Stereospecific and Redox-Sensitive Increase in Monocyte Adhesion to Endothelial Cells by Homocysteine

Otilia Postea, Florian Krotz, Anna Henger, Christiane Keller, Norbert Weiss

Objective—Previous studies have shown that elevated homocysteine (Hcy) levels promote the development of atherosclerotic lesions in atherosclerosis-prone animal models. There is evidence that oxidant stress contributes to Hcy’s deleterious effects on the vasculature. The accumulation and adhesion of monocytes to the vascular endothelium is a critical event in the development of atherosclerosis. We investigated the effects of Hcy on the interaction between human endothelial cells (EC) (EC line EA.hy 926 and primary human umbilical vein EC [HUVEC]) and the monocytic cell line THP-1, and the impact of vascular oxidant stress and redox-sensitive signaling pathways on these events.

Methods and Results—L-Hcy, but not D-Hcy, increases the production of reactive oxygen species inside EC, enhances nuclear factor(NF)-κB activation, and stimulates intercellular adhesion molecule-1 (ICAM-1) RNA transcription and cell surface expression. This leads to a time- and dose-dependent increase in monocyte adhesion to ECs. Pretreatment of ECs with superoxide scavengers (MnTBAP and Tiron) or with an inhibitor of NF-κB activation abolished Hcy-induced monocyte adhesion, ICAM-1 expression, and nuclear translocation of NF-κB.

Conclusions—These findings suggest that reactive oxygen species produced under hyperhomocysteinemic conditions may induce a proinflammatory situation in the vessel wall that initiates and promotes atherosclerotic lesion development.

Key Words: homocysteine ■ endothelial dysfunction ■ reactive oxygen species ■ ICAM-1 ■ NF-κB

A key event in the vascular pathobiology associated with hyperhomocysteinemia is the induction of endothelial dysfunction.1 This can be detected by impaired endothelium-dependent vasodilator function in animal models of mild hyperhomocysteinemia2–4 and in patients with either acutely5 or chronically6 elevated plasma homocysteine (Hcy) levels. It indicates a reduction in bioavailable nitric oxide (NO). This is thought to be caused by increased vascular oxidant stress under hyperhomocysteinemic conditions leading to inactivation of NO and/or to elevated plasma levels of the NO synthase inhibitor asymmetrical dimethylarginine leading to decreased synthesis.8,9 Endothelial dysfunction not only impairs regulation of vasomotion but also affects the regulation of interactions of the endothelium with circulating inflammatory cells, of endothelium-dependent thrombotic and fibrinolytic mechanisms, and of cell growth within the vessel wall.10

Recently it has been shown that exposure of cultured endothelial cells (ECs) to Hcy leads to endothelial activation resulting in increased expression of chemokines11 and adhesion molecules.12–14 Furthermore, increased P-selectin expression by activated EC and/or platelets has been shown in plasma and aortic sections of mildly hyperhomocysteinemic heterozygous cystathionine β synthase-deficient mice.4 In-duction of hyperhomocysteinemia in apolipoprotein E-null mice enhanced the expression of receptors for advanced glycation end products, vascular cell adhesion molecule (VCAM)-1, E-selectin, tissue factor (TF), and matrix metalloproteinase (MMP)-9 in the vasculature.12,15 These molecular events may increase the chemotaxis, adhesion, and transmigration of mononuclear cells to the vessel wall and promote atherosclerotic lesion development. Induction of hyperhomocysteinemia has been shown to promote the development of atherosclerotic lesions and increase their complexity in atherosclerosis-prone mouse models.15–17

The adhesion of monocytes to dysfunctional ECs is a prerequisite for the development and progression of atherosclerotic lesions, because these monocytes may migrate into the subendothelial space where they differentiate into macrophages18,19 and endocyte-activated forms of low-density lipoprotein (LDL) via scavenger receptors to form foam cells, the hallmark of early atherosclerotic lesions.18 Whether incubation of ECs with Hcy results in increased monocyte adhesion to EC has not been shown conclusively as yet.

We examined the effects of Hcy on the interaction of monocytes with cultured ECs and exploited possible molecular mechanisms, especially the impact of vascular oxidant stress and the activation of redox-sensitive signaling events.
Materials and Methods

A detailed Methods section is available at http://atvb.ahajournals.org.

Cell Lines

The human EC line, EA.hy 926 cells, and primary human umbilical vein ECs (HUVECs) were used as models for vascular ECs. The human monocytic leukemia cell line THP-1 was used as a model for monocytes.

Static Adhesion Assay

The adhesion of THP-1 cells to ECs was studied under static conditions as described previously. Briefly, confluent ECs were cultured on cover slides and treated with the test substances. Cells were then fixed with formalin (2%) and permeabilized with submersion in 0.2% Triton X-100. Samples were incubated with the primary antibody against p65 and with the secondary antibody linked to fluorescein isothiocyanate (FITC). Fluorescence intensities were detected using a confocal microscope (LSM 410 Invert; Zeiss) and monitored using a confocal laser scanning microscope. The cellular distribution of p65 was measured as the ratio of its fluorescence in nucleus/cytoplasm.

