Homocysteine Upregulates Vascular Cell Adhesion Molecule-1 Expression in Cultured Human Aortic Endothelial Cells and Enhances Monocyte Adhesion

Matthew D. Silverman,* Ramagopal J. Tumuluri,* Mishel Davis, Gladys Lopez, James T. Rosenbaum, Peter I. Lelkes

Abstract—Elevated plasma homocysteine is an independent risk factor for atherosclerosis. We hypothesized that homocysteine enhances monocyte/human aortic endothelial cell (HAEC) interactions, a pivotal early event in atherogenesis, by upregulating endothelial adhesion molecules. After incubation of cultured HAECs with reduced DL-homocysteine for up to 24 hours, adhesion of human monocytes to homocysteine-stimulated HAECs was significantly upregulated in a time- and dose-dependent fashion. Pretreatment of HAECs with 100 μmol/L homocysteine caused a 4.5-fold increase in the adhesion of normal human monocytes ($P<0.001$). Similarly, adhesion of monocytic U937 cells was maximally elevated by 3.5-fold at 100 μmol/L homocysteine ($P<0.001$). In support of our hypothesis, vascular cell adhesion molecule (VCAM)-1 mRNA expression increased 5-fold in HAECs after 3 hours of treatment with 100 μmol/L homocysteine, as assessed by quantitative reverse transcription–polymerase chain reaction. Neutralizing antibody studies confirmed the involvement of VCAM-1 in mediating monocyte adhesion to homocysteine-stimulated HAECs. Coincubation of HAECs with homocysteine and tumor necrosis factor-α synergistically elevated monocyte adhesion as well as VCAM-1 protein expression, with the latter evaluated by flow cytometry. Preincubation of HAECs with cyclooxygenase inhibitors completely abrogated homocysteine-induced monocyte adhesion, whereas scavenging reactive oxygen species and the elevation of NO caused partial inhibition only. These data support the notion that the proinflammatory effects of homocysteine may have important implications in atherogenesis. **(*Arterioscler Thromb Vasc Biol*. 2002;22:587-592.)**

Key Words: human aortic endothelial cells ■ homocysteine ■ monocyte adhesion ■ vascular cell adhesion molecule-1 ■ cyclooxygenase

Homocysteine (Hcy) is a sulfhydryl amino acid metabolite of dietary methionine. Elevated plasma Hcy is an independent cardiovascular risk factor that is associated with accelerated atherosclerosis and increased cerebrovascular/ischemic heart disease.[1] Without intervention, nearly 50% of the patients with congenital hyperhomocystinuria (ie, total plasma Hcy [tHcy] levels >200 μmol/L versus 7 to 14 μmol/L in normal individuals) will experience a major cardiovascular event by the age of 30 years.[2] However, more frequently seen in the general population are modestly elevated tHcy levels, which are nonetheless strong predictors of existent and future development of vascular pathologies.[3]

Elevated Hcy appears to contribute to cardiovascular disease, in part, by inducing endothelial cell (EC) dysfunction. In vivo, moderately elevated tHcy causes EC damage,[4] exacerbates hypertension-related atherosclerosis,[4] and impairs flow-mediated arterial dilation.[5] In vitro, Hcy-thiolactone is cytotoxic to ECs,[6] and the free reduced thiol (HcyH) alters the endothelial expression of bioactive molecules, such as NO, interleukin (IL)-8, and tissue factor.[7,8] Additionally, HcyH increases leukocyte adhesion to cultured human umbilical vein ECs,[9] and elevated tHcy induces adhesion molecule expression and leukocyte adhesion in rodent aortas.[10]

Atherosclerosis is a chronic low-grade inflammatory disorder[11] in which the adhesion of monocytes to the vascular endothelium and their subsequent migration into the vessel wall are pivotal early events in pathogenesis. Experimental evidence strongly implicates sustained elevated EC expression of adhesion molecules, such as vascular cell adhesion molecule (VCAM)-1, intercellular adhesion molecule

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(ICAM)-1, and E-selectin, as mediators of the subintimal leukocyte accumulation in atherosclerosis.[11] In the present study, we tested the hypothesis that HcyH increases monocyte adhesion to cultured human ECs by upregulating the expression of adhesion molecules on the EC surface. Our results suggest that even moderately elevated HcyH is proinflammatory and upregulates the endothelial expression of VCAM-1. HcyH-induced EC activation is dependent on endothelial cyclooxygenase (COX) activity and is modulated by endothelium-derived NO and reactive oxygen species (ROS).

