice with PBS and incubated with Alexa Flour-594 Phalloidin (10 U) and LPC (100 μ g/mL, dissolved in 3.7% formaldehyde) for 30 min at 4°C in the dark (Lominadze et al. 2006). After incubation, the cells were washed with PBS and digital images of intracellular F-actin were taken with the confocal microscope (Olympus FV1000, objective 100 $\times$) using a HeNe-G laser (596 nm) to excite the dye, whereas emission was observed above 620 nm. The images were compared for the formation of individual stress fibers and presence of actin foci (Lominadze et al. 2006).

In separate experiments, Hcy-induced formation of F-actin in ECs were compared to F-actin formation induced by thrombin. The cells were treated with or without 50 μ M Hcy for 24 h and 0.5 U/mL thrombin for 15 min (Qiao et al. 1995; Trepatt et al. 2005; Tyagi et al. 2007) and analyzed for intracellular F-actin formation using confocal microscopy.

Hcy-induced formation of F-actin fibers was assessed for each well by analyzing the total fluorescence intensity in four random fields with image analysis software (Image-Pro Plus; Media Cybernetics). Four experiments were done to study Hcy-induced F-actin formation and

three experiments were done to study Hcy- and thrombin-induced F-actin formation. All the experiments were done in duplicate (two wells per experimental group). Results are expressed as a percent of the control values.

Western Blot Analysis for ERK Phosphorylation

Hcy-induced phosphorylation of ERK was studied using a method described previously (Moshal et al. 2006). Briefly, MAECs were grown in 6-well plates (Corning, Corning, NY) until they become 80% to 90% confluent. Before experimentation, cells were serum starved by depriving fetal bovine serum to 0.01% in the medium for 16 h. Then cells were incubated with one of the following: Hcy (5 or 50 μ M), Hcy (50 μ M) with muscimol (50 μ M), Hcy (50 μ M) with MEK inhibitors (PD98059 or U0126; 50 μ M each), or Hcy (50 μ M) with GABA (50 μ M) at 37°C for 24 h. Other cells were incubated with one of the following: muscimol (50 μ M), PD98059 or U0126 (50 μ M each), or GABA (50 μ M) at 37°C for 24 h. Cells incubated with low-serum medium were used as a control.

After incubation, the cells were washed twice with ice-cold 1× PBS and lysed with ice-cold lysis buffer (composition: Tris-HCl [50 mM], NaCl [150 mM], Triton X-100 [1%], and EGTA [1 mM], pH 7.4) supplemented with phenylmethylsulfonyl fluoride (1 mM), leupeptin (1 μ g/mL), sodium orthovanadate (200 μ M), and aprotinin (1 μ g/mL). Cell lysates were assayed for protein concentration using Bradford assay (Bradford 1976). Equal amount of protein was resolved on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto a polyvinylidene difluoride (PVDF) membrane. After being transferred, blots were washed with Tris-buffered saline with 0.1% Tween-20 (TBST) for 5 min at room temperature and blocked with 5% BSA in TBST for 1 h at room temperature. The blots were then incubated with polyclonal anti-rabbit phospho-ERK1/2 antibody (1:1000 dilution) overnight at 4°C with gentle agitation. The blots were washed three times (8-min wash each time) with TBST and incubated with appropriate secondary antibody (1:3000 dilution). Proteins of interest were immuno detected using an enhanced chemiluminescence (ECL) plus kit (Amersham Biosciences, Piscataway, NJ). The membranes were stripped and reprobed for ERK2 as loading control. The blots were analyzed with Gel-Pro Analyzer software (Media Cybernetics, Silver Spring, MD) as described earlier (Lominadze et al. 2002). The protein expression intensity was assessed by integrated optical density (IOD), i.e., the area of the band in the lane profile. To account for possible differences in the protein load, the results of the measurements are presented as a ratio of IOD of each band under the study (phosphorylated ERK1/2) to the IOD of the respective total protein (total ERK2).

Statistics

All data are expressed as mean $\pm$ SEM. The experimental groups were compared by one-way analysis of variance (ANOVA). If ANOVA indicated a significant difference ($p < .05$), Tukey's multiple comparison test was used to compare group means. Differences were considered statistically significant if $p < .05$.

