Published in final edited form as:

Biomed Pharmacother. 2008 ; 62(6): 395–400. doi:10.1016/j.biopha.2008.02.008.

PRIMING EFFECT OF HOMOCYSTEINE ON INDUCIBLE VASCULAR CELL ADHESION MOLECULE-1 EXPRESSION IN ENDOTHELIAL CELLS

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Abstract

Hyperhomocysteinemia is an independent risk factor for the development of atherosclerosis, as well as for arterial and venous thrombosis. However, the mechanisms through which elevated circulating levels of homocysteine cause vascular injury and promote thrombosis remain unclear. Here, we tested the hypothesis that homocysteine (Hcy) sensitizes endothelial cells to the effect of inflammatory mediators. Human umbilical vein endothelial cells (HUVEC) were incubated with Hcy 1.0 mM for varying time points, and then treated in the absence or presence of 1.5 U/ml thrombin or 10 ng/ml lipopolysaccharide (LPS). Hcy alone had no effect on the expression of vascular cell adhesion molecule (VCAM)-1. However, Hcy enhanced thrombin- and LPS-mediated induction of VCAM-1 mRNA and protein levels. Consistent with these results, pretreatment of HUVEC with Hcy resulted in a two-fold increase in LPS-mediated induction of leukocyte adhesion. The latter effect was significantly inhibited by anti-VCAM-1 antibodies. Together, these findings suggest that Hcy sensitizes HUVEC to the effect of inflammatory mediators thrombin and LPS, at least in part through VCAM-1 expression and function.

Keywords

Homocysteine; VCAM-1; Endothelium

INTRODUCTION

Hyperhomocysteinemia is an important risk factor for the development of vascular disease, including stroke, acute myocardial infarction, peripheral artery disease and venous

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thrombosis^{1,2}. There is growing evidence that hyperhomocysteinemia-mediated atherosclerosis and hypercoagulability is mediated, at least in part, by endothelial dysfunction. Under in vivo conditions, elevated levels of homocysteine (Hcy) are associated with impaired endothelium-dependent vasodilation^{3,4}, increased oxidative stress, and increased expression of cell adhesion molecules in the endothelial lining of the aorta^{5,6}. In cultured endothelial cells, homocysteine has been shown to promote oxidative stress⁷, induce the expression of chemokines (e.g., monocyte chemoattractant protein (MCP)-1 and interleukin [IL]-8) and cell adhesion molecules (e.g. vascular cell adhesion molecule [VCAM]-1 and E-selectin)^{8,9}, and reduce nitric oxide bioavailability¹⁰. Homocysteine has been shown to enhance neutrophil-endothelial interactions in both cultured human cells and rats in vivo, but no changes to the endothelial surface adhesion molecules ICAM-1, E-selectin, VCAM-1 and P-selectin were found¹¹.

The majority of in vitro studies have explored the effect of short- or long-term exposure of Hcy on cell phenotypes. An important question, which remains largely unexplored, is whether (and to what extent) Hcy modulates endothelial cell response to other signal inputs. Hcy was shown to promote tumor necrosis factor (TNF)- α and IL-1-mediated induction of VCAM-1 in endothelial cells^{5,12}. Harrington et al. demonstrated that pretreatment of pulmonary endothelial cells with Hcy attenuated thrombin-induced barrier dysfunction¹³. The goal of the present study was to determine the effect of Hcy on LPS- and thrombin-mediated activation of endothelial cells, as assayed by VCAM-1 expression and function.

