Aus der Medizinische Poliklinik-Innenstadt der
Ludwig-Maximilians Universität München
Vorstand: Prof. Dr. D. Schlöndorff

INFLUENCE OF HOMOCYSTEINE ON THE INTERACTION BETWEEN CIRCULATING MONOCYTES AND ENDOTHELIAL CELLS

Dissertation
zum Erwerb des Doktorgrades der Humanbiologie
an der Medizinischen Fakultät der
Ludwig-Maximilians Universität zu München

vorgelegt von

Otilia Postea
aus
Bukarest, Rumänien
2005

Mit Genehmigung der Medizinischen Fakultät
der Universität München

Berichterstatter:	Priv.-Doz. Dr. med. Norbert Weiss
Mitberichterstatter:	Priv.-Doz. Dr. med. Christian Kupatt
	Prof. Dr. med. Fritz Krombach
Dekan:	Prof. Dr. med. D. Reinhardt
Tag der mündlichen Prüfung:	15.06.2005

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1. INTRODUCTION

1.1. Overview on the Development and Progression of Atherosclerotic Lesions

Atherosclerosis is a major cause of morbidity and mortality in developed countries and is the underlying basis of most cases of myocardial infarction, stroke and peripheral artery disease.

High plasma concentrations of cholesterol, in particular those of low-density lipoprotein (LDL) cholesterol, are one of the principal risk factors for atherosclerosis, and treatment of hyperlipidemia reduces the risk for cardiovascular disease. [1] Accumulation of lipids within the artery wall is a *conditio sine qua non* for the development of atherosclerotic lesions, followed by the proliferation of vascular smooth muscle cells and production of extracellular matrix leading to a progressive narrowing of the vessel lumen. Therefore, the process of atherogenesis has previously been considered to be mainly a disorder of lipid metabolism and a lipid deposition disease in the vessel wall.

Recent developments in vascular biology, however, have indicated that atherosclerosis is much more than that. Nowadays atherosclerosis is considered a chronic inflammatory disease. Inflammatory processes initiate the formation of early lesions, support the development into more complex lesions and even participate in acute events, like plaque rupture leading to the above mentioned clinical syndromes. [2]

The earliest changes that precede the formation of atherosclerotic lesions take place in the endothelium (Figure 1). These changes include increased endothelial permeability to lipoproteins and other plasma constituents, the upregulation of endothelial adhesion molecules, which include E-selectin, P-selectin, intercellular adhesion molecule 1 (ICAM-1), and vascular cell adhesion molecule 1 (VCAM-1), the migration of leukocytes into the artery wall, which is mediated by oxidized LDL, monocyte chemotactic protein 1 (MCP-1), interleukin-8 (IL-8), platelet-derived growth factor, macrophage colony-stimulating factor, and osteopontin.

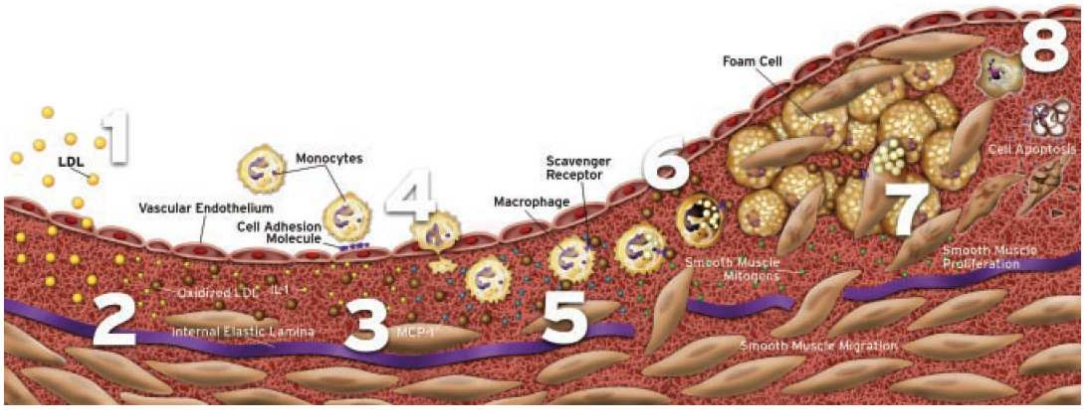

Figure 1. The 7 stages of the development of a atherosclerotic plaque. First LDL moves into the subendothelium and is oxidized by macrophages and smooth muscle cells (SMCs; 1and 2). Release of growth factors and cytokines attracts additional monocytes (3 and 4). Foam cell accumulation and SMC proliferation result in growth of the plaque (6, 7, and 8). [3]

Following activation of the endothelium by atherosclerotic risk factors, monocytes are recruited into the subendothelial space where they differentiate into macrophages. These macrophages endocytose modified forms of low density lipoproteins (LDL) via scavenger receptors to form foam cells, the hallmark of fatty streak lesions. These lesions develop into fibro-fatty plaques, which contain large numbers of macrophages and some CD4+ T cells, and show evidence of smooth muscle migration and proliferation. In human arteries, these fibro-fatty plaques may develop into complex atherosclerotic lesions that are liable to rupture, or may regress when atherosclerotic risk factors are treated. [2]

As fatty streaks progress to intermediate and advanced lesions, they tend to form a fibrous cap that walls off the lesion from the lumen. This represents a type of healing or fibrous response to the injury. The fibrous cap covers a mixture of leukocytes, lipid, and debris, which may form a necrotic core. These lesions expand at their shoulders by means of continued leukocyte adhesion and entry caused by the same factors. The principal factors associated with macrophage accumulation include macrophage colony-stimulating factor, MCP-1, and oxidized LDL. The necrotic core represents the results of apoptosis and necrosis, increased proteolytic activity, and lipid accumulation. The fibrous cap forms as a result of increased activity of platelet-derived growth factor, transforming growth factor β (TGF-β), interleukin-1 (IL-1), tumor necrosis factor α (TNF-α), and osteopontin and of decreased connective-tissue degradation.

Recently, pathologists have advanced the idea of stable and unstable (or vulnerable) atherosclerotic plaques. [4] Stable plaques are characterized by a thick fibrous cap overlying a plaque that does not contain a cholesterol-rich necrotic core. By contrast, unstable plaques have a thin fibrous cap, contain a higher ratio of macrophages to smooth muscle cells, and have a lipid-filled necrotic core. Unstable plaques are more likely to rupture, which exposes the thrombogenic core of the lesion to arterial blood. This leads to platelet aggregation and the formation of an arterial thrombus attached to the vessel wall. Thrombus material can break away from the wall and be transported to a distant site (embolism), where it may lead to blockage of smaller arteries. The clinical consequences of arterial thrombosis are heart attacks and strokes. Indeed, the majority (~60%) of arterial thrombosis is associated with ruptured atherosclerotic plaques. [4]

Another commonly observed feature of atherosclerotic plaques is endothelial cell denudation (plaque erosion) and other changes in the endothelial cells that predispose to arterial thrombosis and its clinical sequelae.

Rupture of the fibrous cap or ulceration of the fibrous plaque can rapidly lead to thrombosis and usually occurs at sites of thinning of the fibrous cap that covers the advanced lesion. Thinning of the fibrous cap is apparently due to the continuing influx and activation of macrophages, which release matrix metalloproteinases and other proteolytic enzymes at these sites. These enzymes cause degradation of the matrix, which can lead to hemorrhage from the vasa vasorum or from the lumen of the artery and can result in thrombus formation and occlusion of the artery.

1.2. Endothelial Dysfunction as Primary Step in Atherosclerosis

The endothelium exerts fundamental control over vascular function, and injury to the endothelium followed by dysfunction is an early key event preceding manifestation of vessel pathology.

Endothelial cells form a monolayer that lines the entire vascular system. The structural and functional integrity of the endothelium is a fundamental requirement for maintenance of vessel wall homeostasis and circulatory function. As a semipermeable membrane, endothelium controls the transfer of small and large molecules into the arterial wall and through the walls of capillaries and

venules. In addition, endothelial cells secrete mediators and express surface molecules that participate to the regulation of vascular tone, inflammation, thrombosis and fibrinolysis.

The key initial event in vascular pathology appears to be damage to the endothelial cells of the artery. The imbalance between endothelial substances responsible for vasorelaxation and vasoconstriction, between procoagulant and anticoagulant factors, between proinflammatory and anti-inflammatory mediators, and between inhibitors and promotors of vascular cell growth, is an early and persistent vascular abnormality in the evolution of atherothrombotic disease in humans. [5] The term endothelial dysfunction is often used to describe several types of potentially reversible changes in the functional state of endothelial cells that occur as a response to environmental stimuli.

Two functional alterations of endothelium deserve comment: endothelial stimulation and endothelial activation.

Endothelial stimulation denotes a rapid (within minutes), reversible response that is independent of new protein synthesis (endothelial cell contraction, expression of adhesive glycoprotein P-selectin).

Endothelial activation depends on new (or altered) protein synthesis and requires longer time (hours or even days) to occur. This response is most frequently induced by inflammatory cytokines, which promote new adhesion molecule expression on the surface of endothelial cells. In addition, the circulating cells can be activated by different stimuli, which induce high levels of adhesion molecules on the surface of these cells.

Adhesion molecules expressed on the cellular surface play vital roles in numerous cellular processes. Some of these include: cell growth, differentiation, embryogenesis, immune cell transmigration and response, and cancer metastasis. Adhesion molecules are also capable of transmitting information from the extracellular matrix to the cell.

Cell adhesion molecules (CAMs) are cell surface proteins involved in the binding of cells, usually leukocytes, to each other, to endothelial cells, or to extracellular matrix. There are four major families of cell adhesion molecules. Most of the CAMs characterized so far fall into three general families of proteins: the immunoglobulin (Ig) superfamily, the integrin family and the selectin family.

The immunoglobulin superfamilies of CAMs are calcium-independent transmembrane glycoproteins. Members of the immunoglobulin superfamily include the ICAMs, VCAM-1, platelet-endothelial-cell adhesion molecule (PECAM-1), and neural-cell adhesion molecule (N-CAM). Each immunoglobulin superfamily CAM has an extracellular domain, which contains several immunoglobulin-like intrachain disulfide-bonded loops with conserved cysteine residues, a transmembrane domain, and an intracellular domain that interacts with the cytoskeleton. Typically, they bind integrins or other members from immunoglobulin superfamily CAMs. Endothelial CAMs play an important role in immune response and inflammation. CAMs are critical to numerous cellular processes and responses. Additionally, they also play a role in various disease states. Transendothelial migration of leukocytes is an example of one of the many roles of adhesion molecules.

The integrin family of CAMs serve as receptors for the ICAMs and VCAMs. Two important integrins, Mac-1α and LFA-1β expressed on leukocytes, are the ligands for ICAM-1 molecule expressed on endothelial cells. The integrins are heterodimeric proteins consisting of an alpha and a beta chain that mediate leukocyte adherence to the vascular endothelium or other cell-cell interactions. Different sets of integrins are expressed by different populations of leukocytes to provide specificity for binding to different types of CAMs expressed along the vascular endothelium.

The selectin family members, P-selectin, L-selectin and E-selectin, are involved in the adhesion of leukocytes to activated endothelium. This adhesion is initiated by weak interactions that produce a characteristic "rolling" motion of the leukocytes on the endothelial surface. P-selectin and L-selectin, acting in concert, have been implicated in the mediation of these initial interactions. Stronger interactions, probably involving E-selectin, follow the initial interactions, leading eventually to extravasation through the blood vessel walls into lymphoid tissues and sites of inflammation.

The leukocyte trafficking consists of four distinct steps. The first is rolling of the circulating leukocyte along endothelial cells (*i.e.*, leukocyte rolling on a blood vessel wall). This step is selectin-mediated. The second step involves the triggering or activation of cell surface adhesion molecules, namely, the integrins.

This can be accomplished through contact with specific extracellular matrix proteins, inflammatory cytokines, or chemokines. The third step involves firm adhesion; the leukocyte firmly attaches to an endothelial cell. This involves arrest of the rolling process and spreading over the endothelial surface, typically a vessel wall. The integrins and their ligands play crucial roles in this step. The fourth step is transmigration of the leukocyte through adjacent endothelial cells in a process called diapedesis. This allows the leukocyte to enter the subendothelial space. PECAM-1 is a crucial player in this step. Transendothelial migration demonstrates cooperativity between leukocyte and endothelial cell adhesion molecules.

The modulation of these molecules to induce adhesion in inflammation can be done using a number of mechanisms, which are dependent on the duration of inflammation, the type of inflammatory stimulus and blood flow conditions. Redistribution of adhesive molecules to the cell surface (P-selectin), induction of adhesion molecules expression on endothelium (ICAM-1, V-CAM-1) and increased avidity of binding (ICAM-1 – LFA-1 interaction) are some of these mechanisms.

In summary, different adhesion molecules and chemokines are implicated in several stages of the interaction between endothelial cells and circulatory cells. The chemotaxis, mediated by chemokines (MCP-1, IL-8), is followed by rolling (P-selectin), firm adhesion (ICAM-1, VCAM-1) and transmigration (PECAM-1) (Figure 2).

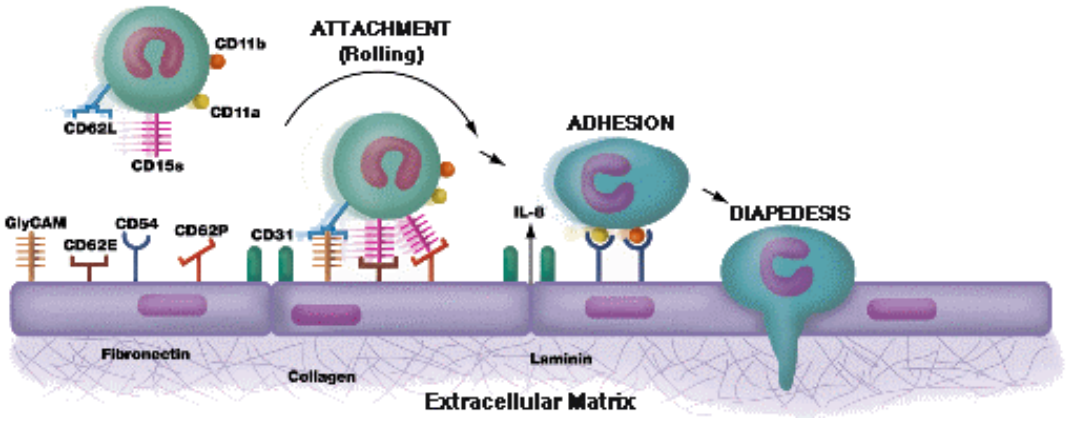

Figure 2. Monocyte arrest on the endothelium. Role of adhesion molecules.

All these mediators are upregulated by several transcription factors, among which, redox-sensitive NF-κB factor occupies an important position.