Methods

Materials

Recombinant human tumor necrosis factor (TNF)-α, anti-human IL-1, polyclonal neutralizing antibody, and anti–VCAM-1 monoclonal antibody (clone BBIG-V1) were from R&D Systems Inc. Anti-human ICAM-1 (clone RR1/1.1.1) and anti-human E-selectin (clone CL26C10B7) monoclonal antibodies were gifts from Boehringer-Ingelheim, Inc. FITC-labeled monoclonal anti-human VCAM-1 antibody (clone 1.G11B1) was from Ancell Immunological Research Products. NS-398 was from Calbiochem, Inc. FCS was from Hyclone Laboratories Inc. EGM-2 Bulletkit growth factor kits were from Clonetics, Inc. Reduced DL-homocysteine (HcyH, as distinguished from total plasma homocysteine [tHcy] or the generic abbreviation for any homocysteine form [Hcy]), culture media MCDB-131 and RPMI-1640, and all other reagents were from Sigma Chemical Co. Cell culture media and all stock solutions had no detectable endotoxin contamination (E-Toxate kit, Sigma).

Cell Isolation and Culture

Human aortic ECs (HAECs), isolated according to Institutional Review Board approved protocols as previously described,[12] were cultured in medium MCDB-131/medium 199 (1:1) containing 10% FCS, EGM-2 growth factor supplements (hydrocortisone omitted), 10 μg/mL gentamicin, and 125 ng/mL fungizone (complete medium) at 37°C in a 5% CO_2/95% air (100% humidity) atmosphere. HAECs were used at passages 5 to 10. Normal human monocytes were isolated with a commercially available kit (Miltenyi Biotec) from blood donated by apparently healthy laboratory personnel according to IRB-approved protocols, and purity was verified to be >95% by cytostaining for morphology and nuclear structure. Monocytes were maintained in MCDB-131/10% FCS and immediately used in adhesion experiments. Human monocytic U937 cells (American Type Culture Collection) were propagated in RPMI-1640/10% FCS and switched into MCDB-131/10% FCS the day before the adhesion assays were performed.

Monocyte Adhesion Assays

Confluent HAEC monolayers grown in 24-well tissue culture plates were incubated for up to 24 hours with 0 to 1000 μmol/L HcyH added from freshly prepared 100× stock solutions in complete medium. Control wells received only vehicle. Cysteine was used as a negative control, and TNF-α was added as a positive control for adhesion molecule induction. To test the possible involvement of diverse intracellular effectors, appropriate pharmacological modulators (detailed below) were administered 30 minutes before HcyH. After HAEC stimulation and washing, 2.5×10^5 monocytes per well were added and allowed to attach at 37°C for 30 minutes. After 3 washes with complete medium, cultures were fixed (0.5% glutaraldehyde/PBS), and attached monocytes were counted on an inverted microscope. Four randomly chosen 1-mm^2 regions in each of quadruplicate wells per condition were evaluated. In some cases, saturating concentrations of neutralizing monoclonal antibodies against ICAM-1, VCAM-1, and E-selectin were added to the HAEC monolayers during the final 30 minutes of the HcyH stimulation, before the monocytes were added. Preliminary antibody dose-response assays verified saturating neutralizing concentrations.

Flow Cytometry

Confluent HAEC monolayers in 12-well plates were stimulated overnight with 0 to 100 μmol/L HcyH, as described above. In some wells, TNF-α (0.1 to 10.0 ng/mL) was added 5 hours before experimental termination. Cells were gently dislodged by brief trypsinization (0.025% [wt/vol] trypsin and 2 mmol/L EDTA in PBS), neutralized and blocked with complete medium, stained with FITC-labeled monoclonal anti–VCAM-1 antibody, and fixed with 2% glutaraldehyde, and cell surface VCAM-1 expression was evaluated by flow cytometry in a FACscan II (Beckton Dickinson, Inc). Ten thousand cells were analyzed per sample by using WinMDI software. Data are expressed as the percentage of VCAM-1–positive cells, after subtracting the small contribution of HAEC autofluorescence. Duplicate samples were evaluated in 4 experiments.

