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

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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. (***Arterioscler Thromb Vasc Biol.* 2006;26:508-513.)**

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 hyperhomocystinemia[2–4] and in patients with either acutely[5] or chronically[6] 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[7] 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 chemokines[11] 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] Induction 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 prerequirement for the development and progression of atherosclerotic lesions, because these monocytes may migrate into the subendothelial space where they differentiate into macrophages[18,19] and endocytose-modified 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.

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Materials and Methods

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

Cell Lines

The human EC line, EA.hy 926 cells,[20] and primary human umbilical vein ECs (HUVECs)[21] were used as models for vascular ECs. The human monocytic leukemia cell line THP-1[22] 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.[23]

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.[24] Briefly, confluent EC 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.[25] 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[26] 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′dichlorodihydrofluorescein diacetate (DCF-DA).[27] Its oxidative conversion to the fluorophore dichlorofluorescein (DCF) was monitored using a confocal laser scanning microscope.[28]

Statistical Analysis

Data are expressed as means±SD. Differences in time- and dose-responses between groups were analyzed with 2-way repeated measures ANOVA with posthoc analysis performed with Fisher's PLSD and Bonferoni/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-Incubated 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.

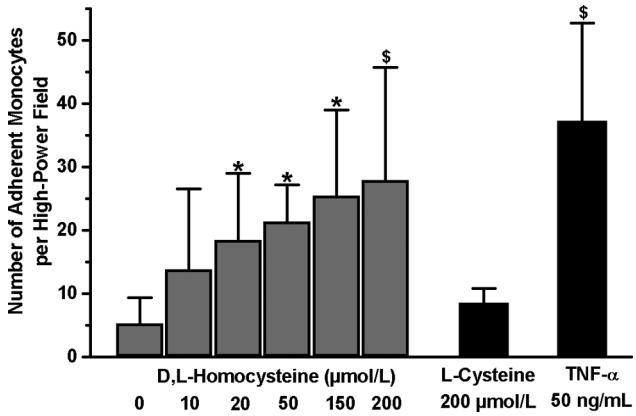

Figure 1. Adhesion of THP-1 monocytes to Hcy-incubated ECs. Six hours of incubation of EA.hy 926 cells with increasing concentrations of D,L-Hcy leads to a dose-dependent increase in adherent monocytes. L-Cys had no effect. TNF-α (50 ng/mL) was used as a positive control. N=5 experiments; *$P<0.05$ vs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

Incubation of EA.hy 926 cells with increasing concentrations of D,L-Hcy (10 to 200 μmol/L) for 6 hours led to a dose-dependent and significant increase in the number of adhering THP-1 cells to EA.hy 926 cells up to 5.3-fold at the highest concentration studied compared with control conditions. Incubation with L-cysteine (L-Cys) (200 μmol/L) had no significant effect. (Figure 1). This could be reproduced using HUVEC (Figure I, available online at http://atvb.ahajournals.org).

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 stereoisoform, EA.hy 926 cells were incubated with 200 μmol/L D,L-Hcy, L-Hcy, or D-Hcy. D,L-Hcy and L-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 1). 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

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

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

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

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

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).

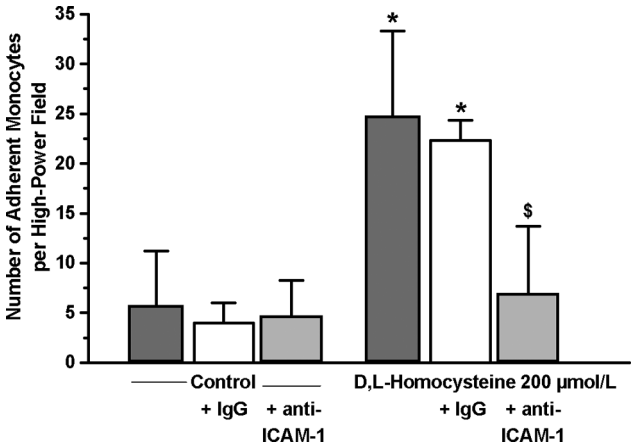

Figure 2. Effect of a blocking ICAM-1 antibody on Hcy-induced monocyte adhesion to ECs. Incubation of EA.hy 926 cells with D,L-Hcy (200 μmol/L) leads to significantly increased monocyte adhesion. This could be blocked by pre-incubation with an anti-ICAM-1 antibody. Isotype matched IgG had no effect. N=3 experiments each; *$P<0.05$ 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 anion scavengers (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 abolished 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-