The presence of atherosclerotic lesions is associated with endothelial dysfunction, manifested as impaired endothelium-dependent regulation of vascular tone. [6-9] In patients with atherosclerotic risk factors, impaired endothelial vasodilator function can be demonstrated early in the process of atherogenesis, even prior to the development of frank atherosclerotic lesions. Its extent is related to the intensity and duration of risk factor exposure, and treatment of risk factors results in reversal or attenuation of endothelial dysfunction. [10-12] These observations suggest that the endothelium is both a target and a mediator of atherothrombosis.

Endothelial vasodilator function may therefore serve as an index integrating the overall stress imposed by vascular risk factors. Indeed, clinical studies have demonstrated that endothelial vasodilator dysfunction is a predictive marker for the future development of coronary artery disease, [12] and is associated with a worse prognosis in the setting of existing coronary artery disease predicting coronary disease progression and cardiovascular event rate. [13,14] These findings underscore the central role of the endothelium in vascular function and its regulation.

One marker of endothelial dysfunction is impairment of endothelium-dependent vasomotor responses, which is indicative of a reduction in the bioavailability of the endothelium derived signalling molecule nitric oxide. [15,16] Nitric oxide is released from endothelial cells in response to increased shear stress [17] and certain biochemical stimuli. [18] Nitric oxide may function as an endogenous anti-atherogenic molecule by maintaining low arterial tone at rest, inhibiting leukocyte–endothelial interactions, attenuating platelet activation, and inhibiting smooth muscle cell proliferation. [19] A reduction in nitric oxide synthesis and/or activity leading to insufficient biological nitric oxide availability may therefore contribute to the initiation and progression of atherosclerosis.

Decreased nitric oxide bioavailability may reflect an absolute deficit of nitric oxide, impaired availability of bioactive nitric oxide or enhanced nitric oxide inactivation.

Decreased nitric oxide synthesis may be due to decreased expression or activity of endothelial nitric oxide synthase, alterations of membrane receptors in the arterial wall that interact with biochemical or physiological stimuli to induce nitric oxide synthesis, decreased availability or affinity of the substrate L-arginine

and cofactors, especially tetrahydrobiopterin, for nitric oxide synthase, and elevated levels of endogenous inhibitors of nitric oxide synthase.

Impaired bioavailability and bioactivity of nitric oxide may be due to enhanced degradation by increased free radicals and/or oxidation-sensitive mechanisms, impaired nitric oxide diffusion from the endothelium to vascular smooth muscle cells followed by decreased sensitivity to its vasodilator action, and impaired interaction of nitric oxide with guanylate cyclase resulting in decreased cyclic GMP production. [19-21]

1.3. Hyperhomocysteinemia as a Cardiovascular Risk

1.3.1. Definition of Hyperhomocysteinemia

In 1969, McCully first described the association of hyperhomocysteinemia and the development of premature atherosclerosis and thromboembolism in children with rare inborn errors of homocysteine metabolism that presented elevated plasma homocysteine levels up to 30 times the normal range. [22,23]

Observations of the natural history of patients with severe hyperhomocysteinemia showed that untreated patients suffer one thrombembolic event per 25 patient-years [23]. A multicenter observational study showed that treatment of hyperhomocysteinemia in these patients leads to a significant reduction in thromboembolic events compared to untreated patients (relative risk 0.09; 95% CI 0.036 to 0.228; $P < 0.0001$). [24]

These data support the hypothesis of a causal relationship between severe hyperhomocysteinemia and cardiovascular risk in patients with these rare genetic disorders.

It was Kilmar McCully who first proposed, based on these observations, that even mildly elevated plasma homocysteine levels, as found in 5 to 10 percent of people in the general population, may play a role in the etiology of vascular diseases.

At present there is no consensus on what levels constitute hyperhomocysteinemia. An increased risk for atherosclerotic vascular disease seems to be associated with fasting homocysteine levels above 12 µmol/L as discussed below. Therefore, it is suggested to refer to fasting plasma

homocysteine levels between 12 and 30 µmol/L as mild or – synonymously used – moderate hyperhomocysteinemia, to levels between 31 and 100 µmol/L as intermediate hyperhomocysteinemia, and to plasma levels > 100 µmol/L as severe hyperhomocysteinemia, respectively [5,25,26].

1.3.2. Case-control Studies on Mild Hyperhomocysteinemia and Vascular Diseases

A great number of retrospective case-control studies and prospective nested case-control studies performed during the last twenty years nearly uniformly showed an association between mildly elevated plasma homocysteine levels and atherothrombotic vascular diseases indicated by an increased prevalence and incidence of coronary artery disease, cerebrovascular disease, and peripheral vascular disease.

Hyperhomocysteinemia persists as a risk factor even after statistical adjustment for conventional risk factors, suggesting that homocysteine's effect on cardiovascular disease seems to be independent from other factors.

Data from a meta-analysis performed by Boushey and colleagues [27] that included data from 27 studies published before 1995 indicated that a 5 µmol/L increase in plasma homocysteine above median levels of 10 µmol/L is associated with a significant and graded increase in the risk of coronary artery disease (odds ratio 1.6; 95 % confidence intervall: 1.4 to 1.7), cerebrovascular disease (odds ratio 1.5; 1.3 to 1.9), and peripheral vascular disease (odds ratio 6.8; 2.9 to 15.8). By comparing elevated homocysteine levels to other established vascular risk factors, the authors calculated that a 5 µmol/L increase in plasma homocysteine levels is equivalent to a 0.5 mmol/L (20 mg/dL) increase in plasma cholesterol levels in increasing the risk for myocardial infarction.

Data of a recent case-control study conducted in nine European centers suggested that the cardiovascular risk associated with elevated homocysteine levels detected both under basal conditions (> 12 µmol/L) and after an oral methionine challenge (net increase > 27 µmol/L over basal levels or absolute increase > 38 µmol/L) is comparable to the risk associated with hyperlipidemia or smoking, but somewhat lower than that of hypertension [25].

Taking together the above mentioned studies, it has been estimated that ten percent of the population's coronary artery disease risk appears to be attributable to plasma homocysteine levels [27].

1.3.3. Prospective Studies on Mild Hyperhomocysteinemia and Vascular Diseases

Prospective cohort studies, which are providing the most rigorous evidence for an association between a potential causal factor and a clinical event, however, yielded some inconclusive results in linking homocysteine to vascular disease.

Most of the prospective studies provided evidence for mild hyperhomocysteinemia as a risk factor for atherothrombotic vascular disease after adjustment for conventional risk factors [28-40], although some studies have not [41-46]. Possible reasons for these conflicting results may derive from different ethnic backgrounds of the study populations, differences in sample size, and most importantly, differences in the lifestyle of the specific study's participants.

The last point appears to be especially important as a source of potential bias owing to multivitamin use by study subjects. For example, the Atherosclerosis Risk in Community Trial, the largest prospective trial with a negative outcome, did not provide detailed information about vitamin supplementation as a potential confounding variable [43-45].

Nevertheless, the definitive reasons for the variable results are still unknown. Several arguments, however, weigh in favor of the positive studies, as summarized in Table 1.

The debate about hyperhomocysteinemia as a cardiovascular risk factor will continue, as long as results of large-scaled intervention trials aimed at reducing cardiovascular events by homocysteine lowering treatment are still pending. Several of these studies are performed at the moment.

Table 1. Arguments for mild hyperhomocysteinemia as a cardiovascular risk factor (modified with permission from Weiss et al., [47])

	References
Clear-cut association between hyperhomocysteinemia and atherothrombosis in all retrospective case-control studies.	[27]
Correlation of the magnitude of cardiovascular risk with plasma homocysteine levels in many studies.	[34,38,39,48,49]
Consistent results of meta-analyses of the retrospective case-control and of the prospective population based studies favoring hyperhomocysteinemia as a vascular risk factor.	[27,39,50-53]
Association between mortality of patients with preexisting coronary or peripheral vascular disease and plasma homocysteine levels independent of traditional risk factors.	[34,54-59]
Semi-quantitative relation of plasma homocysteine levels with the extent of atherosclerosis in coronary, peripheral, and carotid arteries.	[53,60-66]
Mildly elevated plasma homocysteine levels induce endothelial vascular dysfunction, a key event in the pathogenesis of atherothrombosis, and lowering of plasma homocysteine levels may have a favorable effect on vascular function.	[67-80] [80-87]

1.3.4. Causes of Hyperhomocysteinemia and Therapeutic Options

Homocysteine levels may be determined by nutritional and genetic factors, and modified by concomitant disease states and drugs. Plasma homocysteine levels are strongly and inversely correlated with plasma levels of folate and to a lesser extent with vitamin B_6 and vitamin B_{12} levels [88,89].

It has been suggested that inadequate plasma concentrations of one or more of these vitamins contribute to approximately two thirds of all cases of hyperhomocysteinemia. In accordance with these observations, numerous clinical studies, including 12 randomized and placebo controlled intervention studies,

have uniformly shown that supplementation with folic acid alone or in combination with other B-vitamins is able to effectively lower plasma homocysteine levels, at least in patients with normal renal function.

A recently performed meta-analysis of the published controlled clinical trials indicated [90] that folic acid supplementation reduces homocysteine levels by 25 % (95 % CI: 23 to 28 %) with similar effects in a daily dose range of 0.5 to 5 mg. An additional reduction in plasma homocysteine is produced by vitamin B_{12} (mean dose 0.5 mg), whereas vitamin B_6 in a mean dose of 16.5 mg did not have any significant effect.

In addition, elevated plasma homocysteine levels may be increased due to genetic defects, as part of the acute phase response, diabetes, chronic renal failure, cancer, hypothyroidism, and some drugs (Table 2). [91]

Table 2. Causes of mild hyperhomocysteinemia

Nutritional factors
Deficiencies of folic acid, vitamin B_6 or vitamin B_{12}
Vegetarian diet
Chronic alcohol consumption
Genetic defects
Thermolabile variant of methylenetetrahydrofolate reductase
Cystathionine β-synthase deficiency
Diseases
Pernicious anemia
Renal impairment
Hypothyroidism
Diabetes
Malignancies (acute lymphoblastic leukemia, carcinoma of the breast, ovary and pancreas)
Severe psoriasis
Medications
Folate antagonists (methotrexat)
Anticonvulsive drugs (phenytoin, carbamazepine)
Theophylline
Nicotinic acid
Colestipol
Thiazide diuretics

1.4. Chemistry and Metabolism of Homocysteine

Homocysteine occupies a pivotal position in the metabolism of the essential sulfur-containing aminoacid, methionine (Figure 3).

Methionine, in the form of S-adenosyl-methionine, is the major methyl-group donor in mammals. Homocysteine produced from methionine is metabolized either by remethylation to methionine or by transulfuration to cystathionine.

In all tissues studied so far, remethylation can occur via methionine synthase, a vitamin B_{12} dependent enzyme that uses 5-methyl-tetrahydrofolate (5-MTHF, a derivative of folic acid) as a methyl donor. In the liver, remethylation may be achieved additionally by betaine-homocysteine methyltransferase, the methyl donor in this instance being betaine.

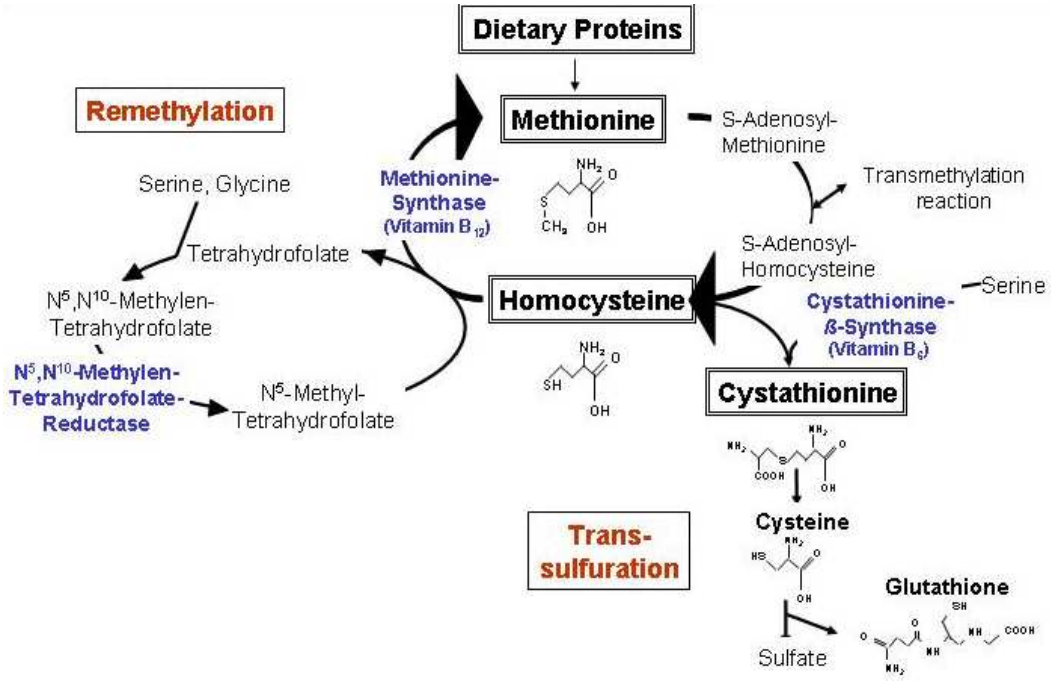

Figure 3. Scheme of homocysteine metabolism.

Transulfuration of homocysteine is initiated by the enzyme cystathionine β synthase (CBS, vitamin B_6 or pyridoxine dependent) that forms cystathionine, which is finally converted to cysteine. The transsulfuration pathway may end in glutathione synthesis, therefore contributing to the maintenance of the cellular redox balance. Endothelial cells do not express CBS, therefore they lack the

transsulfuration pathway. This may be one reason why they cannot compensate homocysteine toxicity by increasing glutathione biosynthesis.

Remethylation and transsulfuration each account for 50% of homocysteine disposal. Remethylation is the major determinant of fasting plasma homocysteine levels, whereas impaired transsulfuration increases homocysteine levels predominantly when methionine levels are elevated (such as post-prandially).

Chemically, methionine contains a sulfide sulfur (R-S-R'), whereas homocysteine and cysteine are sulfhydryl compounds (R-SH). Compounds containing a free sulfhydryl group are known as "thiols."

Other biologically relevant low-molecular-weight thiols are glutathione, coenzyme A, and dihydrolipoic acid. Under aerobic conditions (i.e., in the presence of molecular oxygen as an electron acceptor) and at physiological pH, thiols such as homocysteine, oxidize to form disulfides, according to the general reaction:

$$2\ RSH + O_2 \Leftrightarrow RSSR + H_2O_2.$$

In plasma, this reaction can be catalyzed by transition metals such as copper and cobalt (the former present in the circulation associated with albumin and ceruloplasmin, the latter with cobalamin).

Homocysteine can autooxidize readily via general thiol oxidation mechanism described above and form homocystine, or oxidize other thiols such as cysteine and glutathione to form mixed disulfides, or oxidize free cysteine residues on proteins and peptides to form mixed disulfides (Figure 4).