Competitive Quantitative Reverse Transcription–PCR

Adhesion molecule mRNA levels were evaluated by using a quantitative polymerase chain reaction (PCR) assay, as previously detailed.[12] Briefly, human-specific ICAM-1 and VCAM-1 primers were synthesized by using the published sequences.[13,14] Internal standards were constructed in plasmid vectors by deleting an internal 123- and 231-bp fragment from the targeted cDNA sequence for ICAM-1 and VCAM-1, respectively. Coamplification of constant amounts of cDNA with serial dilutions of internal standards and comparison of relative band densities allowed the precise determination of target message amounts present in HAECs.[12] Band intensities were compared by using Mocha 1.2.10 Image Analysis software (Jandel Corp). Results are presented as picomole equivalents of internal standard concentrations.

Statistical Analysis

Results are expressed as mean±SD for the indicated number of experiments. The significance of variability among the experimental group means was determined by 1-way ANOVA with the use of SigmaStat 2.0 software (Jandel Corp). Differences between experimental groups were considered to be statistically significant at $P<0.05$.

Results

Enhanced Monocyte Adhesion in HcyH-Stimulated ECs

HcyH treatment (18 hours) caused a concentration-dependent increase in the adhesion of monocytic U937 cells to HAEC monolayers (Figure 1). Background U937 monocyte adhesion to unstimulated HAEC monolayers was 9±1 cells/mm^2 (n=34 experiments conducted in quadruplicate wells per experiment). By use of U937 monocytes, stimulation of HAECs with 10 μmol/L HcyH (the concentration of tHcy typically present in the circulation of normal individuals)[1–3] caused no difference in adhesion versus that in unstimulated control cells (10±2 cells/mm^2, $P=0.279$; n=6). However, at higher concentrations, which were representative of moderate (15 to 30 μmol/L) to severe (>30 μmol/L) hyperhomocysteinemia, a significantly increase in monocyte adhesion was observed. This effect was obvious at 20 μmol/L HcyH (17±4 cells/mm^2, $P=0.007$; n=3), and was maximal at 100 μmol/L HcyH (35±7 cells/mm^2, $P<0.001$; n=33). Monocyte adhesion remained elevated at concentrations up to 300 μmol/L HcyH (26±10 cells/mm^2, n=4). At higher concentrations, HcyH was cytotoxic to HAECs, as inferred from enhanced trypan blue uptake, increased LDH release, and disorganization of the EC monolayer with cell rounding and detachment (not shown).

Background adhesion of normal human peripheral blood monocytes was 26±6 monocytes/mm^2 (n=3, Figure 2), likely reflecting mild activation during isolation. Adhesion of normal monocytes to HAECs stimulated with 200 μmol/L HcyH was increased by ≈4.5-fold (to 118±16 monocytes/mm^2, $P<0.001$ versus control cells; n=3), paralleling the increased

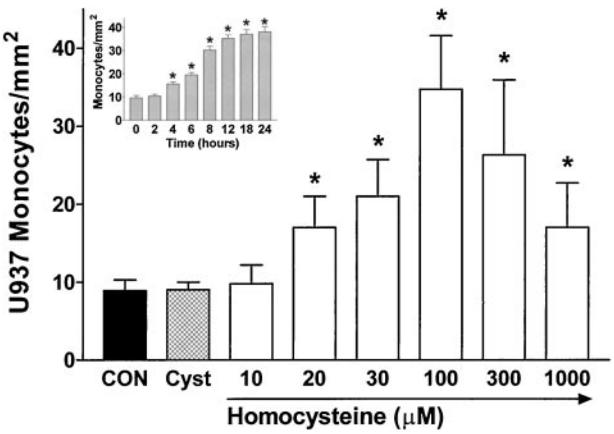

Figure 1. Hcy dose- and time-dependently enhances EC–U937 monocyte interactions. HAECs were stimulated with 0 to 1000 μmol/L DL-homocysteine or with 100 μmol/L cysteine (Cyst) for 18 hours before U937 monocyte addition. Hcy concentrations ≥20 μmol/L significantly increased adhesion, being maximal at 100 μmol/L (n=2 to 33 experiments). Cysteine had no effect (n=3). With 100 μmol/L Hcy, increased adhesion was observed after 4 hours (inset), reached a maximum after 12 to 18 hours of stimulation, and was maintained through 24 hours (n=2). *$P<0.05$ vs control cells.