Flow Cytometry

The expression levels of the adhesion molecules ICAM-1, VCAM-1, E-Selectin, and PECAM-1 on EA.hy 926 cells and HUVECs were quantified by flow cytometry.

Immunofluorescent Detection of NF-κB Translocation

Activation of NF-κB was determined by assessing the distribution of its subunit p65 between cytoplasm and the nucleus of EC in immunofluorescence images as described. Briefly, confluent ECs were cultured on cover slides and treated with the test substances. Cells were then fixed with formalin (2%) and permeabilized by submersion in 0.2% Triton X-100. Samples were incubated with the primary antibody against p65 and with the secondary antibody linked to fluorescein isothiocyanate (FITC). Fluorescence intensities were detected using a confocal microscope (LSM 410 Invert; Zeiss) and the cellular distribution of p65 was measured as the ratio of its fluorescence in nucleus/cytoplasm.

Real-Time Reverse-Transcription Polymerase Chain Reaction

Total RNA isolated from EA.hy926 cells was reverse-transcribed. Quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR) was performed on a TaqMan ABI 7700 Sequence Detection System (Applied Biosystems) as described previously using oligonucleotide primers specific for human ICAM-1 cDNA and for 18S rRNA and cyclophilin A cDNAs as housekeeping genes.

Fluorescent Measurement of Intracellular Reactive Oxygen Species

The intracellular generation of reactive oxygen species (ROS) was evaluated by loading pretreated EA.hy 926 cells with 6-carboxy-2',7'dichlorofluorescein diacetate (DCF-DA). Its oxidative conversion to the fluorophore dichlorofluorescein (DCF) was monitored using a confocal laser scanning microscope. The intracellular generation of ROS was evaluated by loading pretreated EA.hy 926 cells with 6-carboxy-2',7'dichlorodihydrofluorescein diacetate (DCF-DA). Its oxidative conversion to the fluorophore dichlorofluorescein (DCF) was monitored using a confocal laser scanning microscope.

Statistical Analysis

Data are expressed as means±SD. Differences in time- and dose-dependent responses between groups were analyzed with 2-way repeated measures ANOVA with post hoc analysis performed with Fisher’s PLSD and Bonferroni/Dunn procedures. Other data were analyzed by factorial ANOVA and post hoc comparisons. Differences were considered significant when the error level was P<0.05.

Results

Time- and Dose-Dependent Increase in Monocyte Adhesion to Hcy-Infused ECs

Incubation of EA.hy 926 cells with 200 μmol/L D,L-Hcy for 0.5 to 24 hours resulted in a time-dependent increase in the number of adhering THP-1 cells compared with baseline THP-1 monocyte adhesion to unstimulated EA.hy 926 monolayers (baseline: 7.7±1.6 cells per microscopic field). The effect of Hcy on monocyte adhesion was statistically significant (P<0.05) from baseline between 3 and 8 hours of incubation, peaked after 6 hours (28.5±5.9 cells per microscopic field), and declined thereafter (n=3 to 5 experiments). All further experiments were performed with 6 hours of incubation.

Incubation of EA.hy 926 cells with increasing concentrations of D,L-Hcy leads to a dose-dependent increase in adherent monocytes. L-Cys had no effect. TNF-α (50 ng/mL) was used as a positive control. N=5 experiments; *P<0.05 vs control.

Figure 1. Adhesion of THP-1 monocytes to Hcy-incubated ECs. Six hours of incubation of EA.hy 926 cells with increasing concentrations of D.L-Hcy leads to a dose-dependent increase in adherent monocytes. L-Cys had no effect. TNF-α (50 ng/mL) was used as a positive control. N=5 experiments; *P<0.05 vs control.

Stereospecific Effect of L-Hcy on Monocyte Adhesion to ECs

To examine whether the effect of Hcy is stereospecific for the naturally occurring L-isoform, or independent from its stereoisomeric form, EA.hy 926 cells were incubated with 200 μmol/L D,L-Hcy, L-Hcy, or D-Hcy. D,L-Hcy, L-Hcy, and D-Hcy dose-dependently and significantly increased monocyte adhesion to EC up to almost 5-fold compared with control. In contrast, D-Hcy and L-Cys had no significant effect (Figure II, available online at http://atvb.ahajournals.org).