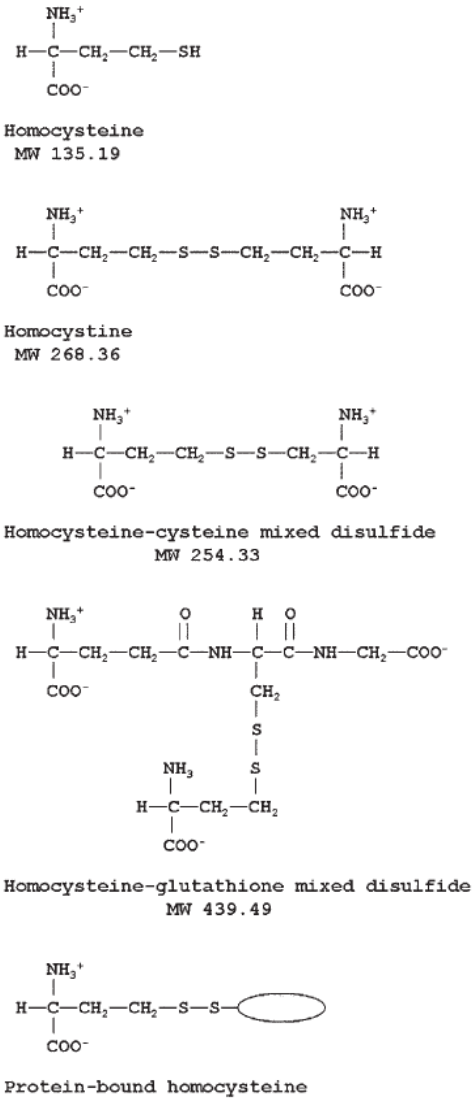

Figure 4. Chemistry of homocysteine and different homocysteine oxidation products. [92]

1.5. Mechanisms of Homocysteine-Induced Alteration of Healthy Endothelium

Experimental data suggests that a key event in the vascular pathology associated with hyperhomocysteinemia is endothelial dysfunction and injury resulting in decreased vasodilatory capacity, activation of circulating leukocytes and platelets, activation of prothrombotic and inhibition of fibrinolytic mechanisms, and stimulation of vascular smooth muscle cell proliferation (Figure 5).

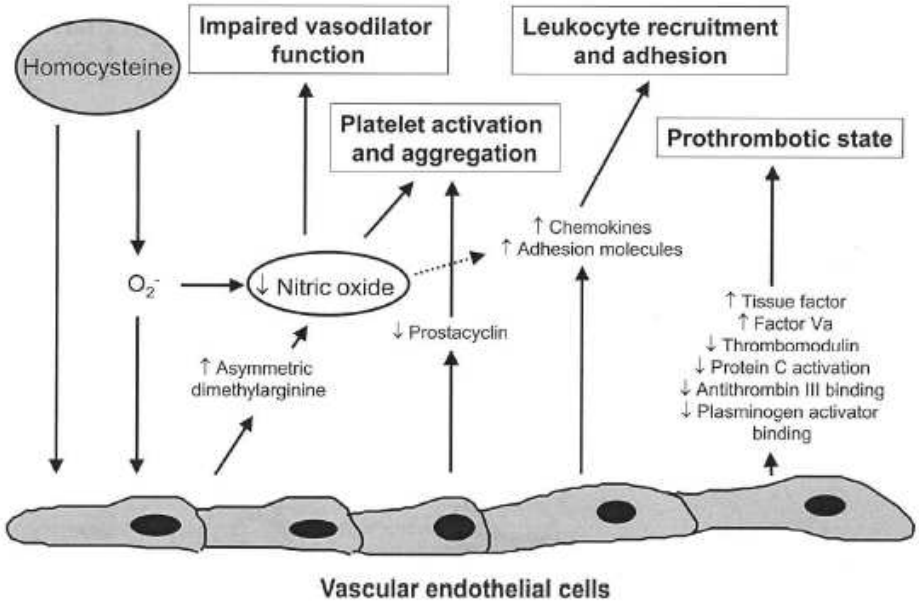

Figure 5. Putative mechanisms and effects of homocysteine-induced endothelial dysfunction. Exposure of endothelial cells to homocysteine leads to reduced bioavailability of nitric oxide resulting in impaired endothelium-dependent vasodilator function. This may be caused either by increased oxidative inactivation of nitric oxide by oxygen-derived free radicals or by increased endothelial synthesis of the endogenous nitric oxide synthase inhibitor asymmetric dimethylarginine. Decreased bioavailable nitric oxide together with a decrease in the endothelial synthesis of prostacyclin promotes platelet activation and aggregation. Elevated homocysteine levels induce the expression of several chemokines and adhesion molecules by endothelial cells that lead to increased recruitment and adhesion of circulating inflammatory cells. This may be caused by decreased bioavailable nitric oxide and/or increased vascular oxidant stress leading to increased activation of the redox-sensitive transcription factor NF-κB that may mediate the inflammatory response. Homocysteine may further promote thrombosis by activating endothelium-dependent prothrombotic mechanisms, such as the expression of tissue factor, or the activity of factor Va, and by inhibiting antithrombotic and fibrinolytic mechanisms. [5]

These cellular events may promote the development of atherosclerotic lesions and atherothrombotic clinical manifestations. Increased oxidant stress in the vasculature by hyperhomocysteinemia may initiate the pathophysiological

changes associated with this cardiovascular risk factor. This may result in the depletion of nitic oxide by oxygen-derived free radicals leading to a decrease in the bioavailability of this antiatherogenic signalling molecule, as outlined below.

1.5.1. Impaired Endothelium-Dependent Vasodilatation as an Indicator of Endothelial Dysfunction in Mild Hyperhomocysteinemia

A key regulatory system of the normal activity of the endothelium involves nitric oxide synthase (NOS), which synthesizes nitric oxide (NO). This simple molecule, besides other effects, acts as an endothelium-derived relaxing factor for vascular smooth muscle cells in response to a variety of stimuli. One of the important physiologic stimuli is shear stress at the vessel wall, which is increased when blood flow increases. The consequent release of NO induces vasodilatation and regulates vascular tone.

Lentz and colleagues used a diet enriched in methionine and depleted in folate to increase plasma homocysteine in cynomolgus monkeys. This experimental hyperhomocysteinemia impaired endothelium-dependent vasodilatation. [93] This observation was confirmed by other studies using rats with diet-induced hyperhomocysteinemia, [94,95] or mice with heterozygous disruption of the cystathionine β-synthase gene ($CBS^{(-/+)}$mice), [96-98] or the combination of genetic and dietary approaches. [99]

Oral methionine administration (0.1 g/kg body weight) increased plasma homocysteine to 25 to 40 µmol/L in young healthy subjects and impaired endothelium-dependent vasodilator function, but preserved endothelium-independent vasodilator responses [67-70,76]. The same dysfunction has been noticed in patients with chronic mild hyperhomocysteinemia [78,79].

It seems that homocysteine, and not methionine or vitamin deficiencies, is responsible for these adverse vascular effects. Mice with a genetic defect in homocysteine transulfuration pathway have mild hyperhomocysteinemia of 1.5 to 1.8-fold the level in wildtype mice, but have normal plasma methionine levels and are provided a chow that yields normal B-vitamin levels. [96-98] Thus, a mild increase in homocysteine alone is sufficient to induce endothelial dysfunction. Several analyses of different species of homocysteine and their time-dependent

concentrations in plasma after oral homocysteine or methionine challenges revealed that peak reduction in flow mediated dilation coincided with maximal concentrations of reduced homocysteine, but not with free oxidized homocystine, protein-bound oxidized homocystine, or related species, [75] suggesting that reduced homocysteine is responsible for vascular dysfunction in vivo.

Decreased accumulation of cyclic guanosine monophosphate, the second messenger of nitric oxide mediated vasodilatation, in aortas from mildly hyperhomocysteinemic $CBS^{(-/+)}$ mice compared to their wildtype littermates, [96] and lower plasma levels of the nitric oxide-derived end-products nitrite and nitrate in hyperhomocysteinemic subjects than in healthy controls [80] indicated that the effects of homocysteine are presumably due to a decrease in bioavailable endogenous nitric oxide. This was also confirmed by the observation that homocysteine impairs endothelium dependent vasodilator function induced by shear stress, acetylcholine or bradykinin but does not impair endothelium independent vasodilatation induced by sodium nitroprusside or nitroglycerin. In accordance with these in vivo findings, homocysteine, but not cysteine, has been shown to decrease the production and/or bioactivity of nitric oxide and of *S*-nitrosothiols by cultured endothelial cells. [97,100,101]

1.5.2. Mechanisms of Reduced Nitric Oxide Bioavailability in Hyperhomocysteinemia

In respiring cells, small amounts of oxygen are reduced to reactive oxygen species. These reactive oxygen intermediates, produced in mitochondria, peroxisomes, and the cytosol, are scavenged by the cellular defense systems, including enzymatic and nonenzymatic antioxidants. A state of moderately increased levels of intracellular reactive oxygen species is referred to as *oxidative stress*. Cells respond to this stress by increasing the levels of antioxidants and altering the intracellular reduction-oxidation (redox) state.

Many diseases are linked to damage from reactive oxygen species as a result of an imbalance between radical-generating and radical-scavenging systems.

The various risk factors for atherosclerosis, including hyperlipidemia, hypertension, diabetes and hyperhomocysteinemia, have in common the

generation of intracellular oxidative stress (Figure 6). All these events support the development and progression of atherosclerotic lesions and thrombus formation.

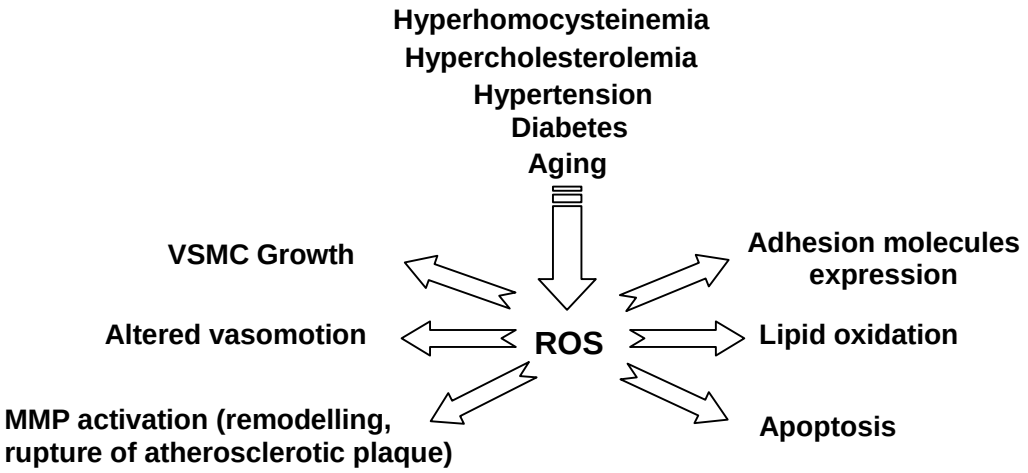

Figure 6. Roles of reactive oxygen species (ROS) in vascular disease. (MMP-matrix metalloproteinase, VSMC-vascular smooth muscle cell)

1.5.2.1. Oxidative Inactivation of Nitric Oxide

Nitric oxide (NO), produced by nitric oxide synthases (NOS), is a short-lived molecule ($t_{1/2}$=seconds) capable of diffusing across membranes and reacting with a variety of targets. Reaction of NO with O_2 in aqueous solutions produces the relatively unreactive nitrate (NO_3^-) and nitrite (NO_2^-) ions as products. The reaction with other species of O_2 generates toxic compounds which disturb the equilibrium of the cellular metabolism.

Depletion of bioavailable nitric oxide by oxidant stress in hyperhomocysteinemia has been suggested as one of the mechanisms that generate the adverse effects of homocysteine on endothelial function (Figure 7). [102]

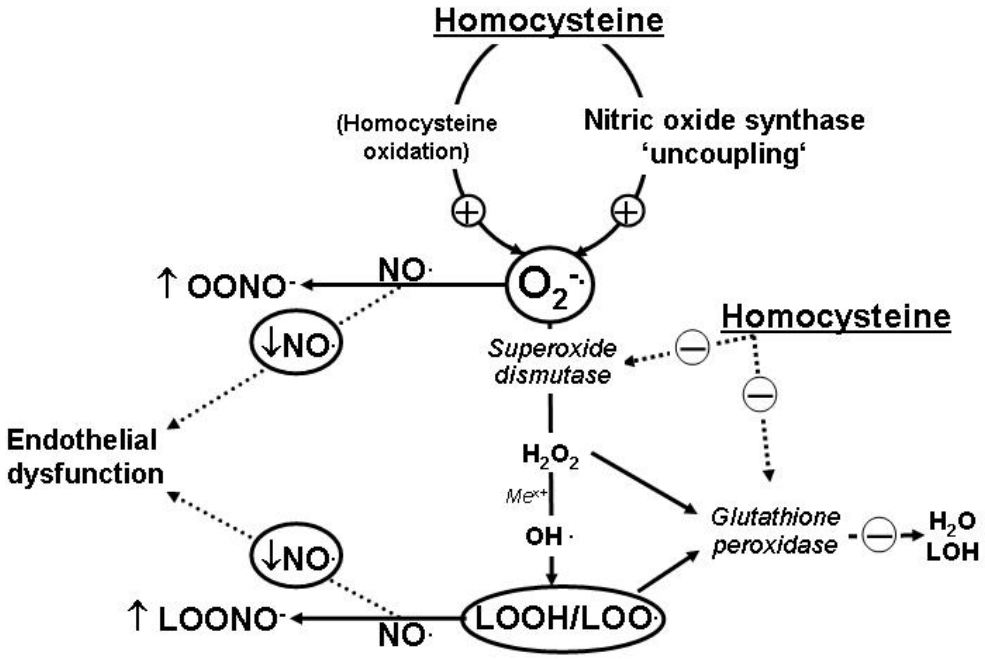

Figure 7. Putative mechanism of homocysteine-induced increased vascular oxidant stress leading to decreased bioavailable nitric oxide. (O_2^{-},superoxide anion radical; $NO^{\cdot}$, nitric oxide; $OONO^{-}$,peroxynitrite; H_2O_2, hydrogen peroxide; Me^{x+}, metal cation; $HO^{\cdot}$, hydroxyl radical; LOOH, lipid peroxide; $LOO^{\cdot}$, lipid peroxyl radical; $LOONO^{-}$, lipid peroxynitrite; H_2O, water; LOH, lipid alcohol.) [5]