RESULTS

Hcy induced a dose-dependant increase in the permeability of the EC layer to albumin as determined by the fluorescence intensity of albumin that leaked through MAECs into the lower wells of the Transwells (Figure 1). The permeability of the MAECs to albumin, induced by the highest dose of Hcy (50 μ M), was increased (128% $\pm$ 9%, 186% $\pm$ 25%, 303% $\pm$ 34 % of control) with treatment time (1, 12, and 24 h, respectively). The permeability of MAEC layer to albumin induced by Hcy (50 μ M) during 24 h was decreased significantly by GABA, muscimol, GM6001, and MEK inhibitors (PD98059 or U0126) (Figure 1). In the absence of

Hcy, treatment of MAECs with GABA, muscimol, GM-6001, or MEK inhibitors (PD98059 or U0126) had no effect on albumin leakage through MAEC layer (Table 1).

The enhanced permeability of the MBECs to albumin, induced by Hcy (50 μ M), was decreased significantly by GABA, muscimol, MEK inhibitors (PD98059 or U0126), and GM6001 (Figure 2). In the absence of Hcy, treatment of MBECs with GABA, muscimol, MEK inhibitors (PD98059 or U0126), or GM-6001 had no effect on MBEC albumin leakage (Table 2).

In separate experiments, Hcy treatment significantly decreased transendothelial electrical resistance compared to untreated control MBECs (Figure 3). This decrease in resistance was significantly less in the presence of muscimol and GABA (Figure 3). Treatment of the MBECs with GABA and muscimol had no effect on transendothelial electrical resistance (Figure 3).

Hcy caused a dose-dependant increase of F-actin formation in MAECs (Figure 4). The formation of F-actin, induced by the highest dose of Hcy (50 μ M), was significantly decreased by GABA, muscimol, or by MEK inhibitors (PD98059 or U0126) (Figure 4). Formation of F-actin in the MAECs treated with GABA, muscimol, or with MEK inhibitors (PD98059 or U0126) alone was not different from that in control group (Table 3). The treatment of ECs with Hcy (50 μ M) for 24 h caused the formation of F-actin ($160\% \pm 6\%$ of control), which was similar to that ($141\% \pm 4\%$ of control) induced by thrombin (0.5 U/mL for 15 min) (Figure 4A). In the parallel series of experiments, treatment of MBECs with Hcy (50 μ M) for 24 h caused the formation of F-actin, which was not different from that induced by thrombin (0.5 U/ml for 15 min) (Figure 5).

Hcy induced a dose-dependant increase of ERK phosphorylation in MAECs that was significantly decreased in the presence of muscimol (Figure 6). Treatment of MAECs with muscimol alone did not alter ERK phosphorylation in the ECs compared to that in the control group (Figure 6).

DISCUSSION

The present study shows that pathological concentrations of Hcy (<12 μ M) can enhance albumin leakage through an EC monolayer. These results coincide with our data showing that Hcy increases albumin leakage of pial vessels in mice (Lominadze et al. 2006). It is known that thrombin-induced intercellular gaps are accompanied by formation of F-actin (Qiao et al. 1995). In the present study, the treatment of the ECs with higher than normal dose of Hcy (50 μ M) for 24 h caused F-actin formation to the level that was caused by thrombin (0.5 U/mL 15 min; Figure 4), which is known to increase F-actin formation (Trepatt et al. 2005). These data suggest that an increased concentration of Hcy in the blood stream has a role in increasing EC permeability similar to thrombin.

Increased content of Hcy may enhance albumin leakage by inducing the formation of F-actin (Figures 4 and 5), which may cause stiffening of the cells and opening of interendothelial junctions (IEJs). Role of Hcy on mouse brain IEJs was clearly shown using ECIS (Figure 3), where, the effect of Hcy was significantly diminished by GABA or muscimol. The results of the present study also coincide with our previous results, where we showed that Hcy may affect EC by activating MMPs (Lominadze et al. 2006; Moshal et al. 2006). The present results show a restoration of Hcy-induced albumin leakage through EC layer in the presence of MMP inhibitor (GM6001), which confirms the role of MMPs in Hcy-induced microvascular leakage (Lominadze et al. 2006). Changes in MMPs may cause subendothelial matrix remodeling by altering collagen/elastin ratio (Shastri et al. 2006) and target focal adhesion molecules and cell junction molecules at the basal side of the ECs, such as cadherins and/or connexins (Mehta and Malik 2006).