MATERIALS AND METHODS

Cell culture

HUVEC were purchased from Clonetics (Hopkinton, MA), and grown in endothelial basal medium (EBM-2 media) and supplemented with the Endothelial Growth Medium (EGM-2 MV; Clonetics). Cells were incubated with DL-homocysteine (Sigma, St-Louis, MO) tested endotoxin-free at different concentrations (0.1 mM, 0.5 mM and 1.0 mM) for 6 days (fresh media with the addition of fresh Hcy every other day). These concentrations were used based on previous reported experiments studying the effects of homocysteine on endothelial cells, which differ from the circulating concentrations in the bloodstream. On day 6, HUVEC (80–90% confluence) were serum-starved in medium EBM-2 without EGM-2 MV and containing the same concentrations of Hcy added with 0.5% fetal bovine serum (FBS; Hyclone, Logan, UT) for 24 hours. Cells were then treated with thrombin 1.5 U/ml (Calbiochem; San Diego, CA) or LPS (Lipopolysaccharide; Sigma, St-Louis, MO) 10 μ g/ml for the times indicated, and harvested for total RNA and protein as previously described¹⁴. Alternatively, serum-starved HUVEC were treated with 1.0 mM Hcy for 2, 4, 6 and 8 hours with or without thrombin 1.5 U/ml for 4 hours after Hcy treatment.

Northern and Western blot analyses

Northern and Western Blot analyses were carried out as previously described¹⁴. Northern blots were hybridized with (³²P) dCTP-labeled human probes for VCAM-1. Full length human VCAM-1 cDNA was used for probing. For Western blot analyses, the following antibody was used: anti-VCAM-1 (rabbit polyclonal antibody cross reacting with human

VCAM-1) (Santa Cruz Biotechnology; Santa Cruz, CA). The Northern and Western blots were quantified using Scion Image (Scion Corp., Frederick, MD).

Real-Time Polymerase Chain Reaction (TaqMan RT-PCR)

HUVEC were harvested for total RNA using Trizol (Invitrogen). Multiplex RT-PCR was carried out according to the protocol provided by the manufacturer for the TaqMan One step PCR Master Mix Reagents Kit (ABI, Foster City, CA). Amplification of the 18S RNA was used as endogenous control. TaqMan analysis and the subsequent data analysis were performed with an ABI PRISM 7700 Sequence Detector System. This system is able to detect the increase of the fluorescent signal released from the fluorogenic probe as the PCR proceeds. For each sample, 100 ng of total RNA was subjected to Real-Time PCR. The following sequences were employed (F, forward primer; R, reverse primer; T, Taqman probe). For (VCAM)-1, F:5'-TCGAGACCACCCAGAATCT-3'; R:5'-GCCTGTGGTGCTGCAAGTC-3'; T:5'-ATATCTTGCTCAGATTGGTGACTCCGTCTCA-3'. The primer design was performed with the Primer Express 1.5 software. The 18S ribosomal RNA endogenous control (VIC labeled probe) was purchased from ABI, Foster City, CA (Part # 4310893E). All experiments were performed in triplicate.

Leukocyte adhesion assay

HUVEC were seeded on 0.25% gelatin-coated plates and cultured in EBM medium containing 10% FBS (Hyclone, Logan, UT) and EGM-2 –MV (without VEGF) (Clonetics). Venous blood was obtained from healthy donors free from medication for at least 10 days prior to the experiments. The protocol was approved by the Institutional Review Board (IRB). Neutrophils were isolated using Ficoll-Paque gradient, as described previously¹⁵. Over ninety-five percent of the isolated cells were neutrophils as determined by Coulter counter and viability was found to be greater than 98% as assessed by trypan blue dye exclusion assay. Prior to neutrophil adhesion assays, HUVEC were pretreated in the absence (control) or presence of Hcy (treated) 1 mM (fresh media with the addition of fresh Hcy every other day) for 7 days. Neutrophil adhesion to HUVEC was measured under static conditions as described previously¹⁵. Briefly, leukocytes were added (1×10^6 neutrophils/ml, 500 μ l/well) to confluent monolayers of HUVEC grown in 24-well plates and treated with 1 or 10 μ g/ml LPS for 4 hours before adding neutrophils. In some experiments, HUVEC confluent monolayers were treated for 15 min with blocking mouse monoclonal anti-human VCAM-1 IgG (α -VCAM-1; 1–10 μ g/ml) (Clone P3C4; Chemicon International, Hampshire, United Kingdom) prior to the addition of neutrophils. Sixty min following the addition of neutrophils, the wells were rinsed three times with DPBS to remove nonadherent neutrophils, and fixed with a 2% paraformaldehyde DPBS solution. Adhesion of neutrophils to confluent HUVEC was assessed with a color video digital camera adapted to a binocular microscope. For each well, 3 fields of view were randomly selected and the neutrophils in each field of view were counted and recorded as the number of adhered neutrophils/mm². Due to slight variations of basal adhesion between experiments, we then reported our data as neutrophil adhesion index (%).