Adhesion Molecule Expression on Hcy-Stimulated ECs

The endothelial expression of adhesion molecules, like “CAMs” or “selectins,” mediates monocyte adhesion to ECs. To explore which adhesion molecule might be involved in Hcy-induced monocyte adhesion to ECs, the expression of different adhesion molecules on ECs was studied in untreated and Hcy-incubated EC (200 μmol/L for 6 hours) using fluorescence-activated cell sorter (FACS) analysis. D,L-Hcy and L-Hcy–incubated EA.hy 926 cells and D,L-Hcy–incubated HUVECs showed a significant increase in ICAM-1 protein expression (Table I). This effect was specific for Hcy but not for other thiols, and was specific for the L-stereoisomer of Hcy, as L-Cys and D-Hcy, respectively, had no effect. VCAM-1, PECAM-1, or E-selectin protein expression was not significantly affected.
cyte adhesion to EA.hy 926 cells (Figure 2) and HUVEC

Blocking ICAM-1 significantly reduced Hcy-induced mono-

antibody against ICAM-1 or an isotype matched control

D,L-Hcy for 6 hours, followed by incubation with a blocking

expression were not stimulated by any Hcy-species used (data

To examine whether increased ICAM-1 protein expression on EA.hy 926 cells is regulated on a transcriptional level, ICAM-1 mRNA levels were monitored by real-time RT-PCR. Incubation of ECs with L-Hcy (200 μmol/L) for 6 hours led to a significant increase in ICAM-1 mRNA levels compared with control (131 ± 7% of control; n=4 experiments; P<0.05). Incubation with L-Cys had no effect (108 ± 8% of control; n=4 experiments).

To confirm the functional relevance of increased ICAM-1 expression on monocyte adhesion to Hcy-incubated ECs, EA.hy 926, and HUVECs were incubated with 200 μmol/L D,L-Hcy for 6 hours, followed by incubation with a blocking antibody against ICAM-1 or an isotype matched control antibody, and monocyte adhesion assays were performed. Blocking ICAM-1 significantly reduced Hcy-induced monocyte adhesion to EA.hy 926 cells (Figure 2) and HUVEC (Figure III, available online at http://atvb.ahajournals.org).

TABLE 1. Relative ICAM-1 Expression on EA.hy 926 Cells and on HUVECs as Determined by Flow Cytometry

<table>
<thead>
<tr>
<th>Condition</th>
<th>ICAM-1 Expression (%) Control on EA.hy 926 Cells</th>
<th>ICAM-1 Expression (%) Control on HUVECs</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>D,L-Hcy 200 μmol/L</td>
<td>129.9±18.4*</td>
<td>172.3±36.4*</td>
</tr>
<tr>
<td>L-Hcy 200 μmol/L</td>
<td>145.6±29.7*</td>
<td></td>
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<tr>
<td>D-Hcy 200 μmol/L</td>
<td>101.0±30.7</td>
<td></td>
</tr>
<tr>
<td>L-Cys 200 μmol/L</td>
<td>109.1±14.5</td>
<td>119.7±12.5</td>
</tr>
<tr>
<td>D,L-Hcy 200 μmol/L+Tiron 4 mmol/L</td>
<td>102.4±6.5†</td>
<td>116.0±1.1†</td>
</tr>
<tr>
<td>D,L-Hcy 200 μmol/L+MnTBAP 50 μmol/L</td>
<td>99.6±5.7†</td>
<td></td>
</tr>
<tr>
<td>D,L-Hcy 200 μmol/L+Bay 11-7082 10 μmol/L</td>
<td>97.2±4.1†</td>
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</table>

N=4 to 6 experiments. *P<0.05 vs control; †P<0.05 vs D,L-Hcy.

Dose-Dependent Generation of Intracellular Reactive Oxygen Species in Hcy-Incubated ECs

Because the adverse effects of Hcy on endothelial function are thought to be mediated at least partly via increased vascular oxidant stress,7 we monitored the intracellular generation of ROS using the redox-sensitive dye DCF-DA.

Incubation of EA.hy 926 cells with increasing concentrations of D,L-Hcy (20 to 200 μmol/L) resulted in a dose-dependent and significant increase in intracellular fluorescence indicative of increased generation of ROS. L-Cys (200 μmol/L) had no effect (Figure 3).

Enhanced NF-κB Translocation in Hcy-Incubated ECs

To further evaluate the signaling pathway involved in increased adhesion molecule expression, NF-κB activation in Hcy-incubated EC was monitored by measuring the translocation of the NF-κB p65 subunit from the cytosol to the nucleus using immunofluorescence techniques. Incubation of EA.hy 926 cells with 200 μmol/L D,L-Hcy resulted in a significantly increased nuclear staining for the p65 subunit. This is shown by a 1.6-fold increase in the fluorescence ratio between nucleus and cytosol in Hcy-treated compared with control cells. In contrast, L-Cys had no effect (Figure 4A). Increased NF-κB activation in Hcy-incubated EC could be reproduced using HUVECs (Figure 4C). The specificity of the increased nuclear staining with an antibody against the p65 subunit of NF-κB could be confirmed using the synthetic inhibitor of NF-κB translocation Bay 11-7082 in combination with Hcy (Figure 4C).

Effect of Scavenging Superoxide Anion on Hcy-Induced Monocyte Adhesion, Adhesion Molecule Expression, and NF-κB Translocation

To examine whether scavenging superoxide anion prevents Hcy-induced monocyte adhesion, endothelial ICAM-1 expression, and NF-κB translocation, EA.hy 926 cells were coincubated with Hcy and either the superoxide scavengers MnTBAP (50 μmol/L) or Tiron (4 mmol/L) for 6 hours. Hcy incubation of ECs resulted in a significant increase in adhering monocytes. This could be completely abolished by coincubation with both superoxide scavenger (Figure IV, available online at http://atvb.ahajournals.org). These findings could be reproduced in HUVECs (see http://atvb.ahajournals.org).