The reaction between superoxide anion (O_2^{-}) and nitric oxide forms highly reactive peroxynitrite ($ONOO^{-}$), which leads to inactivation of nitric oxide's biological activity. [103] Peroxynitrite has a short half-life, which makes its detection difficult in biological systems, [104] but may react with cellular tyrosine residues to form nitrosated end-products like 3-nitrotyrosine detected in aortic tissue from mildly hyperhomocysteinemic $CBS^{(-/+)}$ mice compared with wildtype mice, [96] and in protein lysates from aortic rings cultured in homocysteine containing media. [105] Elevated levels of superoxide anion were observed in aortic tissue from mildly hyperhomocysteinemic mice compared to wildtype mice [96] and in cultured endothelial cells incubated with homocysteine. [106] Scavenging of superoxide anion by either superoxide dismutase or Tiron reversed endothelial dysfunction in hyperhomocysteinemic animal models [96,107] and in isolated aortic rings incubated with homocysteine. [106]

Hydrogen peroxide decomposes to the toxic oxygen species hydroxyl radical, which can react with all biological macromolecules (lipids, proteins, nucleic acids and carbohydrates) causing lipid peroxidation, and to hydroxide anion,

which promotes alkaline tissue damage. The initial reaction of peroxidation generates a second radical, which in turn can react with a second macromolecule to continue the chain reaction. Among the more susceptible targets are polyunsaturated fatty acids. Increased plasma levels of the isoprostane F2a-III, an end-product of the non-enzymatic peroxidation of arachidonic acid was observed in mildly hyperhomocysteinemic $CBS^{(-/+)}$ mice. [96] Increased levels of malondialdehyde, conjugated dienes, and oxidized proteins in hyperhomocysteinemic patients [108] and the correlation of plasma homocysteine with plasma F2 -isoprostanes [109] suggested enhanced in vivo lipid and protein oxidation in humans. Oxidant stress induced by hyperhomocysteinemia leads to direct oxidation of low-density lipoprotein, [110], and may indirectly contribute to oxidative modification of low-density lipoprotein mediated by vascular cells in vitro. [110] Elevated levels of lipid peroxides lead to an increase in peroxyl radicals that can inactivate nitric oxide through the formation of lipid peroxynitrites. An increase in the fluorescence of a hydrogen peroxide-sensitive fluorescent probe, 2',7'-dichlorofluorescein, was observed in cultured endothelial cells [101,111] and cultured vascular smooth muscle cells exposed to homocysteine. [112]

The intracellular redox buffer system has an important role in maintaining endothelial function. Reduced glutathione and glutathione disulfide together with glutathione reductase and glutathione peroxidase play a central role in the cellular defense against oxidant stress. This is confirmed by the findings that nitric oxide synthesis is impaired in glutathione-depleted human endothelial cells. Mice deficient in cellular glutathione peroxidase have endothelial dysfunction due to increased vascular oxidative stress with resulting reduction in the bioavailability of nitric oxide. Treatment of hyperhomocysteinemic $CBS^{(-/+)}$ mice with the intracellular cysteine donor L-2-oxo-4-thiazolidine carboxylate, an agent that increases intracellular levels of reduced glutathione and total thiols and thereby shifts the cellular redox state to a more reduced environment, restores endothelial function. [98] The involvement of cellular glutathione peroxidase in homocysteine induced endothelial dysfunction is demonstrated by the observations that overexpression of cellular glutathione peroxidase in hyperhomocysteinemic $CBS^{(-/+)}$ mice restores the normal endothelium-dependent vasodilator response, and that overexpression of cellular glutathione peroxidase in cultured endothelial cells attenuates the homocysteine induced decrease in nitric oxide release from these

cells. [97] Improvemnent of the cellular antioxidant capacity by increasing the concentration of reduced glutathione or by overexpression of cellular glutathione peroxidase compensates for the adverse effects of homocysteine on endothelial function. The conclusion that the adverse vascular effects of homocysteine are at least partly mediated by oxidative inactivation of nitric oxide is also supported by studies in humans demonstrating that pretreatment with antioxidants prevented endothelial and platelet dysfunction induced in healthy individuals by an oral methionine challenge. [69,71]

Another hypothesis regarding the reduced bioavailability of nitric oxide in hyperhomocysteinemia could be the elevation of the plasma concentration of the endogenous nitric oxide synthase inhibitor - asymmetric dimethylarginine [113] which is generated from the degradation of proteins containing methylated arginine residues. [114] Boger and collaborators [115] correlated increased plasma levels of asymmetric dimethylarginine with endothelial dysfunction in cynomolgus monkeys fed a methionine-rich, folic acid depleted diet free of choline, and in healthy humans after an oral methionine challenge. In vitro studies using endothelial cells exposed to homocysteine or to methionine, have shown that the concentration of asymmetric dimethylarginine in the cell culture medium was increased in a dose- and time-dependent manner and associated with reduced nitric oxide synthesis by endothelial cells. This was due to a reduction of the activity of dimethylarginine dimethylaminohydrolase, the enzyme that degrades asymmetric dimethylarginine in hyperhomocysteinemic conditions. This inhibitory effect of homocysteine on this enzyme could be caused by direct binding and functional interference of the reactive sulfhydryl group of homocysteine with cysteine groups of dimethylarginine dimethylaminohydrolase, as it can be prevented by a thiol antioxidant that protects sulfhydryl groups and by the reducing agent dithiothreitol, [116] or by increased oxidant stress under conditions of elevated homocysteine. [117] These observations complement the hypothesis that homocysteine may impair nitric oxide bioactivity by oxidative degradation.

1.5.2.2. Involvement of Nitric Oxide Synthase in the Generation of Reactive Oxygen Species

Nitric oxide is produced by a group of enzymes called nitric oxide synthases (NOS). These enzymes catalyze the production of nitric oxide and L-citrulline from L-arginine, O_2, and NADPH-derived electrons. Mammalian systems contain three well-characterized isoforms of the enzyme: neuronal NOS (nNOS, also called NOS1), inducible NOS (iNOS or NOS2), and endothelial NOS (eNOS or NOS3). The three main isoforms share structural similarities and have nearly identical catalytic mechanisms. They all require a number of cofactors and functional prosthetic groups for activity including FAD, FMN, heme, calmodulin, and tetrahydrobiopterin. The homodimeric form is required for nitric oxide production, and the subunits have molecular masses of approximately 160 (nNOS), 135 (eNOS), and 130 kDa (iNOS).

Several studies have demonstrated that homocysteine induces increased formation of peroxynitrite. Unlike superoxide, peroxynitrite is both able to initiate lipid peroxidation [118] and to react with DCF [119]. In vitro experiments suggested that homocysteine-induced oxidant stress is stereospecific for the naturally occuring L-isoform, indicating a biochemical rather than chemical basis for the effect. In these experiments, increased lipid peroxidation was observed in endothelial cells incubated with L-homocysteine, but not with D-homocysteine, as measured by total isoprostane $F_{2\alpha}$-III and TBARs levels in the supernatant of endothelial cell cultures. Stereospecificity was also observed for DCF fluorescence in situ, indicating a parallel effect on overall reactive oxygen species content. Enhanced lipid peroxidation and DCF-fluorescence was not observed with either cysteine or glutathione, and was not due to extracellular thiol oxidation, as it could be fully replicated with the oxidized disulfide form of homocysteine, L-homocystine. Mechanistically, this pro-oxidant effect seems to be dependent upon both endothelial nitric oxide synthase and superoxide anion.

Inhibition of endothelial nitric oxide synthase with L-nitroarginine methyl ester (L-NAME) completely blocked the homocysteine-dependent stimulation of reactive oxygen species formation as measured by DCF-fluorescence. Furthermore, L-NAME completely abrogated the homocysteine-dependent

increase in isoprostane $F_{2\alpha}$-III formation. Transfection of endothelial cells with superoxide dismutase cDNA blocked the effect of homocysteine on endothelial cell lipid peroxidation, whereas addition of extracellular superoxide dismutase had no effect. Importantly, neither the addition of extracellular catalase nor loading cells with catalase had any effect on lipid peroxidation. This indicates that hydrogen peroxide is not involved in this process. The reactive oxygen species produced rather seems to be superoxide anion.

Although the precise mechanism by which homocysteine may induce increased superoxide anion and peroxynitrite formation remains to be elucidated, two potential mechanisms include endothelial nitric oxide synthase "uncoupling", in which endothelial nitric oxide synthase (eNOS) is the source of superoxide consecutively peroxynitrite; and increased superoxide production from other enzymatic sources. In both situations some of the excess superoxide reacts with nitric oxide to form peroxynitrite (Figure 8).

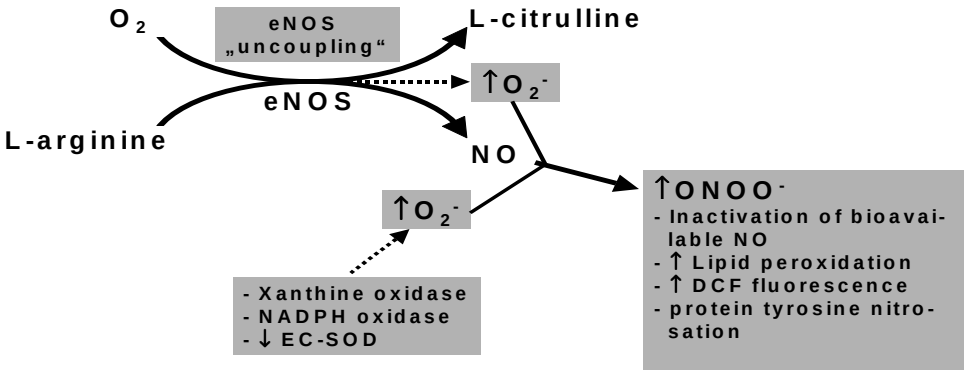

Figure 8. Hypothetical mechanism of nitric oxide synthase-dependent generation of reactive oxygen species in hyperhomocysteinemia. L-homocysteine and L-homocystine, but not the respective D-isoforms, increase membrane lipid peroxidation and DCF-fluorescence in cultured endothelial cells. This effect is dependent on eNOS and superoxide anion (O_2^-), as L-NAME and transfection of endothelial cells with superoxide dismutase cDNA, respectively, blocks hcy's effects. Increased O_2^- may result from "uncoupling" of eNOS and/or from other enzymatic sources. Some of the excess O_2^- reacts with nitric oxide (NO) to form peroxynitrite ($ONOO^-$). This reaction leads to biological inactivation of NO. Furthermore, $ONOO^-$ initiates lipid peroxidation and increases DCF-fluorescence. [92]

Bioavailability of nitric oxide is determined by the balance between its synthesis by eNOS and its inactivation by superoxide anions. The NOS enzymes use 5,6,7,8-tetrahydrobiopterin (BH_4) as a cofactor for transfer of electrons from a heme group within the oxygenase domain to L-arginine to form L-citrulline and NO. If either BH_4 or L-arginine is absent, the electrons from heme reduce oxygen to

form superoxide. This phenomenon called “uncoupling” of eNOS has been also observed during intracellular L-arginine deficiency and increased LDL-cholesterol concentrations. Topal and colleagues [120] examined the participation of eNOS in the homocysteine-induced alterations of NO/O_2^- balance in endothelial cells from human umbilical cord vein. The inhibitory effect of homocysteine on nitric oxide release was associated with increased production of reactive nitrogen and oxygen species and was suppressed by the NOS inhibitor L-NAME. L-NAME markedly decreased reactive nitrogen and oxygen species formation and the ethidium red fluorescence (indicative of superoxide formation) induced by homocysteine. This eNOS-dependent O_2^- synthesis was associated with reduced intracellular levels of both total biopterins and tetrahydrobiopterin and increased release of 7,8-dihydrobiopterin and biopterin in the extracellular medium. Their results have shown that the oxidative stress and inhibition of NO release induced by homocysteine depend on eNOS uncoupling due to reduction of intracellular tetrahydrobiopterin availability. Therefore, eNOS itself can contribute to superoxide anion formation under conditions of elevated homocysteine levels.

1.5.2.3. Elevation of the Oxidation Rate of Homocysteine in Hyperhomocysteinemia

Homocysteine, like other thiol-containing amino acids, added to plasma undergoes auto-oxidation, which is accompanied by the generation of reactive oxygen species, such as hydrogen peroxide or superoxide anion. [110]

An elevation of the oxidation rate of homocysteine and cysteine, expressed as a decreased ratio of reduced to total aminothiols in plasma, has been shown in experimental hyperhomocysteinemia after a methionine load [121-123] and in hyperhomocysteinemic patients. [124-127] This notion is supported by the observation that 1% or less of total homocysteine species consists of free homocysteine in the plasma of healthy humans under physiological conditions, whereas 5-15% consist of homocystine, another 5-15% are mixed disulfides (cysteine-homocysteine or glutathione-homocysteine), and more than 70% is protein-bound [121].

More recent evidence, however, suggests that oxidized forms of homocysteine in plasma arise primarily via disulfide exchange, with only a small

fraction resulting from direct homocysteine oxidation [128]. Hence, the contribution of direct homocysteine oxidation to homocysteine-induced vascular oxidative stress may not be as important as enzymatic mechanisms.

1.5.2.4 Inhibition of Cellular Antioxidant Enzymes by Homocysteine

The cellular defense system against reactive oxygen species includes several antioxidant enzymes and non-enzymatic antioxidants, such as α-tocopherol, ascorbic acid, β-carotene, and glutathione.

Homocysteine has been shown in particular to disrupt the normal function of two important cellular antioxidant enzymes, the cellular isoform of glutathione peroxidase (GPx-1) and superoxide dismutase. As shown in vitro and in vivo studies, homocysteine, but not other low-molecular-weight thiols, decreases both the expression and specific activity of GPx-1. [97,98,100,129,130] This key enzyme for the cellular defense against oxidant stress uses glutathione to reduce hydrogen peroxide and lipid peroxides to their respective alcohols, [131] and may also act as a peroxynitrite reductase [132]. Transition metal ions such as iron and copper catalyze the breakdown of hydrogen peroxide to form hydroxyl radical ($^{\bullet}OH$), which is highly reactive and causes lipid peroxidation, among its numerous effects; and hydroxide anion (OH^-), which promotes alkaline tissue damage. This process is offset in part by catalase and GPx-1-dependent reduction of H_2O_2 to H_2O. Elevated levels of lipid peroxides are accompanied by an increase in peroxyl radicals, which can inactivate nitric oxide through the formation of lipid peroxynitrites [133,134]. Thus a deficiency of GPx-1 may lead to a decrease in bioavailable nitric oxide via at least two mechanisms, an increase in reactive oxygen species and an increase in lipid hydroperoxides.

Extracellular superoxide dismutase is a secreted glycoprotein with an affinity for heparin-like glycosaminoglycans. It is present in the circulation in an equilibrium between the glycosaminoglycans on the endothelial surface and the plasma phase, and acts as the principal enzymatic scavenger of superoxide in the extracellular space. [135,136] The plasma levels of extracellular superoxide dismutase correlate positively with plasma homocysteine levels in homocystinuric patients [137] and in patients with mild hyperhomocysteinemia [138]. This effect is caused by

decreased binding of extracellular superoxide dismutase to endothelial cell surfaces by alterations in endothelial heparan sulfate proteoglycans by homocysteine [139]. This effect may result in a loss of the ability to protect the endothelial surface from oxidative stress, although this hypothesis has not yet been definitively proven.