Cell viability assay with Trypan blue exclusion test

Subconfluent (90% confluence) HUVEC were suspended in 2 ml of medium and 50 μ l of this mixture was incubated with an equal volume of 0.4% Trypan blue (Sigma) for 5 min at room temperature. The cells were counted under light microscopy and assay was performed in triplicate for control and Hcy treated cells. In these assays, viability of Hcy-treated cells was greater than 95%.

Statistical analyses

Data reported are the mean $\pm$ standard deviation of three independent experiments. Paired *t* tests were used to compare individual points where appropriate. $P < 0.05$ was considered as statistically significant.

RESULTS

Homocysteine sensitizes human umbilical vein endothelial cells to agonist-mediated induction of VCAM-1 mRNA and protein expression

To determine the effect of sustained Hcy exposure on subsequent endothelial cell activation, HUVEC were grown in the absence or presence of varying concentrations of Hcy for 7 days and then incubated with or without 1.5 U/ml thrombin or 10 μ g/ml LPS for 4 hours. Cells were harvested for total RNA and processed for Northern blot analyses. Hcy alone had no effect on VCAM-1 expression (Fig. 1A). However, treatment with Hcy resulted in a dose-dependent increase in thrombin-mediated induction of VCAM-1 mRNA, with maximal sensitization occurring at 1.0 mM (Fig. 1A). Neither 0.5 mM cysteine nor 0.5 mM methionine had an effect on VCAM-1 mRNA levels. Short-term exposure to Hcy failed to increase basal or thrombin-inducible VCAM-1 mRNA levels (Fig. 1B). Hcy, but not cysteine or methionine, also resulted in a dose-dependent increase LPS-mediated induction of VCAM-1 (Figs. 1C). To obtain quantitative data, RT-PCR analyses were carried out. In the absence of Hcy preconditioning, VCAM-1 mRNA was induced 266-fold by thrombin and 143-fold by LPS (data not shown). Following 7-day exposure to 1.0 mM Hcy, these effects were enhanced by 40% and 97%, respectively (Fig. 1D). In Western blot analyses, sustained treatment with 1.0 mM Hcy alone did not promote VCAM-1 protein expression (Fig. 2). However, Hcy treatment for 7 days resulted in a dose-dependent increase in thrombin- and LPS-mediated induction of VCAM-1 (100% and 93%, respectively) (Fig. 2).

Homocysteine sensitizes human umbilical vein endothelial cells to VCAM-dependent agonist-mediated induction of neutrophil adhesion

To determine the functional consequences of Hcy preconditioning on VCAM-1 expression, HUVEC were assayed for agonist-mediated leukocyte adhesion. Treatment of HUVEC with 1 μ g/ml or 10 μ g/ml LPS for 4 hours resulted in a 14.9- and 15.1-fold increase in neutrophil adhesion (Fig. 3A). Pretreatment of HUVEC with Hcy 1.0 mM for 7 days alone had no effect on adhesion, but resulted in enhanced LPS-mediated adhesion of neutrophils (40% in 1 μ g/ml-treated cells, and 101% in 10 μ g/ml-treated cells) ($p < 0.001$) (Fig. 3A). Preincubation of control and Hcy preconditioned HUVEC with 10 μ g/ml anti-VCAM-1 antibodies 15 min prior to the addition of LPS 10 ng/ml reduced neutrophil adhesion by 32

and 40% ($p<0.01$), respectively (Figs. 3B and 3C). Treatment with normal mouse IgG (10 $\mu\text{g/ml}$) did not affect the adhesion of neutrophils to HUVEC (data not shown).