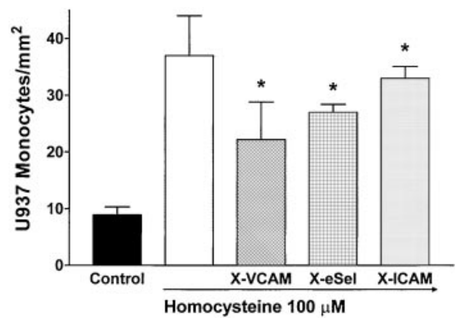

Figure 3. Neutralizing antibody studies. During the last 30 minutes of aortic EC stimulation with DL-homocysteine (100 μmol/L, 18 hours) and just before conducting the U937 monocyte adhesion assay, neutralizing anti–VCAM-1, anti–E-selectin, or anti–ICAM-1 antibodies were added. This resulted in respective 48±16%, 39±17%, and 11±9% inhibitions of Hcy-induced adhesion (n=2 to 5), with anti–VCAM-1 antibody being most effective. *$P<0.05$ vs Hcy-stimulated control cells.

adhesion of U937 cells. Methionine (500 μmol/L), the physiological Hcy precursor, caused a 2.3-fold increase in normal monocyte adhesion (to 60±17 monocytes/mm^2, $P<0.001$ versus control cells, n=3; Figure 2). Folic acid (5 μmol/L) had no effect by itself on subsequent monocyte binding but significantly inhibited HcyH-induced monocyte adhesion by ≈50% (Figure 2). Importantly, stimulation of HAECs with 100 μmol/L L-cysteine, another sulfur-containing amino acid, had no effect on either normal or U937 monocyte adhesion (28±5 and 9±1 cells/mm^2, respectively, versus control cells; n=3 and $P>0.1$ in both cases).

TNF-α stimulation of HAECs, a positive control for the upregulation of EC adhesion molecule expression and induction of monocyte adhesion, markedly increased U937 and normal monocyte adhesion to these cells. With the use of U937 cells, HAECs that had been stimulated with 10 ng/mL TNF-α for 18 hours bound 391±109 versus 9±1 cells/mm^2 background (n=16 experiments, $P<0.001$ versus control cells; data not shown). By use of peripheral blood monocytes, HAECs that had been stimulated with 1 ng/mL TNF-α for 18 hours bound 123±17 versus 26±6 cells/mm^2 background (n=3 experiments, $P<0.001$ versus control cells; Figure 2). With 10 ng/mL TNF-α, HAECs bound 217±17 normal monocytes (n=3, $P<0.001$; data not shown). Simultaneous HAEC stimulation with low-dose TNF-α (1 ng/mL) and HcyH (200 μmol/L) resulted in a modest but significant (40%) increase in monocyte adhesion over the sum effect of both agents applied individually (n=3, $P=0.011$; Figure 2).

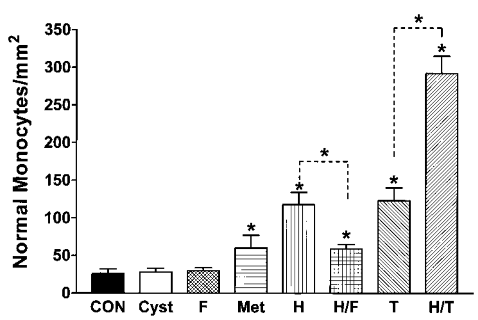

Figure 2. Hcy enhances EC–normal peripheral blood monocyte interactions. Confluent HAEC monolayers were stimulated with DL-homocysteine (H, 200 μmol/L), Cyst (100 μmol/L), methionine (Met, 500 μmol/L), folic acid (F, 5 μmol/L), TNF-α (T, 1 ng/mL), or combinations thereof for 18 hours before incubation with normal peripheral blood monocytes. *$P<0.05$ vs control cells.

