Homocysteine Induces Expression and Secretion of Monocyte Chemoattractant Protein-1 and Interleukin-8 in Human Aortic Endothelial Cells
Implications for Vascular Disease
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Background—Proinflammatory cytokines play key roles in atherogenesis and disease progression. Because hyperhomocysteinemia is an independent risk factor for cardiovascular disease, we hypothesized that homocysteine could be atherogenic by altering the expression of specific cytokines in vascular endothelial cells.

Methods and Results—Northern blot and RNase protection assays showed that DL-homocysteine induced mRNA expression of the proinflammatory cytokines monocyte chemoattractant protein-1 (MCP-1) and interleukin-8 (IL-8) in cultured human aortic endothelial cells (HAECs). Homocysteine had no effect on expression of other cytokines, namely tumor necrosis factor-α, granulocyte-macrophage colony-stimulating factor, interleukin-1β, and transforming growth factor-β. MCP-1 mRNA expression increased 1 hour after homocysteine treatment, reached a maximum within 2 to 4 hours, and declined to basal levels over the next 24 hours. Induction of mRNA expression for both chemokines was observed with as little as 10 μmol/L DL-homocysteine, and maximal expression was achieved with 50 μmol/L DL-homocysteine. Homocysteine also triggered the release of MCP-1 and IL-8 protein from HAECs into the culture medium. The induction was specific for homocysteine, because equimolar concentrations of L-homocystine, L-cysteine, and L-methionine had no effect on mRNA levels and protein release. Furthermore, L-homocysteine induced chemokine expression, but D-homocysteine did not, thus demonstrating enantiomeric specificity. The culture medium from homocysteine-treated HAECs promoted chemotaxis in human peripheral blood monocytes and U937 cells. Anti-human recombinant MCP-1 antibody blocked the migration.

Conclusions—Pathophysiological levels of L-homocysteine alter endothelial cell function by upregulating MCP-1 and IL-8 expression and secretion. This suggests that L-homocysteine may contribute to the initiation and progression of vascular disease by promoting leukocyte recruitment. (Circulation. 2001;103:2717-2723.)

Key Words: homocysteine ■ peptides ■ endothelium ■ cells ■ cardiovascular diseases

Cytokines play critical roles in atherosclerosis and inflammatory diseases. Dysregulation of cytokine expression in vascular cells may contribute to the atherosclerotic process, and we now report that homocysteine can enhance the expression of specific cytokines in human aortic endothelial cells (HAECs). Homocysteine, an intermediary metabolite of methionine, is an independent risk factor for cardiovascular disease.1,2 Whether hyperhomocysteinemia is causal or merely a marker for cardiovascular disease is the subject of active investigation.

Previous in vivo and in vitro studies suggest that homocysteine changes the endothelium from a nonthrombogenic to a thrombogenic phenotype3 and limits the bioavailability of nitric oxide.4 The “response to injury” hypothesis, whereby the vascular endothelium becomes “activated” in response to injurious agents, may apply to homocysteine as well.5 Activated endothelial cells release proinflammatory cytokines,5,6 including chemokines, which recruit monocytes, T lymphocytes, and neutrophils to the site of injury.5,7 We hypothesize that homocysteine stimulates the production of specific cyto-
thiol concentrations were determined spectrophotometrically. Unique length and represented a distinct sequence in human mRNAs custom-designed set of cDNA templates (Pharmingen) with an in thiolactones (Sigma) by a method modified from Hatch et al. Final monocytes, and IL-8, a chemokine for T lymphocytes and neutrophils, suggest that homocysteine may alter endothelial cell function and promote atherosclerosis.

Methods

Cell Culture

Primary cultures of HAECs were established after collagenase treatment of segments of human thoracic aorta, obtained as discarded tissue from donor hearts used in heart transplant surgery (approved by the Institutional Review Board of the Cleveland Clinic Foundation). Cells were grown in medium 199 (Gibco-BRL) supplemented with 150 μg/mL endothelial cell growth factor, 90 μg/mL heparin, 20% FCS (BioWhittaker), and an antibiotic/antimycotic mixture (Gibco-BRL). For all experiments, confluent HAEC cultures (passages 5 to 7) were incubated with fresh culture medium for 24 hours and then treated with homocysteine or other compounds. Endotoxin levels of the DL-homocysteine (ICN Pharmaceuticals) working solutions, prepared in complete medium, were <0.012 ng/mL (Limulus amoebocyte lysate assay; Associates of Cape Cod). L-Homocysteine and t-homocysteine were freshly prepared from their respective thiocarboxylic acid (Sigma) by a method modified from Hatch et al.10 Final thiol concentrations were determined spectrophotometrically.