FACS analysis showed that both Tiron and MnTBAP prevented Hcy-induced ICAM-1 expression in ECs (Table 1), whereas Tiron and MnTBAP had no effect on ICAM-1 expression in control cells (data not shown). Finally, Tiron and MnTBAP ablished the stimulatory effect of Hcy on NF-κB translocation in ECs (Table 2).

Inhibition of NF-κB Translocation by Bay 11-7082 Prevents Hcy-Induced NF-κB Activation, ICAM-1 Expression, and Monocyte Adhesion to HUVECs

To further confirm that increased NF-κB activation mediates increased ICAM-1 expression on ECs and increased monocyte adhesion to ECs induced by Hcy, HUVECs were incubated with D,L-Hcy and the inhibitor of NF-κB activa-
tion, Bay 11-7082 (10 μmol/L). Pharmacological inhibition of NF-κB activation completely suppressed Hcy-induced NF-κB translocation (Table 2), ICAM-1 expression (Table 1) and adhesion of monocytes to HUVECs (9.7±0.4 adherent monocytes per microscopic field on HUVEC incubated with Hcy only; n=3 experiments, P<0.05).

Figure 3. Dose-dependent increase in reactive oxygen species in Hcy-incubated ECs as visualized by intracellularDCF-fluorescence using confocal laser microscopy. Six hours of incubation of EA.hy 926 cells with D,L-Hcy dose-dependently increased intracellular fluorescence. L-Cys had no effect. The thrombin receptor-activating peptide (TRAP) (H-Ser-Phe-Leu-Leu-Arg-Asn-NH₂) was used as a positive control. The right panel shows the mean±SD fluorescence intensity of 3 experiments quantified by image analysis, and the left panel shows representative fluorescence microscopy images. Scale bar is 100 μm. N=3 experiments. *P<0.05 vs control.

Discussion
Atherosclerosis is viewed as an inflammatory process of the vessel wall that initiates and promotes lesion development. This process involves circulating leukocytes, particularly monocytes, that are recruited by and adhere to the activated endothelium, and then migrate into the subendothelial space where they differentiate into macrophages.18,19 These macrophages endocytose modified forms of low-density lipoprotein via scavenger receptors to form foam cells, the hallmark of fatty streak lesions. The lesions develop into fibro-fatty plaques, which contain large numbers of macrophages and some CD4⁺ T cells, and show evidence of smooth muscle cell migration and proliferation. These fibro-fatty plaques may develop into complex atherosclerotic lesions.

Elevated levels of Hcy are associated with an increased risk for atherosclerotic vascular diseases in humans.20 However, the mechanisms by which excess Hcy is harmful to the vasculature are not completely understood as yet. Very high concentrations of Hcy are toxic for ECs,30 whereas pathophysiological relevant concentrations as found in patients with mild hyperhomocysteinemia induce functional endothelial changes summarized as endothelial dysfunction.1 In addition, Hcy has been shown to promote the formation and increase the complexity of atherosclerotic lesions in atherosclerosis-prone animal models.15–17

In vitro studies have shown that Hcy is able to induce mRNA and protein expression of the proinflammatory cyto-

Figure 4. NF-κB translocation in Hcy-incubated EA.hy 926 cells (A) and HUVECs (B, C). Six hours of incubation of ECs with D,L-Hcy 200 μmol/L leads to increased NF-κB activation. This becomes evident by increased nuclear translocation indicated by increased nuclear staining with an anti-p65 antibody. TNF-α was used as positive control. L-Cys had no significant effect. The values are expressed as nucleus/cytosol ratio ± SD fluorescence intensity of 3 to 6 experiments. A. The top row represents fluorescence images, and the bottom row the corresponding transmission images of representative experiments. Each white scale bar represents a length of 10 μm. Control: 0.72±0.27; D,L-Hcy 200 μmol/L: 1.17±0.29; L-Cys 200 μmol/L: 0.87±0.22; TNF-α: 2.14±0.51. *P<0.05 vs control. B. Typical transmission image of a HUVEC cell layer, in which a cytosolically located region of interest (ROI) and a corresponding ROI with nuclear localization were randomly placed before mean fluorescence within these ROIs was recorded. Scale bar is 10 μm. C, Bar graph of mean nucleus/cytosol ratios of all experiments in HUVECs. *P<0.05 vs control.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Nuclear/Cyttoplasmic Ratio of Fluorescence Intensity After Staining With a p65 Antibody</th>
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<tbody>
<tr>
<td></td>
<td>in EA.hy 926 Cells</td>
</tr>
<tr>
<td>Control</td>
<td>0.72±0.27</td>
</tr>
<tr>
<td>D,L-Hcy 200 μmol/L</td>
<td>1.17±0.29*</td>
</tr>
<tr>
<td>D,L-Hcy 200 μmol/L+ Tiron</td>
<td>0.63±0.05†</td>
</tr>
<tr>
<td>D,L-Hcy 200 μmol/L+ MnTBAP</td>
<td>0.75±0.38†</td>
</tr>
<tr>
<td>D,L-Hcy 200 μmol/L+Bay 11–7082</td>
<td>0.77±0.01†</td>
</tr>
</tbody>
</table>