Because normal monocytes and U937 cells behaved qualitatively similarly and because 100 μmol/L HcyH maximally induced U937 monocyte adhesion without affecting EC viability, this cell line and HcyH dose were used in subsequent experiments. Because in kinetic studies (Figure 1, inset) the HcyH-induced monocyte adhesion occurred 4 hours after HAEC stimulation and was maximal between 12 and 24 hours, we stimulated HAECs for 18 hours in subsequent mechanistic studies.

HcyH Upregulates Endothelial Adhesion Molecule Function

Using neutralizing monoclonal antibodies against human ICAM-1, VCAM-1, and endothelial-leukocyte adhesion molecule (ELAM) (5 μg/mL for all), we evaluated their relative involvement in HcyH-induced monocyte adhesion to HAECs (Figure 3). Although anti–ICAM-1 monoclonal antibody caused a modest (11±9%, $P=0.032$; n=5), albeit significant, reduction of HcyH-induced U937 monocyte adhesion, anti–VCAM-1 and anti-ELAM monoclonal antibodies individually inhibited the number of adherent monocytes by 48±16% ($P<0.001$, n=5) and 39±17% ($P=0.004$, n=4), respectively. In TNF-α–stimulated HAECs (10 ng/mL, 18 hours), anti–ICAM-1, anti–VCAM-1, and anti-ELAM monoclonal antibodies inhibited U937 monocyte adhesion by ≈25%, 40%, and 35%, respectively (not shown).

HcyH Upregulates VCAM-1 mRNA and Cell Surface VCAM-1 Protein Expression in HAECs

Consistent with the findings of the functional assays, using quantitative reverse transcription (RT)-PCR, we detected a 4.9±1.2-fold increase in VCAM-1 mRNA in HAECs stimulated with 100 μmol/L HcyH for 3 hours ($P=0.027$, n=2;

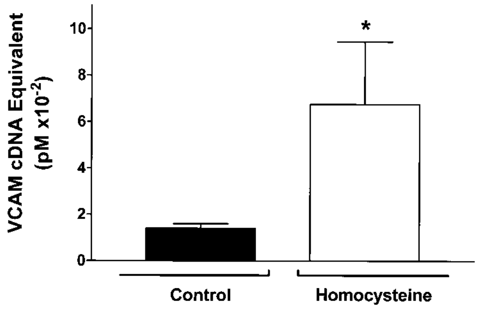

Figure 4. Hcy induces VCAM mRNA in aortic ECs. HAECs were stimulated with 100 μmol/L DL-homocysteine for 3 hours, and VCAM and ICAM-1 mRNA steady-state levels were determined by competitive RT-PCR. Although Hcy treatment resulted in a 4.9±1.2-fold increase in VCAM message, no significant change in ICAM-1 mRNA was detected (n=2, not shown). *$P<0.05$ vs unstimulated control cells.

Figure 4). By contrast, HcyH stimulation had no detectable effect on ICAM-1 mRNA levels in the same cells ($P>0.08$, not shown). By flow cytometry, stimulation with HcyH resulted in a modest, yet statistically significant, increase in VCAM-1 surface protein in HAECs versus control cells. The percentage of VCAM-1$^+$ HAECs were ≈1% in unstimulated cultures and 2%, 3%, and 6% in HAECs that were stimulated for 24 hours with 10, 25, and 100 μmol/L HcyH, respectively (Table). In comparison, 5 hours of stimulation with 0.1, 1.0, and 10.0 ng/mL TNF-α resulted in ≈12%, 23%, and 65% VCAM-1$^+$ cells, respectively. VCAM-1 expression in HAECs was synergistically augmented when the cells were simultaneously exposed to HcyH and low concentrations of TNF-α. For example, in HAECs exposed for 18 hours to 100 μmol/L HcyH and then additionally stimulated with 0.1 ng/mL TNF-α for 5 hours, VCAM-1$^+$ cells increased to ≈28% ($P<0.01$, n=4) compared with 6% and 12% for the same doses of HcyH and TNF-α alone, respectively (Table).