DISCUSSION

The present study was motivated by several considerations. First, the history of signal input is a critical – though largely overlooked – determinant of endothelial cell phenotype. We previously demonstrated that pretreatment of endothelial cells with inflammatory mediators, including LPS and TNF- α significantly modified thrombin signaling¹⁶. Thus, we were interested in determining whether Hcy had a similar effect. Second, we have a longstanding interest in VCAM-1 as a molecular marker of endothelial cell activation^{17–20}. VCAM-1 is a highly inducible gene that has been implicated not only in atherosclerosis but also in many other diseases including sepsis and thrombosis^{21, 22}. Third, hyperhomocysteinemia is a risk factor for atherosclerosis and venous thrombosis. Therefore, an understanding of its mechanism of action may yield important insights into the link between inflammation and coagulation.

Previous studies have demonstrated an association between hyperhomocysteinemia and increased VCAM-1 expression in the intact endothelium⁵. Silverman et al. showed that Hcy alone induced VCAM-1 mRNA by approximately 5-fold in human aortic endothelial cells (HAEC)¹². Preincubation of HAEC with Hcy for 18 hours followed by TNF- α treatment resulted in a synergistic effect on VCAM-1¹². In another report, Hcy (20 h) was shown to augment IL-1-induction of VCAM-1 and E-selectin in HAEC, as well as IL-1-mediated adhesion of U937 monocytes⁹.

In the current study, we failed to detect any effect of Hcy alone on VCAM-1 expression in HUVEC. However, we demonstrated that chronic, but not acute, exposure to Hcy (7 days) sensitizes endothelial cells to the effects of activation agonists (thrombin and LPS) on VCAM-1 protein and mRNA expression. In addition, pretreatment with Hcy resulted in increased LPS-mediated neutrophil adhesion, an effect that was significantly attenuated by anti-VCAM-1 antibodies. These latter results suggest that the sensitization effect of Hcy is functionally relevant.

The finding that Hcy induces LPS-mediated neutrophil adhesion, at least in part, via a VCAM-1-dependent mechanism raises interesting questions about sepsis pathophysiology. LPS induces VCAM-1 expression in cultured endothelial cells, and endotoxemia models are associated with increased VCAM-1 expression in the intact endothelium²³. Moreover, consistent with our data, others have shown VCAM-1 can mediate not only monocyte, but also neutrophil adhesion flow conditions²⁴. VCAM-1-mediated adhesion of neutrophils may involve direct binding to integrin ($\alpha 4\beta 1$ or $\alpha 9\beta 1$)^{24–26}, or VCAM-1 intracellular signaling²⁷. Based on these findings, it is interesting to speculate that hyperhomocysteinemia predisposes patients to an accentuated host response to infection.

The mechanisms underlying Hcy-mediated sensitization of the endothelium remain to be defined. Previous studies have shown that Hcy induces the production of reactive oxygen species and activates NF- κ B in endothelial cells⁷. Other agonists, such as thrombin and

TNF- α induce VCAM-1 via an NF- κ B-dependent mechanism^{28, 29}. Moreover, VCAM-1 is a highly redox-sensitive gene^{30, 31}. Thus, it seems likely that Hcy is potentially mediating its effect, at least partly through a redox/NF- κ B-dependent pathway.

An alternative explanation to our results may be sought from more recent interesting data on the homocysteine thiolactone-mediated hypothesis of vascular disease³². Two novel Hcy metabolites, Hcy-thiolactone and protein N-linked Hcy (N-Hcy-protein) have been recently discovered^{32, 33}. Hcy treatment of Human Endothelial Cells such as HUVEC was shown to cause the metabolic conversion of Hcy to thiolactone (Hcy-thiolactone), a toxic metabolite, followed by its consequent accumulation; Hcy-thiolactone mediates Hcy incorporation into proteins (N-Hcy-proteins) or so called protein homocysteinylation³⁴. The effects of these toxic metabolites on endothelial cells were found to be more important than Hcy, resulting in protein damage and consequent induced toxicity to human endothelium³⁴ and thus contributing to inflammation^{32, 35}. Other authors reported on chronic infusion of baboons with Hcy-thiolactone and subsequent endothelial cell injury³⁶ and thiolactone, but not Hcy, was found to induce gross changes in endothelial cell morphology and to induce cell death³⁷. Pathophysiological consequences of Hcy-thiolactone-dependent protein N-homocysteinylation recognized thus far include protein and cell damage and/or cell death, activation of an adaptive immune response (by enhanced synthesis of autoantibodies against N-Hcy-proteins)^{32, 38} and enhanced thrombosis, manifested by N-homocysteinylation of fibrinogen^{32, 39}. Taken together, the findings underlying the homocysteine thiolactone-mediated hypothesis of vascular disease are consistent with our observation that only a prolonged, chronic exposure, which causes substantial accumulation of N-Hcy-proteins in the cells, leads to enhanced neutrophil adhesion to HUVEC.