N=3 to 6 experiments.
*P<0.05 vs control; †P<0.05 vs D,L-Hcy.
kines monocyte chemoattractant protein-1 and IL-8 in cultured human aortic ECs (HAECs). In contrast, it had no effect on the expression of other cytokines, like tumor necrosis factor (TNF)-α, granulocyte-macrophage colony-stimulating factor, IL-1β, and transforming growth factor (TGF)-β. This cascade of events triggered increased recruitment of monocytes to ECs. The induction of chemokine expression is specific for Hcy, because equimolar concentrations of L-homocystine, L-Cys, and L-methionine had no effect on mRNA levels and protein release. Furthermore, L-Hcy induces chemokine expression, but D-Hcy does not, thus demonstrating enantiomeric specificity. In addition, Hcy has been shown to increase neutrophil adherence to EC in vitro and in vivo. This contact results in neutrophil migration across the endothelial layer, with concurrent damage and detachment of EC. This effect is mediated via binding of leukocyte β2-integrins (Mac-1, CD11b, LFA-1β, CD18) to ECs, because the interaction between EC and leukocytes could be abolished using blocking antibodies against these molecules, although the underlying mechanisms remained unclear.

Whether incubation of ECs with Hcy results in increased monocyte adhesion to ECs has not been shown conclusively as yet. Adhesion of U937 monocyte cells to IL-1β–stimulated, but not to unstimulated, HAECs was slightly increased when both ECs and monocytes were pretreated with Hcy. Our experiments have shown that incubation of ECs with pathophysiologically relevant concentrations of Hcy without any additional stimulation leads to a time- and dose-dependent increase in the adhesion of monocytes (Figure 1).

Previous studies have shown that Hcy stimulates the expression of VCAM-1 and E-selectin in Hcy-incubated HAEC and in aortas of hyperhomocysteinemic mice by mechanisms not elucidated so far. Our present experiments show that increased adhesion of monocytes to Hcy-stimulated ECs is mediated by increased ICAM-1 expression on ECs, shown both on a protein and mRNA level. The functional role of increased ICAM-1 expression on monocyte adhesion to EC has been confirmed by experiments in which a blocking antibody against ICAM-1 augmented Hcy’s effect (Figure 3).

NF-κB is a transcription factor that activates a variety of target genes relevant to the pathophysiology of the vessel wall. These include cytokines, chemokines, and leukocyte adhesion molecules (including ICAM-1). Physiological modulation and pathological activation of the NF-κB system may contribute to the changes in gene expression that occur during atherogenesis. Our experiments show that incubation of EA.hy 926 cells and HUVECs with Hcy results in a significantly increased nuclear staining for the NF-κB p65 subunit, demonstrating increased nuclear translocation. This cannot be obtained with other thiols. Activation of NF-κB by Hcy has previously been shown in several vascular cells including cultured vascular smooth muscle cells and endothelial cells, and in THP-1 macrophages. Increased activation of NF-κB associated with increased expression of ICAM-1 has recently been shown in aortas of mildly hyperhomocysteinemic rats, but not in controls. This confirms the relevance of our findings for the in vivo situation.

Elevated Hcy levels are associated with increased vascular superoxide output and vascular oxidant stress. This effect is specific for Hcy and does not occur with other low-molecular-weight thiols and is stereospecific for the naturally occurring L-isomer of Hcy. These findings seem to rule out extracellular production of ROS as a major source of oxidant stress under conditions of elevated Hcy levels. Both D- and L-Hcy undergo extracellular transition metal catalyzed autooxidation to the same extent, resulting in equivalent amounts of hydrogen peroxide produced. We have shown that incubation of ECs with the L-isomer of Hcy only modifies the endothelial phenotype in a way that promotes monocyte adhesion by a redox-sensitive pathway. This suggests that Hcy needs to be internalized (or synthesized intracellularly) in ECs to promote ROS production and monocyte adhesion, mostly likely involving enzymatic pathways in cellular ROS production. Previously we have shown that Hcy-induced production of ROS in ECs depends on endothelial NO synthase. Incubation of ECs with Hcy reduces levels of the NO synthase cofactor tetrahydrobiopterin. This results in “uncoupling” of NO synthase activity and production of superoxide anion instead of NO.

NF-κB is one of the transcription factors that may be controlled by the redox status of the cell. Generation of ROS seems to be a common step in signaling pathways that lead to IkB degradation and nuclear NF-κB accumulation. This concept is supported by a variety of studies: Diverse agents that can activate NF-κB also elevate levels of ROS. Chemically distinct antioxidants, as well as overexpression of antioxidant enzymes, can inhibit nuclear NF-κB translocation. However, a direct role of ROS in NF-κB activation remains to be proven.