Effect of Homocysteine and TNF-α on VCAM-1 Protein Expression in HAEC

Experiment	% VCAM-1–Positive Cells
Unstimulated control	1.1±0.4
10 μM HcyH	1.6±0.8§
25 μM HcyH	2.8±0.7*
100 μM HcyH	6.2±1.1†
0.1 ng/mL TNF-α	12.4±2.3†
1 ng/mL TNF-α	23.7±4.2†
10 ng/mL TNF-α	67.6±8.9†
100 μM HcyH+0.1 ng/mL TNF-α	27.5±5.3‡
100 μM HcyH+1 ng/mL TNF-α	46.5±7.4‡
100 μM HcyH+10 ng/mL TNF-α	69.2±11.4¶

VCAM-1 surface expression on HAEC exposed to DL-homocysteine (HcyH, 24 h) and TNF-α (last 5 h), either alone or in combination, was assessed by flow cytometry. Results are expressed as percentage of VCAM-1–positive cells and represent means±SD (n=4), as analyzed by ANOVA with Bonferroni's post hoc analysis.

*$P<0.01$ vs unstimulated control.

†$P<0.001$ vs unstimulated control.

‡$P<0.01$ vs corresponding TNF-α concentration alone.

§No significant difference vs unstimulated control.

¶No significant difference vs corresponding TNF-α concentration alone.

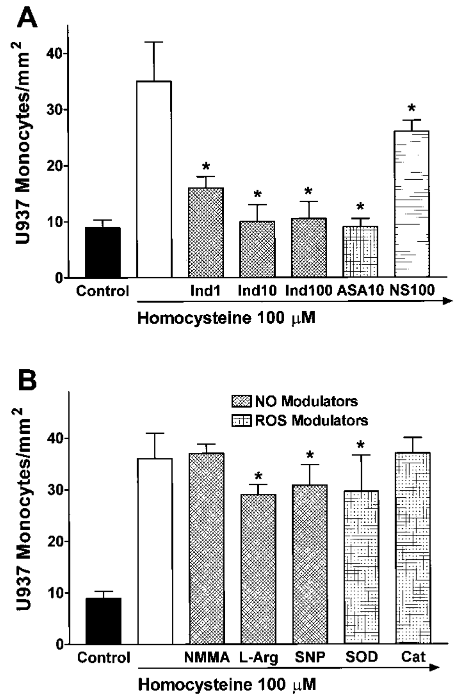

Figure 5. COX, NO, and ROS mediate Hcy-induced monocyte adhesion to HAECs. A, Concomitant incubation of ECs with nonspecific COX-1/COX-2 inhibitors (indomethacin, 1 to 100 μmol/L [Ind1 to 100]; aspirin, acetylsalicylic acid, 10 μmol/L [ASA10]) during DL-homocysteine (HcyH) stimulation prevented induced monocyte adhesion. However, a specific COX-2 inhibitor, NS398 (100 μmol/L [NS100]), only partially inhibited HcyH-induced adhesion (n=3). *$P<0.05$ for inhibitor/HcyH vs HcyH only–stimulated control cells. B, Elevating NO with L-arginine (L-Arg, 1 mmol/L) or with sodium nitroprusside (SNP, 100 μmol/L) resulted in significant (≈20% to 25%) inhibition of concomitant HcyH-stimulated adhesion. The NO synthase competitive inhibitory substrate L-NMMA (100 μmol/L) had no effect. Scavenging superoxide radicals with SOD (500 U/mL) caused a 25% inhibition of HcyH-induced adhesion, whereas the H_2O_2 scavenger, catalase (Cat, 5000 U/mL), had no effect. *$P<0.05$ for inhibitor/HcyH vs HcyH only–stimulated control cells.

Enhanced Monocyte Adhesion to HcyH-Activated ECs Is Abrogated by COX Inhibitors

Indomethacin, a nonselective COX-1/COX-2 antagonist, caused a concentration-dependent inhibition of HcyH-induced monocyte adherence to HAECs (Figure 5A). Importantly, at 10 μmol/L indomethacin, which approximates clinically attainable plasma levels (ie, 3 to 6 μmol/L), HcyH-induced adhesion was completely abrogated ($P>0.1$ versus unstimulated control cells, n=3). Similarly, acetylsalicylic acid (aspirin), another nonselective COX-1/COX-2 inhibitor, completely inhibited the HcyH effect at 10 μmol/L, also a pharmacologically relevant concentration ($P>0.1$, n=2). In contrast, NS-398, a highly selective COX-2 antagonist, only partially inhibited (≈38±8%) HcyH-mediated monocyte adhesion, even at 100 μmol/L, a concentration >10-fold above its reported IC_{50} for COX-2 inhibition ($P<0.001$, n=3).[15] At the concentrations used in the present study, these COX inhibitors neither modulated baseline monocyte adhesion nor were they cytotoxic to HAECs.