In summary, this study has shown that homocysteine increases the effect of proinflammatory mediators (thrombin and LPS), at least in part, via a VCAM-1-dependent mechanism.

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Fig. 1. Hcy sensitizes HUVEC to thrombin- and LPS-mediated induction of VCAM-1 mRNA

A, Northern blot analysis of VCAM-1 mRNA expression in HUVEC preincubated for 7 days in the absence or presence of Hcy (0.1–1.0 mM), cysteine or methionine and treated with (+) or without (–) thrombin (1.5 U/ml) for 4 hours. **B**, Northern blot analysis of VCAM-1 mRNA expression in HUVEC preincubated for 2, 6 or 8 hours in the absence or presence of Hcy, and treated with or without thrombin (1.5 U/ml) for 4 hours. **C**, Northern blot analysis of VCAM-1 mRNA expression in HUVEC preincubated for 7 days in the absence or presence of Hcy (0.1–1.0 mM), cysteine or methionine and treated with (+) or without (–) LPS (10 µg/ml) for 4 hours. **D**, Real-Time PCR assays of VCAM-1 mRNA in HUVEC preincubated for 7 days in the absence (control) or presence of Hcy, and treated with thrombin (1.5 U/ml) or LPS (10 µg/ml) for 4 hours. All experiments were performed in triplicates for each standard and treated samples and data shown are mean $\pm$ SD of 3 experiments; * $p < 0.05$.

Fig. 2. Hcy sensitizes HUVEC to thrombin- and LPS -mediated induction of VCAM-1 protein expression

A, Western blot analysis of VCAM-1 protein from whole cell extract of HUVEC preincubated for 7 days in the absence or presence of Hcy (0.1–1.0 mM), and treated with (+) or without (–) thrombin (1.5 U/ml) for 4 hours. **B,** Western blot analysis of VCAM-1 protein from whole cell extract of HUVEC preincubated for 7 days in the absence or presence of Hcy (0.1–1.0 mM), and treated with (+) or without (–) LPS (10 µg/ml) for 4 hours. Blots shown are representative of at least 2 experiments performed using separately prepared cell extracts.

Fig. 3. Hcy augments LPS-mediated, VCAM-1-dependent adhesion of neutrophils

A, Confluent monolayers of HUVEC were preincubated for 7 days in the absence or presence of Hcy (1.0 mM), treated without (PBS) or with LPS (1 or 10 µg/ml) for 4 hours and then incubated 5×10^5 neutrophils. Data represent mean $\pm$ SEM of 4 experiments; *** $p < 0.001$ as compared to PBS, ††† $p < 0.025$ as compared to LPS 10 µg/ml. **B, C,** Confluent monolayers of HUVEC were preincubated for 7 days in the absence (**B**) or presence (**C**) of Hcy (1.0 mM), treated without (PBS) or with LPS (10 µg/ml) $\pm$ antibody against VCAM-1 for 4 hours and then incubated 5×10^5 neutrophils. Data are means $\pm$ SEM of 6 experiments; ** $p < 0.05$, *** $p < 0.001$ as compared to PBS, ††† $p < 0.025$ as compared to LPS 10 µg/ml. A–C, For each experiment, neutrophil adhesion index was set at 100% for the condition inducing maximal neutrophil adhesion.