Northern Hybridization Assays

Total RNA (10 μg), extracted with RNAeasy (Qiagen), was electrophoresed on agarose gel11 and transferred to nylon membrane (Genescreen, NEN) for Northern blotting. After hybridization11 with 32P-labeled human MCP-1 cDNA probes (740-bp insert from ATCC) and autoradiography, relative intensities were quantified by densitometry or phosphorimaging and normalized to GAPDH mRNA expression. Rat GAPDH cDNA (1.3 kb) was obtained from ATCC.

RNase Protection Assays

A 32P-labeled antisense RNA probe cocktail was transcribed from a custom-designed set of cDNA templates (Pharmingen) with an in vitro transcription kit (Ribouquant, Pharmingen). Each template had a unique length and represented a distinct sequence in human mRNAs for TNF-α, GM-CSF, IL-1β, MCP-1, IL-8, TGF-β, GAPDH, and L-32 ribosomal RNA. The radiolabeled antisense probes were hybridized to equal amounts of total RNA (2 μg) and subjected to RNase digestion with an RNase protection assay kit (Ribouquant, Pharmingen). The RNase-protected fragments were purified, resolved on 4.75% denaturing polyacrylamide gels (National Diagnostics), and autoradiographed. The protected fragments, which migrated slightly ahead of their respective undigested marker probes, were identified by extent of migration in comparison to the sizes of undigested marker probes. The relative intensities were quantified by densitometry or phosphorimaging and normalized to GAPDH and L-32 mRNA. Yeast tRNA served as a negative control.

Measurement of MCP-1 and IL-8 Protein Release From HAECs

HAEC cultures were treated with or without dl-homocysteine or other test compounds for up to 8 hours. MCP-1 and IL-8 protein concentrations were determined in the culture supernatants by ELISA (Quantikine, R&D Systems) and were normalized to total cell protein, which was measured with the BCA protein assay kit (Pierce). Basal concentrations of MCP-1 and IL-8 in the medium from untreated cells were subtracted from the concentrations found in the treated cells at each time point. In some experiments, cells were pretreated with cycloheximide (10 μg/mL) for 30 minutes and then exposed to dl-homocysteine.

MCP-1 Chemotaxis Assays

Chemotaxis in response to MCP-1 was studied both in the human histiocytic lymphoma cell line U937 (ATCC CRL 1593) and in normal human peripheral blood mononuclear cells (PBMCs). Monocytes were isolated from donor blood by Ficoll-Paque (Pharmacia) density gradient centrifugation, followed by adherence to serum-coated culture flasks.12 Adherent cells were detached, washed, resuspended in medium 199, and used in the chemotaxis assays. HAEC cultures were treated with 0, 50, or 500 μmol/L dl-homocysteine or 400 U/mL TNF-α (Boehringer Mannheim) for 2 to 5 hours. Culture medium was removed and transferred to the lower compartment of 48-well Boyden chambers (Neuroprobe) (for U937) or 96-well Chemotx microplates (Neuroprobe) (for monocytes). The Boyden chamber was assembled with polycarbonate filters (8-μm pore size) and preblocked with FCS. Purified PBMCs or U937 cells (1×104 to 2.5×105/50 μL) were added to the upper compartment and incubated for 90 minutes at 37°C. Monocytes adhering to the top surface of the filter were scraped off, and those migrating to the lower chamber and adhering to the underside of the filter were fixed, stained (Hema 3 stain kit, Fisher), and counted (5 fields/well). The U937 cells that had migrated to the lower chamber were counted with a hemocytometer. Conditioned medium from untreated HAECs supplemented (1 to 500 ng/mL) with recombinant human MCP-1 (rhMCP-1, R&D Systems) and medium from TNF-α–treated HAECs served as positive control. Anti–human MCP-1 polyclonal antibody (R&D Systems) was added (70 μg/mL) to culture medium for 30 minutes to neutralize the secreted MCP-1 and to assess MCP-1–specific chemotaxis. Normal rabbit IgG was used as negative control. Medium from untreated cells was used to determine basal migration.

Statistical Methods

Results are expressed as mean±SEM. Probability values were calculated by 1-way ANOVA with Sigmasstat software (Tukey’s test or Fisher’s least significant difference test). In those instances in which the data were not normally distributed, the data were subjected to logarithmic transformation before the parametric analysis. Significant differences were said to exist at a value of P<0.05. Each data point represents the average of 3 or 4 experiments.