Enhanced Monocyte Adhesion to HcyH-Activated ECs Is Modulated by NO and ROS

An interrelationship exits between EC adhesion molecule expression and NO and ROS production.[16,17] Incubation of HAECs with N^G-monomethyl-L-arginine (L-NMMA, 100 μmol/L), a competitive inhibitor of endothelial NO generation, had no effect on basal or HcyH- mediated endothelial adhesiveness. Sodium nitroprusside (100 μmol/L), an exogenous NO donor, and excess L-arginine (1 mmol/L), the physiological substrate for NO production, caused moderate, yet significant, inhibition of HcyH-induced monocyte adhesion (Figure 5B). Scavenging free superoxide radicals with superoxide dismutase (SOD, 500 U/mL) significantly decreased monocyte adhesion in HcyH-treated HAECs (Figure 5). However, catalase (5000 U/mL), which catalyzes the conversion of hydrogen peroxide to water, either with or without SOD present, had no additional inhibitory effect.

Discussion

Although tHcy is a recognized independent cardiovascular risk factor,[1,3] the mechanisms by which it alters the physiology of the vascular wall remain largely unknown. Others have previously demonstrated Hcy-induced EC dysfunction, including altered proliferation[6] and the expression of bioactive molecules.[7,8] To date, however, the few studies investigating the potential role of Hcy in modulating leukocyte-endothelial interactions have largely focused on the potential involvement of ICAM-1, yielding, in part, controversial results.[9,10,18,19] One notable exception is a recent report that demonstrated increased vascular VCAM-1 expression in hyperhomocysteinemic mice.[20]

Our primary finding is that HcyH is proinflammatory and upregulates the expression of HAEC adhesion molecules, specifically VCAM-1, resulting in enhanced monocyte adhesion. Although this primary effect is modest in terms of VCAM-1 steady-state mRNA and cell surface protein expression, it nonetheless is significant, resulting in ≈500% increased adhesion of normal and U937 monocytes to HcyH-stimulated HAECs. Methionine, the biological precursor to Hcy, but not cysteine, induces endothelial adhesiveness, indicating the specific action of HcyH. Additionally, folic acid, a coenzyme critical in metabolizing Hcy, reduced HcyH-induced monocyte adhesion by ≈50% (Figure 2). This finding is in line with recent reports wherein folate inhibited HcyH-induced VCAM-1 in murine atherosclerotic lesions,[20] ameliorated moderately elevated plasma tHcy levels, and reduced neointimal formation in rats made hyperhomocysteinemic by methionine or HcyH feeding.[21] Importantly, we found that HcyH acts in synergy with low concentrations of TNF-α in upregulating VCAM-1 expression, suggesting that even modest elevations of tHcy might increase the basal activation state of ECs and, thereby, precondition the vessel wall to concomitant or subsequent injury by known proatherogenic/inflammatory agonists.

Neutralizing antibodies against VCAM-1 decreased HcyH-induced monocyte binding to HAECs by ≈50%, indirectly confirming increased functional VCAM-1 expression. Because VCAM-1 expression is focally elevated in ECs in vascular regions prone to atherogenesis,[11,20] our findings might reflect a link between elevated tHcy levels and enhanced monocyte infiltration in developing atherosclerotic lesions. The partial efficacy of blocking antibodies against E-selectin and, to a lesser extent, ICAM-1 suggests that other adhesion molecules are also involved in the atherogenic activation of ECs by HcyH.

Consistent with the functional assay results, HcyH increased VCAM-1 gene and protein expression in HAECs. By quantitative RT-PCR, we observed a 5-fold increase in VCAM-1 mRNA levels in HAECs that had been treated with 100 μmol/L HcyH for 3 hours. By flow cytometry, HcyH significantly increased the $VCAM^+$ fraction of HAECs. This could have important implications in atherogenesis, a chronic low-grade inflammatory state involving multiple bioactive mediators.