In summary, experimental data suggest that elevated levels of homocysteine lead to increased vascular generation of reactive oxygen species, primarily by a mechanism involving nitric oxide synthase, and secondarily due to increased homocysteine oxidation. These effects are further amplified by alterations in the function of important cellular antioxidant enzymes. The resultant vascular oxidant stress may lead to vascular dysfunction.

1.5.3. Recruitment and Adhesion of Circulating Mononuclear Cells to the Endothelium as Marker of Homocysteine-Induced Endothelial Dysfunction

In addition to an impairment of endothelium-dependent vascular reactivity, homocysteine-induced endothelial dysfunction may be manifest as an activation of endothelial cells leading to increased expression of chemokines and adhesion molecules resulting in increased recruitment of circulating inflammatory blood cells and their increased adhesion at the endothelium. All these events are known to play an important role in the pathogenesis of early atherosclerotic lesions and their progression.

Cellular adhesion molecules, particularly members of the selectin family and immunoglobulin superfamily, are intimately involved in the recruitment of leukocytes to sites of inflammation, including developing atherosclerotic lesions, and their role in the pathogenesis of atherosclerosis has now been clearly demonstrated. Plasma levels of cellular adhesion molecules have been associated with many conventional and emerging cardiovascular risk factors and acute coronary events. They have also been proposed to be of potential value in the prediction of future cardiovascular risk, and as potential targets for therapeutic intervention.

Incubation of cultured endothelial cells with homocysteine has been shown to increase the expression of the chemoattractant proteins MCP-1 and IL-8

leading to increased chemotaxis of human peripheral blood monocytes and U937 cells. [140] Previous works showed that homocysteine increased P-selectin expression by activated / dysfunctional endothelial cells and / or platelets in mildly hyperhomocyst(e)inemic $CBS^{(-/+)}$ mice determined by ELISA for soluble P-selectin and immunostaining of aortic section [98] events which can be avoided by antioxidant therapy.

Induction of hyperhomocyst(e)inemia in apoE-null mice (by a diet enriched in methionine but depleted in folate and vitamins B_6 and B_{12}) enhanced the expression of receptors for advanced glycation end products (RAGE), VCAM-1, tissue factor (TF) and MMP-9 in the vasculature. This went along with an increase in atherosclerotic lesion area and complexity [141], pointing out that hyperhomocysteinemia may promote atherosclerotic lesion progression via an enhanced vascular inflammatory response. The mechanisms underlying these observations are not yet clear.

Reduced bioavailability of nitric oxide promoting P-selectin expression [98] and/or activation of the redox sensitive transcription factor NF-κB regulating adhesion molecules expression, tissue factor and RAGE expression [141] may be involved in the vascular inflammatory response to hyperhomocysteinemia.

1.5.4. Reduced Endothelial Progenitor Cell Recruitment and Proliferation and Increased Endothelial Cell Apoptosis in Hyperhomocysteinemia

Studies from several groups have shown that homocysteine decreases the proliferation rate of cultured vascular endothelial cells and induces programmed cell death in cultured endothelial cells which might contribute to endothelial cell injury in hyperhomocysteinemia. [129,142,143]

Chen and collaborators investigated whether homocysteine has influences on endothelial progenitor cells number and activity. [144] Incubation of isolated human mononuclear cells with homocysteine decreased the number of endothelial progenitor cells in a dose and time dependent way, a maximum effect could be observed when incubating with 200 µmol/L for 24 hours. In addition, homocysteine dose and time dependently impaired endothelial progenitor cells capacity to adhere and to induce vasculogenesis in vitro.

Zhang and colleagues [142] showed that homocysteine induced programmed cell death in human umbilical vein endothelial cells as measured by the TdT-mediated dUTP nick end labeling assay, DNA ladder formation, induction of caspase 3-like activity, and cleavage of procaspase 3. Homocysteine-induced cell death was specific to homocysteine, was not mediated by oxidative stress, and was mimicked by inducers of the unfolded protein response (UPR), a signal transduction pathway activated by the accumulation of unfolded proteins in the lumen of the endoplasmic reticulum.

These data are confirmed by another study, which shows that homocysteine induces upregulation of Fas in endothelial cells in a dose-dependent manner. Homocysteine-induced Fas expression was inhibited by the treatment with catalase. In addition, homocysteine inhibited expression of the caspase-8 inhibitor FLICE-inhibitory protein (FLIP). These data suggest that upregulation of Fas expression and downregulation of FLIP may be a mechanism through which homocysteine induces endothelial cell apoptosis. [145]

1.5.5. Inhibition of Endothelial Cell Thromboresistance by Homocysteine

The mechanisms by which homocysteine may contribute to atherogenesis may involve thrombogenic mechanisms: homocysteine increases thromboxane-mediated platelet aggregation in vitro likely by reducing nitric oxide bioavailability [146] or by decreasing the biosynthesis of prostacyclin, [147,148]; increases tissue factor expression [149] and the activation of factor V to factor Va, [150] thus favouring thrombin generation through initiation and propagation of the coagulation cascade. In addition, homocysteine may inhibit inactivation of factor Va by activated protein C through homocysteinylation of the cofactor, and may reduce the binding of antithrombin III to the endothelial surface through inhibition of heparan sulphate expression. [151] Homocysteine decreases the expression of endogenous anticoagulants and inhibits fibrinolysis [152,153]. It modulates thrombomodulin expression and activity, and thrombomodulin-dependent activation of the anticoagulant protein C by thrombin. [154] All these observations suggest that hyperhomocysteinemia promotes thrombosis. However, most of the in vitro studies mentioned above were performed with homocysteine concentrations

above pathophysiologically relevant levels, or were reproduced with cysteine, and, thus, could be related to the non-specific reactivity of the sulfhydryl group of homocysteine.

Support of the hypothesis that mild hyperhomocysteinemia induces a prothrombotic state in vivo is derived from animal studies and clinical observations. Dietary folate deficiency in rats induces platelet hyperreactivity and increases macrophage tissue factor activity, effects that correlate with increases in homocysteine levels. Plasma homocysteine concentrations induced by methionine challenge in rats correlated with platelet aggregation and increased synthesis of the proaggregant thromboxane A2. Increased thromboxane A2 synthesis has also been documented in homocystinuric patients. [155] In addition, plasma homocysteine concentrations were found to correlate with plasma fibrinogen levels in mildly hyperhomocysteinemic patients with coronary heart disease, [63] and increased levels of von Willebrand factor were found in mildly hyperhomocysteinemic patients with premature arterial disease and in healthy individuals after methionine challenge. [156] Together with a reduction of the antiplatelet effects of nitric oxide by homocysteine, these findings further support the view that increased platelet activation and aggregation may be involved in the development of thrombosis in hyperhomocysteinemia.

1.6. Influence of Homocysteine on Circulating Mononuclear Cells

In vivo and in vitro studies have shown that homocysteine not only influences the endothelium but also the phenotype of circulating mononuclear cells. The changes at both levels may contribute to accelerated formation and promotion of atherosclerotic lesions.

Beauchamp et al. [157] have shown that homocysteine increased - in a time- and dose-dependent manner - lipoprotein lipase mRNA expression and secretion in J774 macrophages, the levels of c-fos mRNA and enhanced nuclear protein binding to the AP-1 sequence of the lipoprotein lipase gene promoter. The experiments demonstrated that homocysteine-induced lipoprotein lipase mRNA expression requires PKC activation. These observations suggest a new mechanism by which homocysteine may exert its proatherogenic effects in human diabetes.

Another set of experiments revealed that VEGF mRNA was upregulated by homocysteine in a dose- and time-dependent manner in THP-1 macrophages accompanied by an increase in VEGF secretion. Importantly, other sulfur compounds, such as methionine and cysteine, showed no effect on VEGF expression, indicating that homocysteine specifically induced VEGF. These findings suggest that hyperhomocysteinemia could promote the development of atherosclerotic lesions through VEGF induction in macrophages. [158]

The induction of oxidant stress in hyperhomocysteinemia was confirmed by the finding that human neutrophils exposed to homocysteine released superoxide anion to the extracellular medium, and that this effect could be inhibited by superoxide dismutase and diphenyleneiodonium (DPI), an inhibitor of NADPH oxidase activity. In addition, homocysteine increased intracellular H_2O_2 production by neutrophils, enhanced the activation and phosphorylation of mitogen-activated protein kinases (MAPKs), specifically p38-MAPK and ERK1/2, and increased migration of neutrophils. This is the first evidence that homocysteine enhances the oxidative stress of neutrophils, and underscores the potential role of phagocytic cells in vascular wall injury through superoxide anion release under hyperhomocysteinemic conditions. [159]

In vivo experiments using methionine feeding in C57BL/6 mice resulting in hyperhomocysteinemia showed that superoxide anion radical levels in peritoneal macrophages and NF-κB binding activities were higher in the methionine group than in the control group. These data suggest that hyperhomocysteinemia may intensify disturbances in peroxidation and inflammatory mediator activation in peritoneal macrophages, which could be a possible mechanism of homocysteine's atherogenic effects. [160]

1.6.1. Increased Recruitment and Chemotaxis of Homocysteine-Stimulated Monocytes to Unstimulated Endothelial Cells

Homocysteine-mediated leukocyte-endothelial cell interaction is one potential mechanism by which hyperhomocysteinemia may lead to the development of atherosclerosis due to promotion of vascular inflammation.

In vitro studies performed by Koga and collaborators revealed a significant increase in monocyte (U937) adhesion to HAEC when only U937 cells were treated with homocysteine or when both cell types were treated with homocysteine. The process seemed to be mediated by VCAM-1. [161]

Monocyte chemoattractant protein-1 (MCP-1), which is a potent chemokine that stimulates the migration of monocytes into the intima of arterial walls, seems to be modulated by homocysteine at the expression level. Wang et al. [162,163] showed that homocysteine induces monocyte chemoattractant protein-1 expression in vascular smooth muscle cells and THP-1 macrophages by activating NF-kB. The same group has also shown that homocysteine stimulates the expression of the MCP-1 receptor (CCR2) in human monocytes, an effect that is possibly mediated by oxygen-derived free radicals. [164]

1.6.2. Possible Involvement of Inducible Nitric Oxide Synthase Enzyme in Homocysteine-Induced Oxidative Stress in Monocytes

Isolation of iNOS, a 130 kDa cytoplasmatic protein, was first reported in macrophages where its activity was found to be inducible in response to stimuli such as proinflammatory cytokines or endotoxin. Expression of iNOS has now been reported in a large number of cell types, and in most circumstances, the enzyme is inducible. The iNOS gene is under transcriptional control although activity is also influenced by a variety of other control mechanisms that affect mRNA stability, translation and degradation of the protein, and availability of substrate and cofactors. iNOS activity is not responsive to changes in intracellular Ca^{2+} levels and thus this isoform is capable of a high output and long-lasting release of nitric oxide far exceeding that of the other isoforms. iNOS produces physiological concentrations of nitric oxide in the nanomolar range whereas the Ca^{2+}-dependent isoforms produce picomolar concentrations of nitric oxide. iNOS, like the other two members of NOS family, requires a number of cofactors and prosthetic groups for activity including FAD, FMN, heme, calmodulin, and tetrahydrobiopterin.

The iNOS expression in mouse macrophages was shown to be induced by activated NF-κB [165,166]. The promoter of iNOS contains two consensus

sequences for the binding of NF-κB, which mediates LPS-inducibility [165]. The heterodimer of p65/p50 has been reported as a NF-κB complex responsible for LPS-induced iNOS expression.

1.6.3. Activation of NF-κB in Monocytes and Endothelial Cells by Oxidant Stress

The transcription factor NF-κB is activated by a variety of stimuli and regulates diverse gene expressions and biological responses. The stimuli include bacterial endotoxin, lipopolysaccharide (LPS), ionizing radiation, and carcinogens that are often associated with inflammatory diseases or tumor development. Recent observations support that NF-κB activation also may be important in pathogenesis of chronic diseases such as atherosclerosis and diabetes. [167] Activated NF-κB was found in atherosclerotic lesion [168]. Physiological modulation and pathological activation of the NF-κB system may contribute to the changes in gene expression that occur during atherogenesis. Diverse stimuli activate NF-κB, through the phosphorylation and activation of the IκB kinase (IKK) complex. This complex consists of IKK-α and IKK-β heterodimers, a number of IKK-γ subunits, and possibly other components whose significance is less certain. The activated IKK complex specifically phosphorylates IκBs, which are then rapidly polyubiquitinated, targeting them for degradation by the proteosome. Following release from the inhibitor, NF-κB dimers translocate from the cytoplasm to the nucleus, where they bind target genes and stimulate transcription.

NF-κB activates a variety of target genes relevant to the pathophysiology of the vessel wall, including cytokines, chemokines, and leukocyte adhesion molecules, as well as genes that regulate cell proliferation and mediate cell survival. For example, human monocytic cell (THP-1)-derived macrophages incubated with pathophysiologic concentrations of homocysteine were shown to have significantly enhanced expression of MCP-1 mRNA (up to 2.6-fold) and protein (up to 4.8-fold) due to increased translocation of NF-κB. [163]

NF-κB is one of the transcription factors that may be controlled by the redox status of the cell. [169] Indeed, generation of reactive oxygen species may be a common step in all of the signalling pathways that lead to IκB degradation and

NF-κB nuclear accumulation. Support for this concept comes from a variety of studies showing that the diverse agents that can activate NF-κB also elevate levels of reactive oxygen species and that chemically distinct antioxidants, as well as overexpression of antioxidant enzymes, can inhibit NF-κB activation. [169]

Au-Yeung et. al [188] showed that oxidant stress due to hyperhomocysteinemia results in increased activation of the redox-sensitive transcription factor NF-κB in endothelial cells. Other studies have revealed that pathophysiological levels of homocysteine can alter human monocyte function by upregulating MCP-1 and IL-8 expression and secretion via enhanced formation of intracellular ROS originated from NAD(P)H oxidase source via calmodulin or protein kinase C signaling pathways. Homocysteine-induced ROS generation subsequently activates mitogen-activated protein kinase (p38 and ERK1/2) and NF-κB in a PPARγ activator–sensitive manner. [170] However, a direct role of reactive oxygen species in signalling to NF-κB remains to be proven.

2. HYPOTHESIS OF THE STUDY

The purpose of this thesis is to show potential mechanisms by which pathophysiologically relevant concentrations of homocysteine influence the normal interaction between cultured endothelial cells and cultured mononuclear cells, and thereby promote vascular inflammation.

The specific hypotheses for these in vitro experiments can be summarized as follows:

- homocysteine enhances the expression of adhesion molecules on endothelial cells and monocytes (i.e. P-selectin, E-selectin, ICAM-1, VCAM-1, PECAM-1, TF, RAGE, β-2 integrins) leading to increased adhesion of mononuclear cells to the endothelium;
- these effects are mediated by increased intracellular reactive oxygen species and/or a depletion of bioavailable NO and enhanced NF-κB activity;
- only the naturally occurring L-stereoisomer of homocysteine can promote the activation of endothelial cells and monocytes.