Results

Effect of Homocysteine on Cytokine mRNA Expression

To determine whether homocysteine modulates the expression of MCP-1 mRNA, HAEC cultures were treated with 500 μmol/L dl-homocysteine, and total RNA was isolated at various times from 0.5 to 24 hours. Northern blot analysis revealed that homocysteine induced the expression of MCP-1 steady-state mRNA above basal levels within 1 hour, was maximal between 2 and 4 hours, and then declined to near baseline by 24 hours (Figure 1A). Thus, steady-state MCP-1 mRNA levels were significantly increased in homocysteinetreated HAECs compared with untreated cells.

Because endothelial cells express a variety of cytokines that could play roles in atherogenesis, we tested the effect of homocysteine on expression of TNF-α, GM-CSF, IL-1β, IL-8, and TGF-β mRNAs. HAECs were treated with increasing concentrations of dl-homocysteine (10 μmol/L to 10 mmol/L) for 3 hours. Total RNA was extracted and

kines important in recruitment of leukocytes to sites of vascular injury.

We show that pathophysiological concentrations of homocysteine induce mRNA expression and trigger protein release for monocyte chemoattractant protein-1 (MCP-1) and inter-leukin (IL)-8 in cultured HAECs. The expression of tumor necrosis factor-α (TNF-α), granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-1β, and transforming growth factor-β (TGF-β) was unaffected by homocysteine. Stimulated expression and release of MCP-1, a chemokine for monocytes, and IL-8, a chemokine for T lymphocytes and neutrophils, suggest that homocysteine may alter endothelial cell function and promote atherosclerosis.
Homocysteine and Chemokine Expression

To differentiate between the abilities of the D and L enantiomers of homocysteine to induce chemokine expression, HAECs were treated with 50 μmol/L L-homocysteine, D-homocystine, or DL-homocysteine for 3 hours. As shown in Figure 2B, D-homocysteine did not significantly induce MCP-1 or IL-8 mRNA expression, whereas L-homocysteine induced both messages, as did D-homocysteine.

Effect of Other Sulfur Compounds on Cytokine Expression

To rule out the possibility of a “general thiol effect” on the induction of MCP-1 and IL-8 mRNA, L-cysteine, D-homocysteine, and L-methionine were tested for their ability to induce cytokine expression. All compounds were studied at 50 μmol/L, the concentration of D-homocysteine that gave near-maximal expression for MCP-1 and IL-8 mRNAs. After 3 hours of treatment, there was no increase in steady-state mRNA levels for MCP-1 or IL-8 with either L-cysteine, D-homocysteine, or L-methionine (Figure 2A). In addition, these sulfur metabolites had no effect on the expression of TNF-α, GM-CSF, IL-1β, and TGF-β. The nonbiological thiol β-mercaptoethanol (50 μmol/L) induced MCP-1 mRNA expression 7-fold while only marginally increasing IL-8 expression (Figure 2A). Both MCP-1 and IL-8 mRNAs were induced by 50 μmol/L hydrogen peroxide, as observed previously.13,14

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Effect of Homocysteine on MCP-1 and IL-8 Protein Release From HAECs

Dl-Homocysteine (50 μmol/L) promoted the release of MCP-1 and IL-8 protein from HAECs within 15 minutes (Figure 3A and 3B). A time-dependent accumulation (6- to 8-fold above baseline) occurred, with MCP-1 release plateauing within 2 to 4 hours and IL-8 within 7 to 8 hours (Figure 3C and 3D). With 500 μmol/L Dl-homocysteine, there was a slightly greater release of both chemokines from HAECs immediately after treatment (Figure 3B and 3D). Continued treatment with high concentrations of Dl-homocysteine for 4 to 8 hours, however, did not cause further accumulation of MCP-1 and IL-8 in the culture medium (Figure 3B and 3D). Other sulfur compounds (50 μmol/L of L-cysteine, L-methionine, L-homocysteine) did not affect MCP-1 and IL-8 release (Figure 3A and 3B). Release was also specific for L-homocysteine (Figure 2B, bottom). These studies demonstrate that low concentrations of homocysteine not only

analyzed by multiprobe RNase protection assays. As shown in Figure 1B, MCP-1 and IL-8 mRNAs were induced by homocysteine, whereas TGF-β mRNA, which was expressed in control HAECs, remained unaffected by homocysteine treatment. TNF-α, GM-CSF, and IL-1β mRNAs were not expressed by HAECs in the presence or absence of homocysteine. Thus, homocysteine appears to be selective in its ability to upregulate cytokine expression in these cells.