We used several pharmacological approaches to elucidate some of the possible mechanisms of HcyH action. Protein kinases C and A reportedly mediate cytokine-induced upregulation of EC adhesion molecule expression.[22,23] However, in our system, neither inhibition of protein kinase C (10 to 50 μmol/L H7) nor inhibition of protein kinase A (50 μmol/L H89) nor elevation of intracellular cAMP in HAECs with use of the membrane-permeant mimetic dibutyryl cAMP had any effect on either baseline or HcyH-enhanced EC-monocyte interactions (not shown). Activated ECs can secrete IL-1, an inflammatory cytokine that can act in an autocrine fashion to induce adhesion molecules. However, anti–IL-1 neutralizing antibodies had no effect on monocyte adhesion (not shown), ruling out the possibility that the HcyH effect is mediated by autocrine IL-1.

By contrast, HcyH-induced monocyte binding to HAECs was completely abrogated by pretreating the endothelial monolayers with clinically relevant concentrations of common COX inhibitors. Aspirin and indomethacin, which are nonselective COX-1/COX-2 inhibitors, concentration-dependently inhibited the proinflammatory effect of HcyH. Specific inhibition of COX-2 with NS-398, even at concentrations more than an order of magnitude greater than its IC_{50},[15] resulted in only partial (≈40%) inhibition of HcyH-induced adhesion. This suggests a greater relative importance of the COX-1 isoform in mediating this effect. Because the COX enzymes are responsible for the generation of several classes of vasoactive substances (ie, prostaglandins, thromboxanes, and prostacyclin), careful dissection of the molecular pathways downstream from COX will yield more insight into the specific mechanisms of HcyH-mediated inflammation.[24] It is interesting to speculate that the commonly prescribed daily aspirin tablet, issued as a cardioprotective measure, might have the additional benefit of slowing atherogenesis by inhibiting HcyH-induced monocyte adhesion to the arterial lining. This could reflect a more general suppression of an otherwise proatherogenic EC phenotype.

Elevated plasma tHcy perturbs endothelial NO expression and activity in the vasculature.[7,10,25] Although it is uncertain whether Hcy can modulate NO synthase expression,[7,26] it clearly has the potential to complex with and inactivate released NO,[7,25] potentially forming cytotoxic peroxynitrate radicals. In vivo, HcyH- and tHcy-mediated NO downregulation is accompanied by measurably impaired vascular function.[10,27] Elevated levels of NO downregulate cytokine-induced EC adhesion molecule expression.[16,28] In our hands, L-arginine and nitroprusside attenuated (≈25% inhibition), but did not abrogate, HcyH-induced monocyte adhesion to HAECs. This is in line with evidence that elevated NO mitigates vasomodulatory aberrations in the vasculature[28] and can partially reduce HcyH-induced leukocyte adhesion in vivo.[10] In preliminary gene array studies of HcyH-stimulated HAECs, we saw no change in mRNA expression for the 3 known NO synthase isoforms (not shown). Thus, Hcy-induced endothelial VCAM-1 expression appears in part to be due to downregulated NO activity/bioavailability.

Hcy alters EC redox potentials, in part by downregulating intracellular glutathione peroxidase activity, resulting in accumulation of intracellular ROS.[29] Additionally, superoxide can directly quench the activity of NO.[30] In the present study, SOD pretreatment of ECs reduced HcyH-induced monocyte adhesion by ≈25%, whereas catalase was ineffective, suggesting that superoxide radicals, but not free hydrogen peroxide, may contribute to HcyH-induced adhesion molecule expression.

In summary, we demonstrated that HcyH markedly increases the adhesiveness of HAECs to monocytes and acts in synergy with inflammatory cytokines, such as TNF-α. HcyH-induced upregulation of VCAM-1 mRNA and of functional protein on the EC surface is mediated by multiple signaling pathways, with their relative importance being COX-1>COX-2>NO=ROS. The finding that clinically relevant concentrations of proven COX inhibitors can completely abrogate HcyH-induced monocyte adhesion to ECs further supports their usefulness as part of the pharmacological arsenal that we are amassing against cardiovascular disease.

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