Our experiments support the hypothesis that increased binding of monocytes to ECs induced by Hcy is mediated by increased vascular oxidant stress, NF-κB translocation, and ICAM-1 expression: Hcy incubation of ECs leads to increased generation of ROS (Figure 3). This has functional relevance as scavenging of ROS by antioxidants abolished Hcy-induced NF-κB translocation (Table 2), endothelial ICAM-1 expression (Table 1), and monocyte adhesion to ECs (Figure IV). The role of NF-κB in these redox-sensitive signaling events is further underlined by data that show that inhibition of NF-κB activation abolishes Hcy-induced endothelial ICAM-1 expression (Table 1) and monocyte adhesion to ECs.

In conclusion, these data indicate that increased vascular oxidant stress in hyperhomocysteinemia not only leads to a decrease in the bioavailability of NO but also activates redox-sensitive signaling pathways that induce a proinflammatory state in the vessel wall promoting adhesion molecule expression and monocyte recruitment. These may be additional mechanisms by which Hcy promotes the development of atherosclerotic lesions.

Acknowledgments

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References


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ONLINE DATA SUPPLEMENT – MATERIAL AND METHODS

Chemicals and Cell Culture Ware

D,L-Hcy and L-Hcy thiolactone hydrochloride were purchased from Sigma, D-Hcy thiolactone hydrochloride and L-cysteine (L-Cys) were from Fluka. L-Hcy and D-Hcy were synthesized from their respective thiolactones by hydrolysis in 0.1 mol/L NaOH. Final thiol concentrations were determined spectrophotometrically using Ellman’s reagent. Recombinant human tumor necrosis factor (TNF-α) and Tiron (4,5-Dihydroxy-1,3-benzenedisulfonic acid disodium salt pyrocatechol-3,5-disulfonic acid disodium salt) were from Sigma. MnTBAP (Mn(III)tetrakis (4-benzoic acid) porphyrin chloride) and BCECF/AM (2’,7’-bis-(2-carboxyethyl)-5-(6’)-carboxyfluorescein acetoxymethyl ester) were from Calbiochem and prepared as 1 mg/mL stock in dimethylsulfoxide (DMSO). Mouse anti-human monoclonal ICAM-1 (clone 8.4A6), E-selectin (clone1.2B6) and PECAM-1 (clone JC70A) antibodies and the respective murine IgG1 isotype control were from Sigma, monoclonal mouse anti-human VCAM-1 (clone 1.4C3) and rabbit anti-mouse IgG coupled with FITC were from DAKO. The thrombin-receptor activating peptide (TRAP, H-Ser-Phe-Leu-Leu-Arg-Asn-NH₂: 10µM) was purchased from Bachem. (E)-3-(4-Methylphenylsulfonyl)-2-propenenitrile (BAY 11-7082, 10 µmol/L in DMSO), an inhibitor of cytokine-induced IκB-α phosphorylation that blocks NF-κB activation² was purchased from Sigma.

- 1 -
Cell culture media (DMEM and RPMI-1640), Fetal Bovine Serum (FBS), and antibiotics (penicillin G sodium and streptomycin sulphate) were from Gibco BRL, distributed by Invitrogen. Accutase was obtained from PAA Laboratories. CellFix and CellWash buffer were from Becton Dickinson. All other reagents were from Sigma. Cell culture ware was obtained from Nunc Labware.

**Cell Lines**

EA.hy 926 cells\(^3\), derived by fusing human umbilical vein endothelial cells with the permanent human cell line A549, were a gift from Dr. Cora Edgell, University of North Carolina, Chapel Hill, NC, USA. Passages 30 to 40 were used in this study. These cells are widely used to study endothelial cell specific gene expression and endothelial cell - leukocyte interactions. EA.hy 926 cells express endothelial cell (EC) markers, adhesion molecules\(^4\) and secrete chemokines.\(^5,6\) Cells were maintained in DMEM containing 4,500 mg/L d-glucose, 10% heat-inactivated FBS, and antibiotics (100 units/mL penicillin G sodium and 100 µg/mL streptomycin sulfate). Culture plates were maintained in a humidified incubator at 37°C with a 5% CO\(_2\) atmosphere. Cells were subcultured after treatment with 0.05% trypsin and 0.53 mmol/L disodium EDTA. Cells were seeded in culture flasks or in 2-well cover slides, and allowed to grow to confluence before experimental treatment.