Significant decrease in Hcy-induced albumin leakage and F-actin formation by GABA or its agonist, muscimol, suggests that Hcy may directly affect EC properties through binding to GABA_A receptor. This binding suppressed the activity of GABA_A receptor (Shastry et al. 2006), making Hcy an excitatory neurotransmitter. Our studies show that mouse EC also express GABA_A receptor, and its activity can be suppressed by Hcy (Tyagi et al. 2007a). The results of the present study suggest that the activation of GABA_A receptor may prevent Hcy-induced EC leakage and therefore highlight the functional role of GABA_A receptor in endothelium and its possible involvement in Hcy-induced EC layer permeability.

Mitogen-activated protein kinase kinase (MEK) is a protein kinase that is phosphorylated and activated by Raf. Once activated, MEK phosphorylates and activates ERK (Kolch 2000). PD9805 and U0126 used in the present study are chemically unrelated MEK/ERK inhibitors with different mechanisms of action (Alessi et al. 1995; Favata et al. 1998). Whereas PD98059 binds to the inactive forms of MEK and prevents its activation by upstream activators such as Raf (Alessi et al. 1995), U0126 binds to the activated form of MEK (Favata et al. 1998). It is known that formation of F-actin can occur through ERK signaling (Bourguignon et al. 2005; Chandrasekar et al. 2003). Furthermore, ERK signaling pathway is involved in expression of GABA_A receptor (Bulleit and Hsieh 2000; Kalluri and Ticku 2002). Our data show that Hcy-induced albumin leakage may be regulated by ERK signaling (Figure 6). Furthermore, these results, in combination with the data showing decreased albumin leakage through EC layer in the presence of GABA or muscimol, suggest that competitive binding of Hcy to GABA_A receptor on EC surface involves the activation of MEK/ERK signal. In our previous study, we showed that increased levels of Hcy enhance phosphorylation of ERK and activate MMP-9 (Moshal et al. 2006). The present study indicates the role of MMPs in Hcy-induced albumin leakage through the EC layer. Increased content of Hcy enhances EC layer permeability, which involves inhibition of GABA_A receptor activity and formation of F-actin through ERK signaling. Our data related to the muscimol-induced increase ERK phosphorylation confirm the result by others showing that muscimol activates ERK signaling (Obrietan et al. 2002). However, muscimol-induced ERK phosphorylation was not as robust as the one induced by 50 μ M Hcy. The treatment of the cells with muscimol prior to addition of Hcy (50 μ M) did not increase ERK phosphorylation to the extent that was caused by the same dose of Hcy. This suggests that muscimol competes with Hcy for GABA_A receptor binding sites and induces ERK phosphorylation.

Increased blood Hcy concentration may increase binding of Hcy to endothelial GABA_A receptors. Cytoskeletal ezrinradixin-moesin (ERM) family protein radixin has been identified as an essential clustering factor that anchors GABA_A receptor with actin cytoskeleton of a cell (Tsukita and Yonemura 1999; Loebrich et al. 2006). A role of radixin in ERK signaling pathway has been suggested (Jung et al. 2005). In the present study the role of radixin was not evaluated; however, the role of GABA_A receptor in radixin-mediated ERK signaling pathway can't be ruled out during Hcy-induced endothelial monolayer leakage, which may cause the formation of F-actin in ECs. F-actin formation may increase the rigidity of the cells, widen the IEJ, and may even increase gaps between the cells (Ehringer et al. 1999; Gordon et al. 2005; Lominadze et al. 2004, 2006). The enlarged IEJ gaps may be enough to allow albumin (diameter ~5 nm) to pass through the EC monolayer.

Increased blood Hcy concentration is typical for many cardiovascular and cerebrovascular diseases (Seshadri et al. 2002; Tyagi et al. 1999). Almost all of these diseases are accompanied with increased ECs permeability. We provide the evidence that elevated levels of Hcy have a role in increasing EC layer permeability through its binding to endothelial GABA_A receptor and suppressing its activity.

Limitations of the Present Study

The results of the present study show that elevated content of Hcy increases EC layer permeability to albumin through IEJs. However, the design of our experiments did not permit us to rule out transcellular migration of albumin (Mehta and Malik 2006). In addition to affecting IEJs, Hcy may alter transcytosis affecting caveolae-involved an absorptive or fluid-phase pathways, or formation of transendothelial channels. The experiments to address the role of Hcy in transendothelial albumin transport are in progress.

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FIG. 1.