After normalization against L-32 ribosomal RNA and GAPDH mRNA, we observed that as little as 10 μmol/L homocysteine induced the expression of MCP-1 mRNA above control (Figure 1B). Interestingly, the maximum level of MCP-1 mRNA expression (3-fold) was observed with 50 μmol/L Dl-homocysteine. Higher levels caused a progressive decline in expression (Figure 1B). The dose-dependent effect of homocysteine on IL-8 mRNA expression was quite different (Figure 1B). There was a significant elevation of IL-8 mRNA in cells treated with 10 μmol/L homocysteine, reaching maximal expression at 50 μmol/L. Unlike the expression of MCP-1 mRNA, however, the level of IL-8 mRNA remained high at all concentrations >50 μmol/L (Figure 1B). The significance of these observations is that relatively low concentrations of Dl-homocysteine, within the pathophysiological concentration range, can modulate the expression of 2 proinflammatory chemokines, MCP-1 and IL-8, in cultured HAECs.
upregulate steady-state levels of mRNA for MCP-1 and IL-8 but also trigger the release of the respective proteins from HAECs.

To determine whether homocysteine stimulates de novo synthesis of MCP-1 and IL-8, HAECs were incubated with cycloheximide for 30 minutes before treatment with 50 μmol/L DL-homocysteine. As shown in Figure 3E, cycloheximide treatment alone caused a small increase in MCP-1 protein release. The stimulation of MCP-1 protein release by DL-homocysteine, however, was dramatically inhibited with cycloheximide pretreatment. This result suggests that homocysteine can enhance the translation of MCP-1 protein in HAECs. A similar inhibition was observed on homocysteine-induced IL-8 protein release from HAECs with cycloheximide pretreatment (data not shown).

**Chemotactic Activity of Secreted MCP-1**

Migration of PBMCs and U937 cells toward media from DL-homocysteine-treated HAECs (50 μmol/L) increased ~4.5- and 1.8-fold, respectively, compared with media from untreated cells (Figure 4A and 4C). Media from HAECs treated with 500 μmol/L DL-homocysteine for 2 hours showed a similar increase in migration of U937 cells. Migra-
Discussion

There is considerable evidence that atherogenesis is an inflammatory process. Cytokines play critical roles as multipotent mediators of inflammation by modulating key functions of vascular cells. In this report, we show that homocysteine induces both mRNA expression and protein secretion of the proinflammatory cytokines MCP-1 and IL-8 in cultured HAECs. Other cytokines studied were unaffected by homocysteine. The effect is specific for L-homocysteine, and most significantly, the secreted MCP-1 is chemotactically active. Because MCP-1 and IL-8 are major chemokines for leukocyte trafficking and have already been identified in atheromatous plaques, our findings suggest a potential role for homocysteine in atherogenesis and the progression of vascular disease. Thus, homocysteine appears to work through a mechanism involving upregulation of MCP-1 and IL-8 expression and recruitment of leukocytes across an inflammatory site in the vascular endothelium.

The concentrations of L-homocysteine (10 to 50 μmol/L) used in this study are comparable to pathophysiological levels observed in homocystinuric subjects (up to 100 μmol/L). Recent studies have reported cellular responses that are induced by clinically relevant levels of homocysteine (up to 100 μmol/L) but not by L-cysteine. Wang et al showed that homocysteine inhibited endothelial cell proliferation, presumably after its conversion to S-adenosylhomocysteine and subsequent hypomethylation of p21WAF1. Hajjar’s group reported that homocysteine, but not cysteine, inhibited the binding of tissue plasminogen activator to annexin II, probably by mechanisms involving thiolation of Cys9. Lipton et al found that 10 to 100 μmol/L L-homocysteine in the presence of elevated glycine stimulated N-methyl-d-aspartate receptors in neurons, resulting in neurotoxicity from excessive Ca2+ influx and generation of reactive oxygen species. We also show that clinically relevant concentrations of L-homocysteine (10 to 50 μmol/L) promote MCP-1 and IL-8 expression and secretion.

The induction of MCP-1 and IL-8 expression appears to be specific for L-homocysteine, because L-homocysteine, an oxidized form of homocysteine, and L-methionine, the precursor of homocysteine, did not induce chemokine expression. L-Cysteine was also ineffective. Could the atherogenicity of homocysteine be related to the high pK of its sulfhydryl group? The higher pK of homocysteine relative to cysteine suggests greater reactivity in nucleophilic exchange reactions. This may explain the tolerance of such high levels of cysteine in vivo. Although β-mercaptoethanol induces expression of MCP-1 but not IL-8, it should be noted that the thiol pKs of homocysteine and β-mercaptoethanol are similar, and this could also relate them mechanistically. The fact that β-mercaptoethanol was a more potent inducer than homocysteine could be explained by its greater transportability than homocysteine and its ability to activate nuclear factor-kB. Additional studies are needed to address the possibility that homocysteine-induced chemokine expression, too, may be mediated by activation of specific transcription factors.