Human umbilical vein EC (HUVEC) were isolated and cultured in medium 199 mixed with EC growth medium (PromoCell; 1:1, vol/vol) containing 10% heat-inactivated FBS, and antibiotics as described previously.\(^7\) Passages two and three were used for the experiments. The human monocytic leukemia cell line, THP-1 (ATCC number TIB-202), was used as a model for monocytic cells.\(^8\) Cells were cultured in RPMI-1640 supplemented with 10% FBS and antibiotics (100 units/mL penicillin G sodium and 100 µg/mL streptomycin sulphate).
**Fluorescent Labeling of Cells**

For quantitative adhesion assays THP-1 cells were fluorescently labeled with BCECF/AM as previously described. Briefly, THP-1 cells were harvested and washed three times in serum-free Hank’s balanced salt solution (HBSS). $1 \times 10^7$ cells/mL were incubated with $1 \mu$mol/L BCECF/AM (prepared as 1 mg/mL stock in DMSO) for 30 min. at 37°C and 5% CO$_2$. Cells were then washed three times with HBSS containing 2 mmol/L Ca$^{++}$, 2 mmol/L Mg$^{++}$, 20 mmol/L HEPES and 5% FBS to remove excess dye and resuspended in RPMI-1640 supplemented with 5% FBS at a density of $5 \times 10^5$ cells/mL. Non-fluorescent BCECF/AM is lipophilic and is cleaved by intracellular esterase to yield the highly charged fluorescent BCECF that is retained by viable cells.

**Static Adhesion Assay**

Adhesion of THP-1 cells to EA.hy 926 cells and HUVEC was studied under static conditions. Briefly, EC were cultured to confluence in 2-well cover slides and treated with varying concentrations of D,L-Hcy, L-Hcy, D-Hcy and L-Cys, and the respective positive (TNF$\alpha$, 50 ng/mL) and negative controls (plain medium) for the indicated periods of time. After incubation, EC were washed twice with phosphate buffered salt solution containing calcium and magnesium (PBS$^{++}$). BCECF-labeled THP-1 cells ($5 \times 10^5$ cells/well) were coincubated with EC for 30 minutes at 37°C under slight agitation on a rotating platform. After incubation, non-adherent cells were removed by gentle washing of each well three times with Hank’s balanced salt solution (containing 2 mmol/L Ca$^{++}$, 2 mmol/L Mg$^{++}$, 20 mmol/L HEPES, HBSS$^{++}$). Adherent cells were fixed with 2% glutardialdehyde in HBSS$^{++}$ and visualized using a fluorescence microscope. Ten microscopic fields were randomly selected, photodocumented and fluorescent cells were counted.

In selected experiments, EC were incubated for 6 hours with Hcy and the respective controls, followed by 1 hour incubation with IgG1 (for blocking the unspecific sites) and another hour
with an anti-human ICAM-1 antibody (2 µg/mL) or the respective isotype control before addition of THP-1 cells. For studying the effects of scavenging superoxide anion, EC were simultaneously incubated for 6 hours with Hcy or plain medium, and with MnTBAP (50 µmol/L) or TIRON (4 mmol/L), respectively, before performing static adhesion assays.

**Flow Cytometry**

The expression levels of the adhesion molecules ICAM-1, VCAM-1, E-selectin, and PECAM-1 on EA.hy 926 cells were quantified by flow cytometry. After incubation with the varying test substances, cell monolayer were washed with PBS and incubated with Accutase until the cells were detached from the culture dish. Cells were treated with CellFix, and pelleted by centrifugation. After resuspension in CellWash buffer, cells were labeled with the respective FITC-labeled antibodies (anti-human ICAM-1, PECAM-1, E-Selectin, VCAM-1), washed, and the fluorescence of 10,000 cells was measured on a FACScan flow cytometer (Becton Dickinson). Data analysis was performed with CellQuest software (Becton Dickinson). The median of the specific fluorescence intensity was used as a marker for expression of the respective epitope. Nonspecific fluorescence was detected by using isotype-matched nonbinding antibodies and subtracted.

**Immunofluorescent Detection of NF-κB Translocation**

Activation of NF-κB was determined by assessing the distribution of its subunit p65 between cytoplasm and the nucleus of EA.hy 926 cells or HUVEC in immunofluorescence images as described previously. Confluent cells were cultured in 2-well cover slides and treated with the test substances as indicated. Cells were then fixed with formalin (2%) and subsequently permeabilized by submersion in 0.2% Triton X-100. The samples were then incubated with the primary antibody against p65 and finally treated with the secondary antibody linked to FITC. To quantify distribution of the p65 subunit between cytosol and nucleus of cells,
confocal images of cells were collected which allowed for distinction between the nucleus and the perinuclear region. In the microscopic transmission mode, five pairs of regions of interest (ROI) were placed in every image, of which one ROI was placed in the cytosolic compartment, the corresponding one in the nucleus of a cell. The size of a ROI was defined at 5µm^2. After placement of ROIs the microscopic mode was switched to fluorescence within the very same image. The mean relative fluorescence in every ROI was then measured and the value of the ROI within the nucleus divided by the value of the ROI in the corresponding cytosolic compartment. For every condition of an experiment 10 different images were analyzed and the mean value of the ratios counted as one experiment. Fluorescence intensities were detected using a confocal microscope (LSM 410 Invert, Zeiss) and the cellular distribution of p65 was measured as the ratio of its fluorescence in nucleus/cytoplasm.