3. MATERIALS AND METHODS

3.1. Chemicals and Cell Culture Ware

D,L-homocysteine (D,L-hcy) and L-homocysteine thiolactone hydrochloride were from Sigma Chemical Co, D-homocysteine thiolactone hydrochloride and L-cysteine (L-cys) were from Fluka. L-homocysteine thiolactone hydrochloride and D-homocysteine thiolactone hydrochloride were hydrolyzed to L-homocysteine (L-hcy) and D-homocysteine (D-hcy), respectively, using the following protocol. 15.36 mg of the thiolactone were dissolved in 1 mL H_2O. After that 1 mL of 0.1 M NaOH was added and the solution was incubated 15 minutes at 37°C. The pH was adjusted with 1N NaOH to 9. [171,172] Final thiol concentrations were determined spectrophotometrically using Ellman's reagent. D,L-homocysteine and L-cysteine were freshly prepared as 100 mmol/L stock solutions in DMEM 2% FBS.

Recombinant human tumor necrosis factor (TNF-α), Tiron (4,5-dihydroxy-1,3-benzenedisulfonic acid disodium salt pyrocatechol-3,5-disulfonic acid disodium salt), L-sepiapterin (S(-)-2-Amino-7,8-dihydro-6-(2-hydroxy-1-oxopropyl)-4(1H)-pteridinone), lipopolysaccharides from Escherichia coli O26:B6 were from Sigma Chemical Co.

Monoclonal mouse anti-human antibodies against ICAM-1 (clone 8.4 A6), E-selectin (clone1.2 B6) and PECAM-1 (clone JC 70A) and the respective murine IgG1 isotype control were from Sigma Chemical Co, monoclonal mouse anti-human antibody against VCAM-1 (clone 1.4 C3) and rabbit anti-mouse IgG antibody coupled with FITC were from DAKO. Monoclonal mouse anti-human antibodies against LFA-1β (CD18; clone R3.3), and Mac-1α (CD11b; clone LM2/1) were from Bender MedSystems.

BCECF/AM (2',7' – bis - (2 - carboxyethyl) – 5 - (6') - carboxyfluorescein acetoxymethyl ester) prepared as 1 mg/mL stock in dimethylsulfoxide (DMSO) and 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA) prepared as 100 mmol/L stock in DMSO were from Calbiochem. L-012 (8-amino-5-chloro-7-phenylpyridol (3,4-d) pyridazine-1,4 (2H,3H) dione) was obtained from Aventis.

The cell-permeable superoxide dismutase (SOD) mimetic and peroxynitrite scavenger MnTBAP (Mn(III)tetrakis (4 - benzoic acid) porphyrin

chloride) was from Calbiochem and prepared as 5 mg/mL stock in water. The cell-permeable SOD mimetic Tiron (4,5-Dihydroxy-1,3-benzenedisulfonic acid disodium salt) was purchased from Sigma.

E3-((4-Methylphenyl)sulfonyl)-2-propenenitrile (BAY 11-7082 10 μmol/L in DMSO; Sigma Chemical Co.) was used to decrease nuclear translocation of NF-κB. BAY11-7082 inhibits cytokine-induced IκB-α phosphorylation and thereby blocks NF-κB activation. [173]

Cell culture media (DMEM and RPMI-1640), fetal bovine serum (FBS), and antibiotics (penicillin G sodium and streptomycin sulfate) were from Gibco BRL, distributed by Invitrogen. Medium 199 was from Biomol, and endothelial growth medium from PromoCell. Accutase was obtained from PAA Laboratories. CellFix and CellWash buffer was from Becton Dickinson. All other reagents were from Sigma Chemical Co.

Cell culture ware was obtained from Nunc Labware.

3.2. Cell Lines

Experiments were performed using the endothelial cell line *EA.hy 926* cells and the monocytic cell line *THP-1.* To reproduce the most important findings using primary human endothelial cells, selected experiments were repeated using freshly isolated human umbilical vein endothelial cells (*HUVEC*).

EA.hy 926 cells [174], derived by fusing human umbilical vein endothelial cells with the permanent human cell line A549/8, 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 markers like endoglin, tissue factor, thrombomodulin [175], adhesion molecules [175] and secrete chemokines [176,177]. Cells were maintained in DMEM containing 4,500mg\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℃ 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.

Primary human umbilical vein endothelial cells (*HUVEC*) were isolated and cultivated in 25 cm^2 culture plates in medium 199 containing penicillin/streptomycin and 10% FBS mixed with endothelial cell growth medium (1:1, vol/vol) as previously described. [178] 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 [179]. Cells were cultured in RPMI-1640 supplemented with 10% FBS and antibiotics (100 units/mL penicillin G sodium and 100 µg/mL streptomycin sulfate).

3.3. Fluorescent Labeling of Monocytes

For quantitative adhesion assays, THP-1 cells were fluorescently labeled with BCECF/AM. [161] BCECF/AM is a variable mixture of at least two compounds, which are cell-permeable ester derivatives of BCECF. BCECF/AM is non-fluorescent, colorless and lipophilic. It is intracellularly cleaved by esterases to yield the highly charged fluorescent BCECF that is retained by viable cells. This compound has been used to monitor intracellular pH changes in mammalian fibroblasts, gastric cells, lymphocytes and myocytes. Typical loading buffer contains 1-10 µmol/L BCECF/AM in serum-free media, which is free of amino acids and buffers containing primary or secondary amines.

THP-1 cells were washed three times in serum-free Hank's balanced salt solution (HBSS) and pelleted by centrifugation with a Beckman Coulter centrifuge (GH 3.8) for 5 minutes, 800 rpm, at room temperature. 1 x 10^7 cells/mL were incubated with 1 µmol/L BCECF/AM in serum free HBSS (prepared as 1 mg/mL stock in anhydrous DMSO and stored at -20°C) for 30 minutes at 37°C and 5% CO_2. Cells were then washed three times with HBSS containing 2 mmol/L Ca^{2+}, 2 mmol/L Mg^{2+}, 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 x 10^5 cells/mL. BCECF/AM is also used for apoptotic tests. An influence of this dye

when is used under the conditions described above, on the viability or activation of cells has not been reported so far. [161]

3.4. Static Adhesion Assay

Adhesion of THP-1 cells to treated endothelial cells was studied under static conditions. [180] Briefly, endothelial cells were cultured to confluence in 2-well cover slides and treated with varying concentrations of D,L-homocysteine, L-homocysteine, D-homocysteine and L-cysteine, and the respective positive (TNFα, 50 ng/mL) and negative controls (plain medium) for the indicated periods of time. After incubation, endothelial cells were washed twice with phosphate buffered salt solution containing calcium and magnesium (PBS^{++}) for avoiding detachment of cells from the culture plate.

BCECF-labeled THP-1 cells (5×10^5 cells/mL/well) were coincubated with endothelial cells for 30 minutes at 37°C under slight agitation on a rotating platform. After coincubation, non-adherent cells were removed by gentle washing of each well three times with $HBSS^{++}$ (containing 2 mmol/L Ca^{2+}, 2 mmol/L Mg^{2+}, 20 mmol/L HEPES). 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, endothelial cells were incubated for 6 hours with homocysteine and the respective controls, followed by one hour incubation with an anti-human ICAM-1 antibody (2 µg/mL) or the respective isotype control antibody before addition of THP-1 cells. The monoclonal anti-ICAM-1 (CD54) antibody (clone 8.4A6) used is an IgG1 isotype and recognizes the epitope localized in the D2 domain of this cell surface molecule and is recommended to study ICAM-1 mediated monocyte adhesion. [181]

To evaluate the role of the cellular redox state in the adhesion of mononuclear cells on homocysteine incubated endothelial cells, endothelial cells cultured in 2-well cover slides were coincubated with D,L-homocysteine 200 µmol/L and 50 µmol/L MnTBAP or 4 mmol/L Tiron, respectively for 6 hours at 37°C before static adhesion assays were performed. MnTBAP is a cell-permeable superoxide dismutase (SOD) mimetic and peroxynitrite scavenger but does not

scavenge nitric oxide (NO). Tiron is a cell-permeable superoxide scavenger. After, adhesion assays were performed as described above, adherent cells were counted.

In another set of experiments THP-1 cells were incubated 4 hours with varying test substances including homocysteine to induce phenotypic changes. After several washing steps, THP-1 cells were labeled with BCECF and the functional role of monocyte incubation with homocysteine was studied by coincubating them with native endothelial cells and quantifying adherent monocytes as described above.

In selected experiments, THP-1 cells were preincubated for 4 hours with sepiapterin followed by 4 hours incubation with homocysteine. The role of the cellular redox state in the adhesion of homocysteine incubated mononuclear cells on unstimulated endothelial cells was tested by 4 hours coincubation with Tiron and homocysteine. For blocking studies THP-1 cells were incubated for 4 hours with homocysteine, followed by 1 hour incubation with IgG1 (for blocking the unspecific sites) and another hour with an anti-human LFA-1β antibody (2 μg/mL) or the respective IgG isotype control.

3.5. Determination of Cell Adhesion Molecules Expression by Flow Cytometry

The expression levels of the adhesion molecules ICAM-1, VCAM-1, E-selectin, and PECAM-1 on homocysteine-treated EA.hy 926 cells and HUVECs were quantified by flow cytometry. Cell monolayers cultured in P60 dishes were washed with phosphate-buffered saline without calcium and magnesium (PBS^{--}) and incubated with Accutase™ for 5 minutes at 37℃ until the cells were detached from the culture dish. Accutase™ is a mixture of proteolytic and collagenolytic enzymes from non-mammalian source (free of viruses common in trypsin) used for detaching adherent cells. After collecting from the plates, the cells were treated with CellFix™ (Becton Dickinson), pelleted by centrifugation and resuspended in PBS + 1% bovine serum albumin for blocking unspecific sites. After resuspension, cells were reacted with mouse anti-human ICAM-1, anti-human VCAM-1, anti-human PECAM-1 and anti-human E-selectin antibodies. After washing procedure,

the cells were labeled with rabbit anti-mouse FITC-labeled immunoglobulins, washed, and the fluorescence of ten thousands cells was measured on a FACScan flow cytometer (Becton Dickinson).

The expression levels of β_2-integrins LFA-1β and Mac-1α on THP-1 cells were also quantified by flow cytometry. THP-1 cells were washed with PBS^{--}, treated with CellFix, and pelleted by centrifugation. After resuspension in CellWash buffer, cells were labeled with the respective FITC-labeled antibodies (anti-human LFA-1β and Mac-1α).

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 antibody (Mouse IgG1, Kappa - MOPC-21) and subtracted.

3.6. Measurement of Intracellular Reactive Oxygen Species Generation in Homocysteine-Treated Endothelial Cells and Monocytes

The intracellular generation of reactive oxygen species was evaluated by monitoring the oxidative conversion of the fluorophore dichlorofluorescein (DCF) as described previously [182]. DCF is used for the detection of reactive oxygen species (ROS) and for the determination of the degree of overall oxidative stress. [183] The diacetate of DCF (DCF-DA) is a cell-permeable modification of this fluorogenic probe, which rapidly enters cells due to its lipophicity. Intracellularly, the ester bonds are cleaved, resulting in a more hydrophilic compound (DCF) which is retained in the cells. Briefly, EA.hy 926 cells, grown to confluence on two-well cover slides, were incubated with the test substances for 6 hours and then loaded for 15 minutes at 37°C at darkness with the membrane-permeable dye DCF-DA (10 µmol/L) in modified Tyrode's buffer (135 mmol/L NaCl, 2.7 mmol/L KCl, 1.8 mmol/L $CaCl_2$, 0.28 mmol/L NaH_2PO_4, 0.49 mmol/L $MgCl_2$, 5.5 mmol/L D-Glucose, 20 mmol/L HEPES, pH = 7.4). After washing the cells several times, the intracellular 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 were expressed as arbitrary fluorescent units relative to control conditions. Thrombin-receptor activating peptide (TRAP, Bachem) 10 µmol/L was used as positive control. The peptidic sequence of TRAP is H-Ser-Phe-Leu-Leu-Arg-Asn-NH_2.

To monitor the intracellular generation of reactive oxygen species inside THP-1 cells, THP-1 cells were incubated with the test substances for 4 hours. After washing, cells were incubated in modified Tyrode's buffer containing 10 µmol/L DCF-DA for 15 minutes at 37°C, in the dark. THP-1 cells loaded with DCF-DA and incubated for 15 minutes at 37°C with 10 mmol/L H_2O_2 were used as positive control for the method. The fluorescence intensities were measured by flow cytometry (excitation 488 nm, emission >515 nm). Nonspecific fluorescence was detected by using unstained cells and substracted.

The superoxide release from homocysteine-incubated THP-1 cells was detected by the L-012 chemiluminescence assay. [184] Briefly, THP-1 cells were incubated with the test substances for 4 hours. After washing, cells were resuspended in modified Tyrode's buffer at the density of 50.000 cells per 330 µl. In each tube 30 µl of L-012 (100 µmol/L) was added just before measuring the luminescence. The kinetics of oxidative burst in homocysteine-incubated THP-1 cells was recorded with a Lumat 9507 on addition of L-012. Photon emission was expressed as percent increase in relative light units versus control conditions.

3.7. Isolation of Total RNA and Real-time RT-PCR for ICAM-1 RNA Expression by Endothelial Cells

EA.hy 926 cells were cultured to confluence in P100 dishes and incubated for 6 hours at 37°C with the test substances. After washing with PBS the cells were lysed directly in a culture dish by adding 3 mL of TRIZOL Reagent (Gibco BRL), and passing the cell lysate several times through a pipette.

TRIZOL is a monophasic solution of phenol and guanidine isothiocyanate. Addition of chloroform followed by centrifugation, separates the solution into an aqueous phase and an organic phase. RNA remains exclusively in the aqueous phase. After transfer of the aqueous phase, the RNA is recovered by precipitation with isopropyl alcohol. At the end, RNA is dissolved in 60 µl (30 µl/tube) RNase-

free water by passing the solution a few times through a pipette tip and incubated for 10 minutes at 60℃. The RNA concentration was determined spectrophotometrically and the samples were stored at -80℃.