We also demonstrate that L-homocysteine promotes a substantial release of MCP-1 and IL-8 protein from HAECs. Inhibition of chemokine release by cycloheximide shows that homocysteine stimulates the translation of MCP-1 and IL-8, whereas the rapidity of the release indicates that homocysteine may also trigger chemokine secretion from endogenous stores. Cycloheximide treatment for 1 hour reduces homocysteine-induced MCP-1 release dramatically, to ~25% of the value seen with cycloheximide alone. This demonstrates that MCP-1 release requires de novo protein synthesis, although there is likely to be a small contribution from endogenous stores. Thus, the homocysteine-induced mRNA levels probably reflect an upregulation of transcription or increased mRNA stability.

To explain mechanistically the induction of MCP-1 and IL-8 expression by homocysteine in HAECs, 2 possibilities should be considered. First, H2O2 and other reactive oxygen species that are generated extracellularly during auto-
oxidation of homocysteine could mediate the observed effects through oxidative stress. In fact, as we and others have found, both MCP-1 and IL-8 mRNA expression can be triggered by reactive oxygen species, including H2O2. Thus, by use of supraphysiological homocysteine concentrations (>1 mmol/L), auto-oxidation could result in oxidative stress, thereby inducing chemokine expression. We found, however, that at high homocysteine concentrations (ie, >50 μmol/L), MCP-1 mRNA expression actually declined. Furthermore, our observation that L-homocysteine but not D-homocysteine induces MCP-1 and IL-8 expression suggests that the mechanism must involve ≈1 stereospecific step. Trace-metal–catalyzed auto-oxidation of thiols, including D- and L-homocysteine, is likely to be nonstereospecific. Thus, if the mechanism of chemokine induction involves solely the generation of extracellular reactive oxygen species, then both D- and L-homocysteine should have similar effects. Also, in mild hyperhomocysteinemia, in which concentrations of plasma total homocysteine usually do not exceed 30 μmol/L, the amount of H2O2 generated would probably be of little consequence, given the overwhelming antioxidant defense system in the blood. Although this argues against the oxidative stress hypothesis, additional studies with catalase and superoxide dismutase are needed. Alternatively, thiols themselves can act directly within the cell to induce gene expression. Two facets of this study support the hypothesis that homocysteine is also acting within the cell on as yet unidentified molecular targets. First, we were able to induce chemokine expression at relatively low homocysteine concentrations (10 to 50 μmol/L), but we found that equimolar concentrations of cysteine were ineffective. Second, because the D enantiomer of homocysteine did not induce chemokine expression, stereospecific processes such as transport and/or enzyme catalysis may be involved in L-homocysteine–induced expression of MCP-1 and IL-8. Homocysteine molecular targeting may occur through alteration of the redox status within the cell. A reduction in intracellular glutathione and decreased activity and expression of glutathione peroxidase after homocysteine treatment may lead to the expression of redox-sensitive transcription factors. Homocysteine also induces endoplasmic reticulum stress in endothelial cells by a mechanism that may involve altered redox potential. We are currently investigating altered redox status in homocysteine-treated endothelial cells as a mechanism for increased chemokine expression. Recent evidence suggests that in addition to activation and chemotaxis of neutrophils, IL-8 promotes directed migration of T lymphocytes. Both neutrophils and T lymphocytes are important players in inflammation and pathogenesis of atherosclerosis. MCP-1 is known to induce migration of monocytes at subnanomolar concentrations and to recruit a subset of T cells. Because the gradient for MCP-1 is soluble rather than haptotactic, it would be difficult to maintain high levels of the protein on the luminal surface of the endothelial cells because of blood flow in vivo. Therefore, the MCP-1 accumulating at the abluminal surface of the endothelium after activation establishes an MCP-1 gradient across the endothelial monolayer, potentiating transendothelial migration of monocytes. This theory has been shown to be true in vitro. Accordingly, our finding that the MCP-1 released from homocysteine-activated endothelial cells is biologically active and capable of promoting chemotaxis provides a role for homocysteine in vascular disease. Because monocyte infiltration is one of the key events in atherogenesis, we propose that homocysteine induces the accumulation of MCP-1 and IL-8 and causes recruitment and directed migration of leukocytes through the endothelium. In summary, we have shown that homocysteine induces the expression of the proinflammatory chemokines MCP-1 and IL-8 in HAECS. Our findings suggest a novel role for homocysteine in the pathogenesis of vascular disease.

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