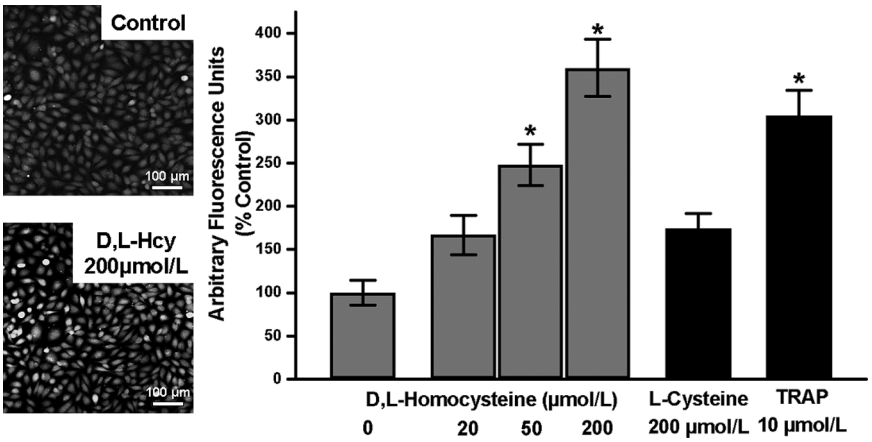

Figure 3. Dose-dependent increase in reactive oxygen species in Hcy-incubated ECs as visualized by intracellular DCF-fluorescence using confocal laser microscopy. Six hours of incubation of EA.hy 926 cells with D,L-Hcy dose-dependently increased intracellular fluorescence. L-Cys had no effect. The thrombin receptor-activating peptide (TRAP) (H-Ser-Phe-Leu-Leu-Arg-Asn-NH_2) 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.

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 coincubated with Hcy and Bay 11-7082 versus 25.3±1.3 adherent monocytes on HUVEC incubated with Hcy only; n=3 experiments, $P<0.05$).

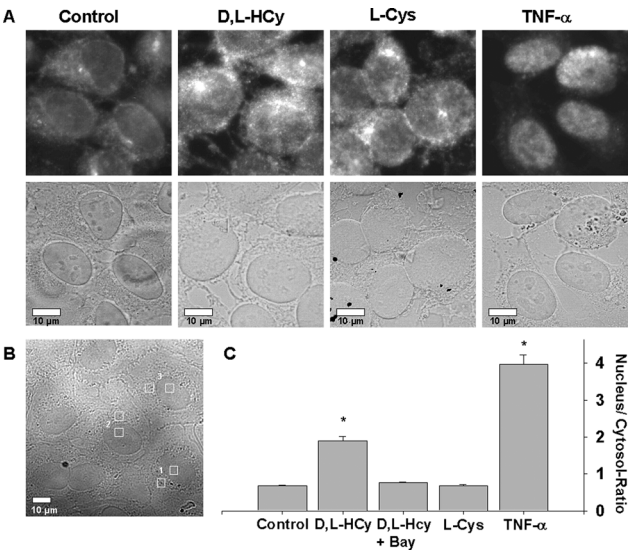

Figure 4. NF-κB translocation in Hcy-incubated EA.hy 926 cells (A) and HUVECs (B, C). Six hours of incubation of ECs with D,L-Hcy 200 μmol/L leads to increased NF-κB activation. This becomes evident by increased nuclear translocation indicated by increased nuclear staining with an anti-p65 antibody. 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.

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.[29] 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 pathophysiologically 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-

TABLE 2. Nuclear Translocation of NF-κB in Endothelial Cells as Determined by Staining With a p65 Antibody

	Nuclear/Cytoplasmic Ratio of Fluorescence Intensity After Staining With a p65 Antibody	
Condition	in EA.hy 926 Cells	in HUVECs
Control	0.72±0.27	0.69±0.04
D,L-Hcy 200 μmol/L	1.17±0.29*	1.90±0.23*
D,L-Hcy 200 μmol/L+Tiron	0.63±0.25†	0.73±0.05†
D,L-Hcy 200 μmol/L+MnTBAP	0.75±0.38†	
D,L-Hcy 200 μmol/L+Bay 11–7082		0.77±0.01†

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).[11,31] 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.[11] This cascade of events triggers 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.[11] In addition, Hcy has been shown to increase neutrophil adherence to EC in vitro and in vivo.[32] 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 β_2-integrins (Mac-1α, 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 monocytic cells to IL-1β–stimulated, but not to unstimulated, HAECs was slightly increased when both ECs and monocytes were pretreated with Hcy.[13] 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.[12,13] 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).[33] 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[34] and endothelial cells,[35] and in THP-1 macrophages.[36] 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.[37] 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-isoform of Hcy.[38] 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-isoform 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.[38] 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.[39]

NF-κB is one of the transcription factors that may be controlled by the redox status of the cell.[40] Generation of ROS seems to be a common step in signaling pathways that lead to IκB 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.[41] 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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