Real time RT-PCR was performed as previously described [185] using the dsDNA-binding dye SYBR Green I and the TaqMan ABI 7700 Sequence Detection System (AppliedBiosystems) for real time quantification [185]. Briefly, 1 µg of total RNA was reverse transcribed in a 40 µl reaction, containing 8 µl first strand buffer (5x), 2 µl 100 mmol/L DTT (both Life technologies), 0.8 µl 25 mmol/L dNTP (Amersham Pharmacia), 1 µl RNase inhibitor (RNasin, Promega), 0.5 µl Microcarrier (Molecular Research Center), 0.86 µg random hexamers (2 mg/mL stock, Roche), and 172 U reverse transcriptase (Superscript I, Life technologies) for one hour at 42℃. Sequence specific amplification was done using heat activated TaqDNA polymerase (Amplitaq Gold, Applied Biosystems) and oligonucleotide primers specific for human ICAM-1 cDNA (forward primer: 5' TGGCAACGACTCCTTCTCG; reverse primer: 5' AGCTGTAGATGGTCACTGTCTGCA, 300 nmol/L each) on a TaqMan ABI 7700 Sequence Detection System (AppliedBiosystems). After an initial denaturation step for 10 minutes at 95℃, the samples were cycled 40 times at 95℃ for 15 seconds and 60℃ for 60 seconds [186] 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 ddH_2O were negative in all runs. All measurements were performed in duplicates.

3.8. 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 in immunofluorescence images as described [187].

Briefly, confluent EA.hy 926 cells were cultured in 2-well cover slides and treated with the test substances as indicated. Cells were then washed with PBS^{++}, fixed with 2% formalin solution and subsequently permeabilized by submersion in 0.2% Triton X-100. After washing again three times with PBS^{++} and blocking the unspecific sites with Blocking Buffer for 10 minutes at room temperature, under slight agitation, the samples were then incubated with the primary antibody (rabbit anti human p65 subunit) 45 minutes at 37℃, and finally treated with the secondary antibody (FITC labeled, goat anti rabbit) for 30 minutes at 37℃.

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.

3.9. Statistical Analysis

Values are reported as means ± SEM. Differences in time-responses and dose-responses to the test substances between groups were tested with two-way repeated measures ANOVA with posthoc anaylsis performed using Fisher's test and Bonferonni/Dunn procedures. Other data were analyzed by factorial ANOVA and posthoc comparisons. Analyses were performed using the STATVIEW software package (Abacus Concepts). Statistical significance was defined as a *P* value less than 0.05.

4. RESULTS

4.1. Effects of Homocysteine on Endothelial Cells

4.1.1. Time- and Dose-Dependent Increase in Monocyte Adhesion to Homocysteine-Incubated Endothelial Cells

Incubation of EA.hy 926 cells with 200 µmol/L D,L-homocysteine for 30 minutes to 24 hours resulted in a time-dependent increase in the number of adhering THP-1 cells compared to baseline THP-1 monocyte adhesion to unstimulated EA.hy 926 monolayers (baseline: 7.6 ± 1.7 cells per microscopic field). The effect of homocysteine on the adhesion was statistically significant from baseline between 3 and 8 hours of incubation, peaked after 6 hours (28.2 ± 1.1 cells per microscopic field), and declined thereafter. All further experiments were performed with incubation times of 6 hours.

Incubation of EA.hy 926-cells with increasing concentrations of D,L-homocysteine (10 to 200 µmol/L) for 6 hours leads to a dose-dependent and significant increase in the number of adhering THP-1 cells to endothelial cells up to a 4.5 fold at the highest concentration studied compared to control conditions (control: 5.9 ± 1.2 cells per microscopic field, D,L-homocysteine 200 µmol/L: 33.1 ± 5.6 cells per microscopic field; n = 5 experiments; *$P < 0.05$). Incubation with L-cysteine had no significant effect (Figure 9).

These findings were reproduced using primary human endothelial cells (HUVEC) incubated with 200 µmol/L homocysteine (control: 7.5 ± 0.96 cells per microscopic field, D,L-homocysteine 200 µmol/L: 26.33 ± 0.75 cells per microscopic field; n = 3 experiments; *$P < 0.05$).

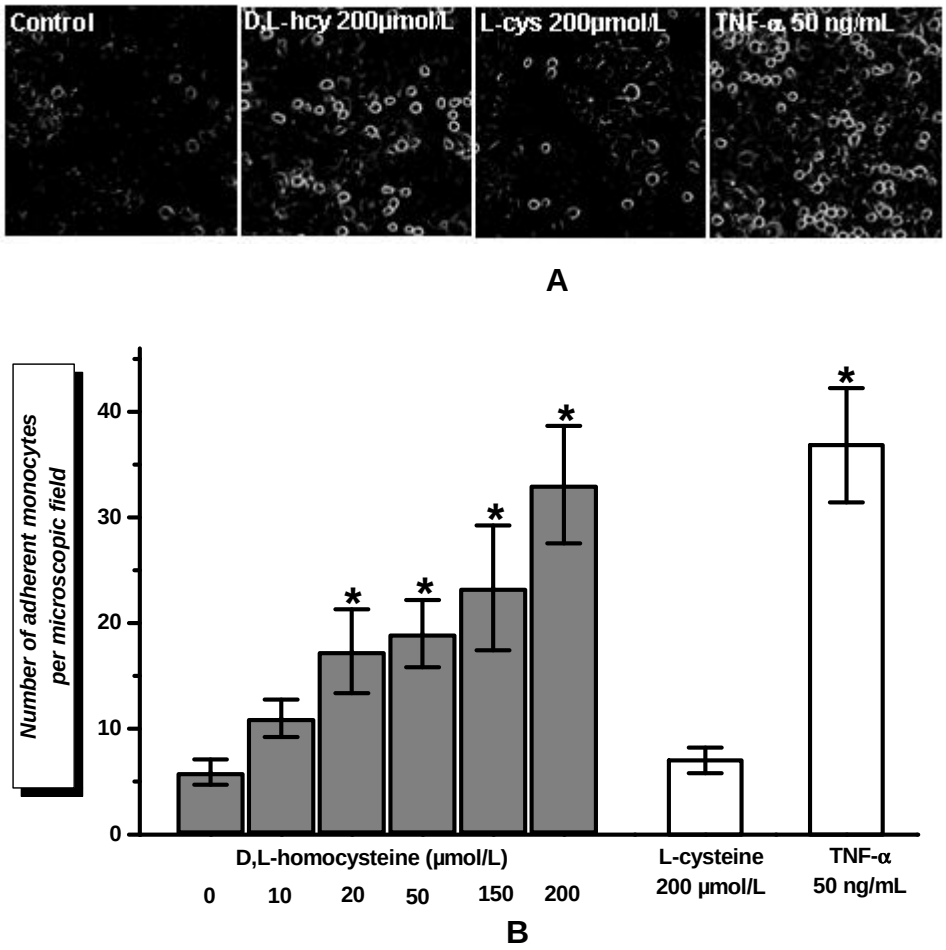

Figure 9. A – Representative photomicrographs of static adhesion assays. TNF-α and homocysteine stimulation of EA.hy 926 cells lead to a significant increase in adhesion of fluorescence labeled monocytes. **B** – Quantitative analysis of dose-dependent adhesion of monocytes to homocysteine-incubated EA.hy 926 cells. Six hours incubation of endothelial cells with increasing concentrations of D,L-homocysteine leads to a dose-dependent increase in adherent monocytes up to four-fold compared to control conditions. L-cysteine had no effect. TNF-α (50 ng/mL) was used as positive control. (n = 5 experiments; **P* < 0.05 vs. control)

4.1.2. Stereospecific Increase in Monocyte Adhesion to Homocysteine–Incubated Endothelial Cells

To examine whether the effect of homocysteine is stereospecific for the natural occurring L-isoform, or independent from the stereoisoform, endothelial cells were incubated with 200 µmol/L D,L-, L-, or D-homocysteine.

Incubation of endothelial cells with D,L-homocysteine and L-homocysteine dose-dependently and significantly increased monocyte adhesion to endothelial cells up to almost fivefold compared to control. In contrast, D-homocysteine had no significant effect, indicating that the stimulatory effects of homocysteine are specific for the naturally occurring L-stereoisomer. (Figure 10)

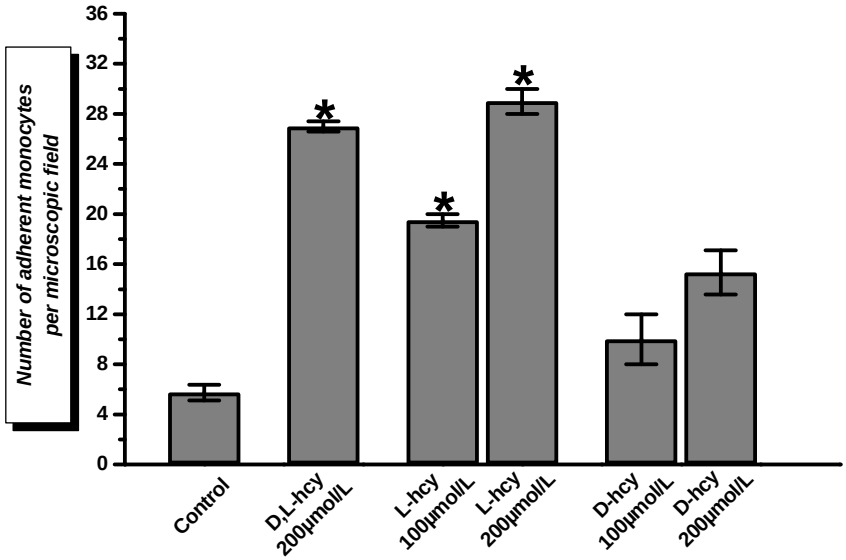

Figure 10. Stereospecific effect of L-homocysteine on monocyte adhesion to EA.hy 926 cells. Incubation of endothelial cells with different stereoisomers of homocysteine only lead to significantly increased monocyte adhesion using the naturally occuring L-homocysteine, and D,L-homocysteine, but not using D-homocysteine. (n = 4 experiments; **P*<0.05 vs. control)

4.1.3. Adhesion Molecules Expression on Homocysteine–Stimulated Endothelial Cells

The endothelial expression of adhesion molecules, like "CAMs" or "selectins", are known to mediate monocyte adhesion to endothelial cells. To explore which adhesion molecule might be involved in homocysteine-induced monocyte adhesion to endothelial cells, the expression levels of different adhesion molecules on endothelial cells were studied in untreated and homocysteine-incubated endothelial cells (200 µmol/L for 6 hours) using FACS analysis.

D,L- and L-homocysteine-incubated EA.hy 926 cells and HUVECs showed a significant increase in ICAM-1 protein expression (Table 3). This effect was specific for homocysteine but not for other thiols, and was specific for the L-stereoisomer of homocysteine, as D-homocysteine and L-cysteine (200 µmol/L for 6 hours) respectively, had no effect on either of the adhesion molecules tested. VCAM-1, PECAM-1, or E-selectin protein expression was not stimulated by any homocysteine species used (data not shown).

Table 3. Relative ICAM-1 expression on EA.hy 926 cells and HUVECs as determined by flow cytometry. Values indicate mean ± SEM, n = 4 experiments, ***P* < 0.05 vs. control.

Condition	***ICAM-1 expression (% Control) on EA.hy 926 cells***	***ICAM-1 expression (% Control) on HUVECs***
Control	100 ± 0	100 ± 0
D,L-homocysteine 200µmol/L	130.6 ± 6.5 *	163.4 ± 9.8 *
L-homocysteine 200µmol/L	146.1 ± 12.9 *	
D-homocysteine 200µmol/L	101.2 ± 12.4	

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

To confirm the functional relevance of increased ICAM-1 expression on monocyte adhesion to homocysteine-incubated endothelial cells, EA.hy 926 and HUVECs, respectively, were incubated with 200 µmol/L D,L-homocysteine for 6 hours, followed by one hour incubation with IgG1 Ab (for blocking the unspecific sites) and then one hour with a blocking antibody against human ICAM-1 or the respective isotype control, and monocyte adhesion assays were performed.

Preincubation with an anti-ICAM-1 antibody markedly reduced homocysteine-induced monocytes adhesion to endothelial cells (from 26.33 ± 0.75 to 12.18 ± 0.51 in HUVECs, and from 25.75 ± 0.854 to 11.25 ± 2.394 in EA.hy 926 cells, respectively; n = 3 experiments, $^{\$}P < 0.05$), but did not bring it back to control levels. (Figure 11)

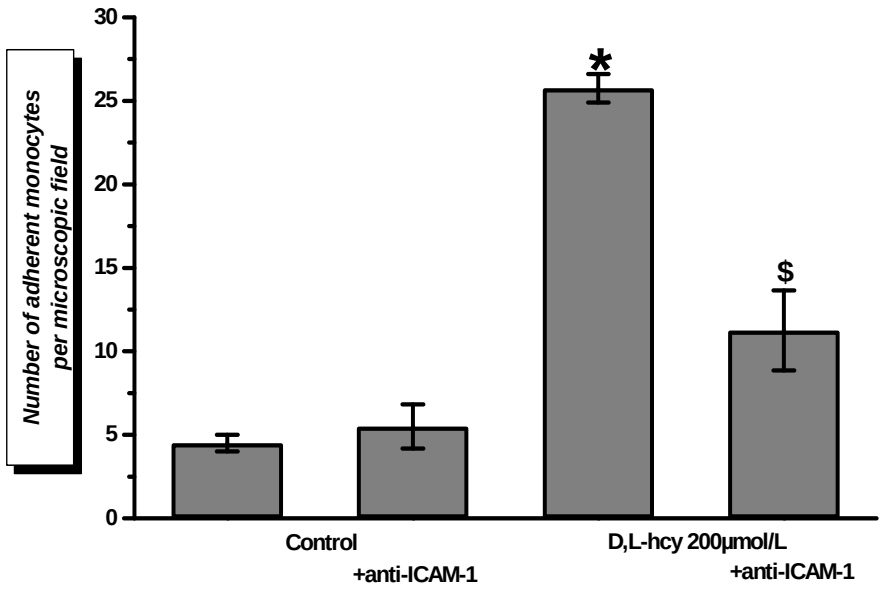

Figure 11. Monocyte adhesion to homocysteine-incubated EA.hy 926 cells could be blocked by an anti-human ICAM-1 antibody. (n = 4 experiments,*P<0.05 vs. control, $^{\$}$P<0.05 vs. D,L-homocysteine 200µmol/L)

These experiments show that an increase in ICAM-1 expression is at least partly responsible for homocysteine's effects on monocyte adhesion to endothelial cells.

4.1.4. Dose-Dependent and Stereospecific Generation of Intracellular Reactive Oxygen Species in Homocysteine-Incubated Endothelial Cells

One well-supported but not universally accepted hypothesis suggests that elevated levels of homocysteine lead to increased vascular oxidant stress, which may result in endothelial dysfunction.

To monitor the intracellular generation of reactive oxygen species, EA.hy 926 cells grown on cover slides were incubated with increasing concentrations of D,L-homocysteine (20 to 200 µmol/L), L-cysteine (200 µmol/L) or sham treated, loaded with the redox-sensitive dye DCF-DA, and the intracellular fluorescence intensities were measured after 6 hours using a confocal laser scanning microscope. Homocysteine-incubation resulted in a dose-dependent and significant increase in intracellular fluorescence indicative of increased generation of reactive oxygen species (RFU 248 ± 24% of control at 50 µmol/L D,L-

homocysteine and 360 ± 33% of control at 200 µmol/L D,L-homocysteine; $P < 0.05$). L-cysteine had no significant effect (RFU 174 ± 18% of control). Thrombin-receptor activating peptide (TRAP, 10 µmol/L) was used as a positive control. (Figure 12).