**Real-time RT-PCR**

Total RNA was isolated from EA.hy 926 cells using the TRIZol Reagent (Gibco BRL). 1 µg of total RNA was reverse transcribed in a 40 µL reaction as previously described. Real-time RT-PCR was performed on a TaqMan ABI 7700 Sequence Detection System (AppliedBiosystems) using heat activated TaqDNA polymerase (Amplitaq Gold, Applied Biosystems), oligonucleotide primers specific for human ICAM-1 cDNA (forward primer: 5’ TGGCAACGACTCCTTCTCG and reverse primer: 5’ AGCTGTAGATGGTCACTGTCTGCA, 300 nmol/L each), and the dsDNA-binding dye SYBR Green I. For all quantitative analyses cDNA content of each sample was compared with another sample following the ΔΔCT technique or standard curves, respectively. 18S rRNA and cyclophilin A, which served as housekeeping genes, were amplified in parallel with the genes of interest. Primers for the genes of interest were designed in PrimerExpress® and searched against the public databases to confirm unique amplification products. Controls
consisting of ddH₂O were negative in all runs. All measurements were performed in duplicates.

**Fluorescent Measurement of Intracellular Reactive Oxygen Species**

The intracellular generation of reactive oxygen species was evaluated by monitoring the oxidative conversion of 6-carboxy-2’,7’-dichlorodihydrofluorescein diacetate (DCF-DA) to the fluorophore dichlorofluorescein (DCF)¹⁴ as described previously.¹⁵ Briefly, EA.hy 926 cells grown on cover slides, were incubated with the test substances for 6 hours. A thrombin-receptor activating peptide TRAP (H-Ser-Phe-Leu-Arg-Asn-NH₂; 10µM) was used as a positive control. After washing, cells were incubated in modified Tyrode’s buffer (135 mmol/L NaCl, 2.7 mmol/L KCl, 1.8 mmol/L CaCl₂, 0.28 mmol/L NaH₂PO₄, 0.49 mmol/L MgCl₂, 5.5 mmol/L D-Glucose, 20 mmol/L HEPES, pH = 7.40) containing 10 µmol/L DCF, for 15 minutes at 37°C in the dark. The fluorescence intensities (excitation 488 nm, emission >515 nm) were measured in several regions of interest using a confocal laser scanning microscope (LSM 410 Invert, Zeiss). Values obtained are expressed as arbitrary fluorescent units relative to control conditions.

**Statistical Analysis**

Values are reported as means ± SEM. Differences in time- and dose-responses between groups were tested with two-way repeated measures ANOVA with posthoc analysis performed with Fisher’s PLSD and Bonferonni/Dunn procedures. Other data were analyzed by factorial ANOVA and posthoc comparisons. Statistical significance was defined as a \( P \) value < 0.05.
References


Stereospecific and Redox-Sensitive Increase in Monocyte Adhesion to Endothelial Cells by Homocysteine

ONLINE DATA SUPPLEMENT - RESULTS

Figure I: Adhesion of THP-1 monocytes to Hcy-incubated HUVEC. Six hours incubation of HUVEC with of D,L-Hcy (200 µmol/L) lead to a four-fold increase in adherent monocytes whereas L-Cys had no effect. N = 3 experiments; control: 7.5 ± 0.2; D,L-Hcy 200 µmol/L: 25.3 ± 1.3 cells per microscopic field, respectively; n = 3 experiments; P < 0.05).
Figure II: Stereospecific effect of L-Hcy on monocyte adhesion to EC. Incubation of EA.hy 926 with different stereoisomers of Hcy only leads to significantly increased monocyte adhesion when using the naturally occurring L-Hcy, and D,L-Hcy, but not when using D-Hcy. TNF-α 50 ng/mL was used as positive control. N = 3 experiments; *P < 0.05 vs. Control.
**Figure III:** Effect of an blocking ICAM-1 antibody on Hcy-induced monocyte adhesion to HUVEC. Incubation of HUVEC with D,L-Hcy (200 µmol/L) leads to significantly increased monocyte adhesion to EC. This could be blocked by preincubation with an anti-ICAM-1 antibody. N = 3 experiments each; *P < 0.05 vs. Control; $P < 0.05$ vs. D,L-Hcy.
**Figure IV:** Coincubation of EA.hy 926 cells with D,L-Hcy and the superoxide scavengers MnTBAP or Tiron completely prevented Hcy-induced monocyte adhesion. N = 4 experiments; *P < 0.05 vs. Control, $P < 0.05 vs. D,L-Hcy.

This finding could be reproduced in HUVEC. Coincubation with D,L-Hcy and Tiron completely reversed Hcy-induced increase in monocyte adhesion to HUVEC (control: 7.5 ± 0.2 cells per microscopic field; D,L-Hcy 200 µmol/L: 25.3 ± 1.3 *; D,L-Hcy 200 µmol/L + Tiron 4 mmol/L: 9.7 ± 0.6 $; n = 3 experiments each; * P < 0.01 vs. control; $ P < 0.05 vs. D,L-Hcy).