Hcy-induced albumin leakage through the mouse aortic endothelial cell monolayer. Fluorescence intensity of bovine serum albumin conjugated with Alexa Flour-488 detected in lower chambers of Transwells. * $p < .05$ versus 5 μ M of Hcy. † $p < .05$ versus the lower dose of Hcy. # $p < .05$ versus 50 μ M of Hcy. Number of experiments $n = 5$ for all groups.

FIG. 2.

Hcy-induced albumin leakage through the mouse brain microvascular endothelial cell monolayer. Fluorescence intensity of bovine serum albumin conjugated with Alexa Fluor-488 detected in lower chambers of Transwells. Hcy, GABA, muscimol, and MEK inhibitors, PD98059 and U0126, were used at concentration of 50 μ M each, whereas GM6001 was 1 μ M. * $p < .05$ versus Hcy. Number of experiments $n = 4$ for all groups.

FIG. 3.

Changes in relative resistance of the confluent mouse brain endothelial cell layers after treatment with Hcy (●), GABA and Hcy (○), muscimol and Hcy (▴), GABA (▽), and muscimol (▴). Cells in the control group (*) were grown in medium alone. Hcy, GABA, and muscimol were used at concentration of 50 μ M each. Number of experiments $n = 4$ for all groups.

FIG. 4.

Hcy-induced F-actin formation in mouse aortic endothelial cells. (A) Examples of images of Hcy- and thrombin-induced F-actin formation (objective 100 $\times$). (B) Total fluorescence intensity changes of F-actin staining induced by Hcy (5 and 50 μ M) and inhibition of 50 μ M Hcy effect by GABA, muscimol, PD98059, and U0126 (50 μ M each). * $p < .05$ versus 5 μ M of Hcy. # $p < .05$ versus 50 μ M of Hcy. Each data point represents the results of duplicate determinations from four separate experiments ($n = 4$).

FIG. 5. Hcy-induced F-actin formation in mouse brain endothelial cells. (A) Examples of images of Hcy- and thrombin-induced F-actin formation (objective 100 $\times$). (B) Total fluorescence intensity changes of F-actin staining induced by 50 μ M of Hcy (24 h) and 0.5 U/ml of thrombin (15 min). Each data point represents the results of duplicate determinations from four separate experiments ($n = 4$).

FIG. 6.

Hcy-induced ERK phosphorylation in MAECs. (A) A representative Western Blot for Hcy-induced ERK1/2 phosphorylation and its inhibition in the presence of muscimol is shown (*top*). Membranes were reprobbed for total ERK2 (*bottom*). Phosphorylation of ERK in the cells treated with medium alone was used as a control group. (B) ERK1/2 phosphorylation induced by 50 μ M Hcy. The data are presented as ratios of integrated optical density (IOD) of each phosphorylated ERK1/2 band to IOD of the respective total ERK2. Bars represent averages $\pm$ SE from three different experiments ($n = 3$). * $p < .05$ versus control; # $p < .05$ versus 50 μ M of Hcy.

TABLE 1

Effect of GABA, muscimol, GM6001, PD98059, or U0126 on albumin leakage through the mouse aortic endothelial cell monolayer

GABA (50 μ M)	Muscimol (50 μ M)	GM6001 (1 μ M)	PD98059 (50 μ M)	U0126 (50 μ M)
115 $\pm$ 13	121 $\pm$ 19	111 $\pm$ 12	108 $\pm$ 8	124 $\pm$ 21

Note. Data are presented as percent of control. Number of experiments $n = 4$.

TABLE 2

Effect of GABA, muscimol, PD98059, U0126, or GM6001 on albumin leakage through the mouse brain endothelial cell monolayer

GABA (50 μ M)	Muscimol (50 μ M)	PD98059 (50 μ M)	U0126 (50 μ M)	GM6001 (1 μ M)
107 $\pm$ 12	101 $\pm$ 11	108 $\pm$ 8	111 $\pm$ 13	121 $\pm$ 14

Note. Data are presented as percent of control. Number of experiments $n = 4$.

TABLE 3

Effect of GABA, muscimol, PD98059, U0126, or GM6001 on F-actin formation in the mouse aortic endothelial cells

(50 μ M) GABA	(50 μ M) Muscimol	(50 μ M) PD98059	(50 μ M) U0126
109 $\pm$ 6	111 $\pm$ 6	98 $\pm$ 1	94 $\pm$ 3

Note. Data are presented as percent of control. Number of experiments $n = 4$.