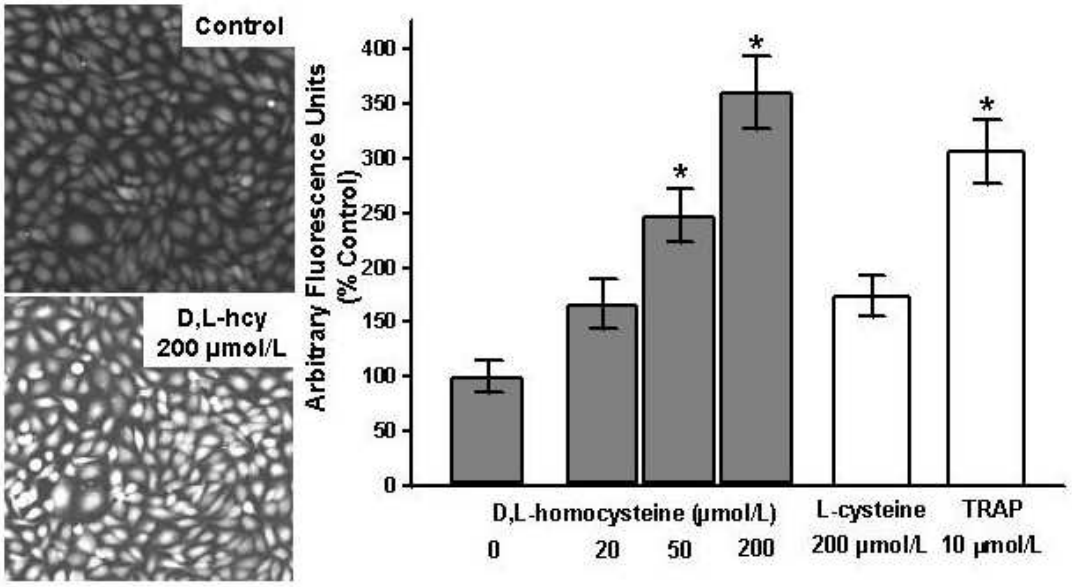

Figure 12. Dose-dependent increase in reactive oxygen species in homocysteine-incubated EA.hy 926 cells. Six hours incubation of endothelial cells with homocysteine dose-dependently increases the fluorescence of the reactive oxygen species-sensitive dye DCF in cultured endothelial cells. EA.hy 926 cells were incubated with different concentrations of homocysteine and L-cysteine, respectively, for 6 hours. TRAP 10 µmol/L was used as a positive control. After loading with DCF-DA, intracellular DCF-fluorescence was visualized using confocal laser microscopy and quantified by image analysis. The right panel shows the mean ± SEM fluorescence intensity of three experiments, and the left panel shows representative fluorescence-microscopy images. (n = 3 experiments, *$P < 0.05$ vs. control)

4.1.5. Enhanced NF-κB Translocation in Homocysteine-Incubated Endothelial Cells

Previous experiments suggested that oxidant stress to EC due to hyperhomocysteinemia may result in increased activation of the redox-sensitive transcription factor NF-κB [188].

To study whether or not this may be involved in increased adhesion molecule expression, NF-κB activation in homocysteine-incubated endothelial cells was studied by measuring the translocation of the NF-κB p65 subunit from cytosol to the nucleus using immunofluorescence techniques. Incubation of EA.hy 926 cells with 200 µmol/L D,L-homocysteine for 6 hours resulted in significantly

increased nuclear staining for the p65 subunit (nucleus/cytosol ratio 2.12 ± 0.31* comparing with control 1.52 ± 0.34, *P < 0.05 vs. control), showing increased nuclear translocation. In contrast, L-cysteine had no effect (nucleus/cytosol ratio 1.24 ± 0.24). (Figure 13)

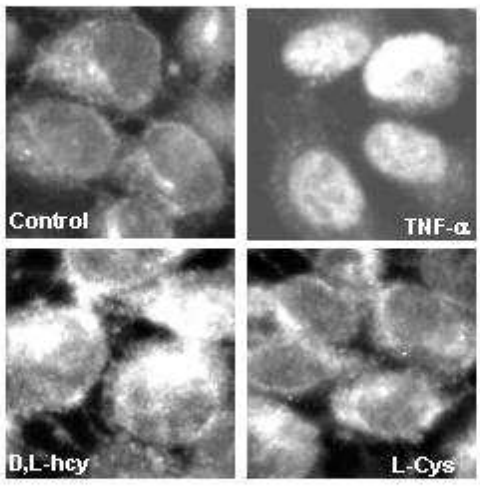

Figure 13. Homocysteine increases nuclear NF-κB translocation in endothelial cells. Six hours incubation of EA.hy 926 cells with D,L-homocysteine 200 µmol/L leads to increased nuclear NF-κB translocation, as evident by increased nuclear staining with an anti-p65-antibody. L-cysteine had no significant effect. TNF-α 50 ng/mL was used as positive control. The values are expressed as nucleus/cytosol ratio ± SEM fluorescence intensity of three experiments *P< 0.05 vs. control. (Control 1.52 ± 0.34; TNF-α 3.31 ± 0.51; D,L-homocysteine 2.12 ± 0.31*; L-cysteine 1.26 ± 0.24)

Increased NF-κB activation in homocysteine-incubated endothelial cells could be reproduced using HUVEC (Figure 14). The specificity of the increased nuclear staining with an antibody against the p-65 subunit of NF-κB could be shown by using the synthetic inhibitor of NF-κB translocation Bay 11-7082 in combination with homocysteine.

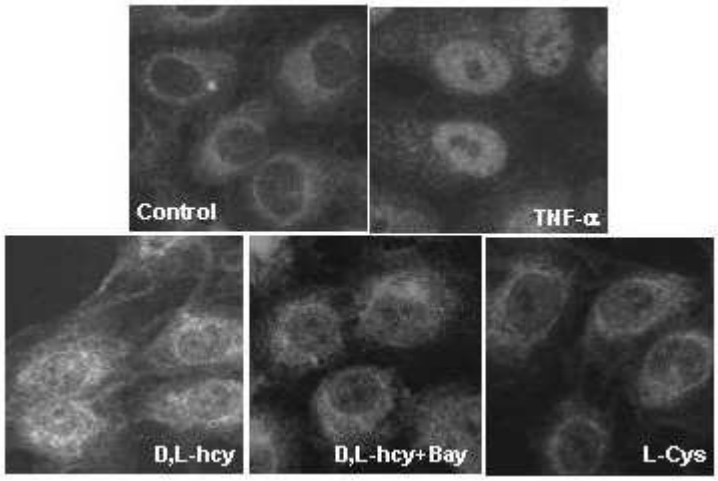

Figure 14. Homocysteine increases nuclear NF-κB translocation in endothelial cells. Six hours incubation of HUVEC with D,L-homocysteine 200 µmol/L leads to increased nuclear NF-κB translocation, as evident by increased nuclear staining with an anti-p65-antibody. L-cysteine had no significant effect. Bay 11-7082 (10 µmol/L) inhibited homocysteine-induced NF-κB translocation. TNF-α 50 ng/mL was used as positive control. The values are expressed as nucleus/cytosol ratio ± SEM fluorescence intensity of three experiments *P< 0.05 vs. control; $^{\$}P$<0.05 vs. D,L-homocysteine. (Control 0.69 ± 0.02; TNF-α 3.96 ± 0.28; D,L-homocysteine 1.9 ± 0.14*; D,L-homocysteine+Bay 0.77 ± 0.01$^{\$}$; L-cysteine 0.70 ± 0.01)

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

To test the influence of oxidative stress on the adhesion of THP-1 cells to homocysteine-incubated endothelial cells, endothelial monolayer was coincubated with homocysteine and MnTBAP (superoxide dismutase mimetic complex) or Tiron.

Coincubation of EA.hy 926 cells with homocysteine and with either MnTBAP or Tiron completely prevented homocysteine-induced increase in monocyte adhesion to endothelial cells (Figure 15).

Coincubation of HUVECs with homocysteine and Tiron almost completely prevented homocysteine-induced increase in monocyte adhesion to endothelial cells (from 26.33 ± 0.75 to 9.67 ± 0.35 cells per microscopic field).

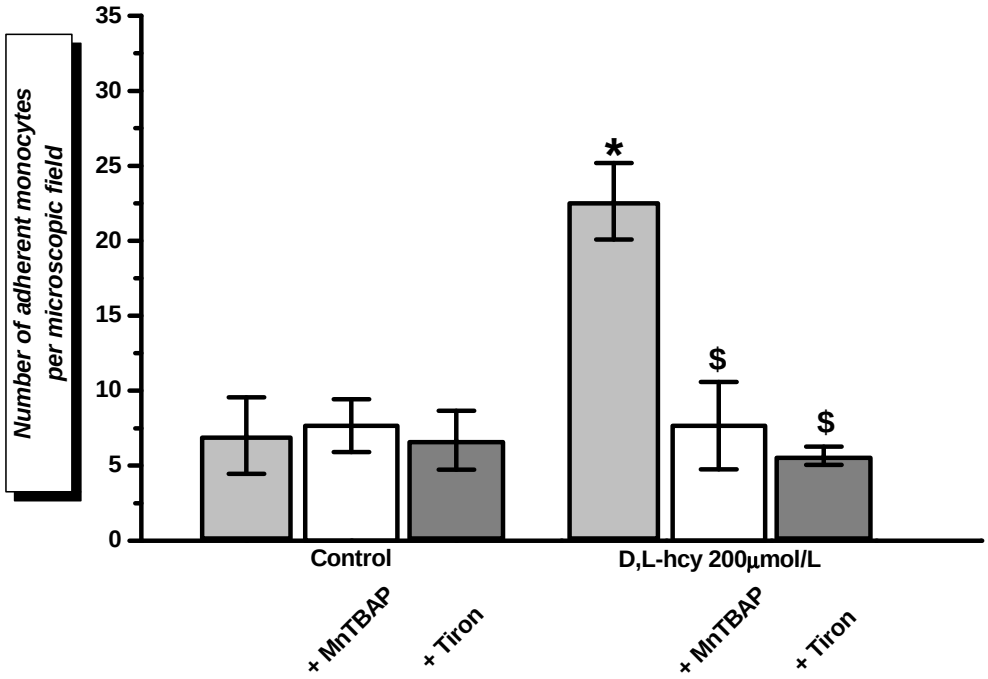

Figure 15. Incubation of EA.hy 926 cells with D,L-homocysteine together with the superoxide dismutase mimetic complex MnTBAP or Tiron abolished homocysteine's stimulatory effect on monocyte adhesion. (n = 4 experiments, *P < 0.05 vs. control $^{\$}P$ < 0.05 vs. D,L-homocysteine 200 µmol/L)

Scavenging of superoxide anion also prevented homocysteine-induced increase in endothelial ICAM-1 expression (Table 4).

Table 4. ICAM-1 expression on endothelial cells coincubated with homocysteine and antioxidants as determined by flow cytometry. Values indicate mean ± SEM, n = 4 experiments, ***P** < 0.05 vs. control, $^{\$}P$ < 0.05 vs. D,L-homocysteine.

Condition	***ICAM-1 expression (% Control) on EA.hy 926 cells***	***ICAM-1 expression (% Control) on HUVECs***
Control	100 ± 0	100 ± 0
Control+Tiron	98.7 ± 5.8	99.4 ± 2.7
Control+MnTBAP	99.6 ± 5.1	
D,L-homocysteine 200µmol/L	130.6 ± 6.5 *	163.4 ± 9.8 *
D,L-homocysteine 200µmol/L+Tiron	102.4 ± 3.8 $	106.0 ± 0.1 $
D,L-homocysteine 200µmol/L+MnTBAP	101.6 ± 4.1 $	

In addition, pretreatment of endothelial cells with the superoxide scavenger Tiron abolished the increased nuclear translocation of NF-κB induced by homocysteine in EA.hy 926 cells (Figure 16) and in HUVEC (Table 5).

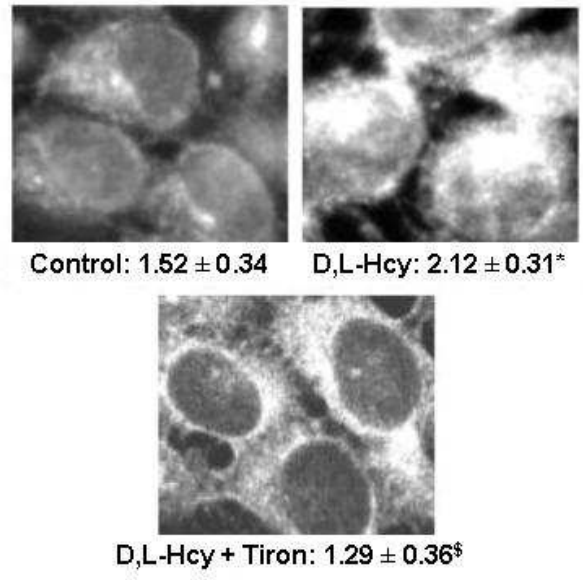

Figure 16. Incubation of EA.hy 926 cells with D,L-homocysteine together with Tiron abolished homocysteine-induced translocation of NF-κB from cytoplasm to the nucleus. The values are expressed as nucleus/cytosol ratio ± SEM fluorescence intensity of three experiments. *P < 0.05 vs. control; $^{\$}P$ < 0.05 vs. D,L-homocysteine.

Table 5. Incubation of HUVECs with D,L-homocysteine together with Bay 11-7082 or Tiron abolished homocysteine-induced translocation of NF-κB from cytoplasm to the nucleus. TNF-α was used as positive control. The values are expressed as nucleus/cytosol ratio ± SEM fluorescence intensity of three experiments. (***P*<0.05 vs. control, $^{\$}P$<0.05 vs. D,L-homocysteine)

Condition	***Nucleus/Cytoplasm Ratio***
Control	0.69 ± 0.02
TNF-α	3.96 ± 0.28
D,L-homocysteine 200 µmol/L	1.90 ± 0.14 *
D,L-homocysteine 200 µmol/L+Tiron	0.73 ± 0.03 $
D,L-homocysteine 200 µmol/L+ Bay 11-7082	0.77 ± 0.01 $
L-cysteine 200 µmol/L	0.70 ± 0.01

These findings indicate that the effects of homocysteine on endothelial cell activation leading to increased monocyte adhesion are at least partly due to increased superoxide anion production. Antioxidant treatment restores the normal function of endothelial cells and stops promoting the redox-sensitive NF-κB signaling cascade induced by reactive oxygen species formation under hyperhomocysteinemic conditions.

4.1.7. Inhibition of Homocysteine-Induced NF-κB Activation in Endothelial Cells Prevents ICAM-1 Expression and Monocyte Adhesion

To further confirm that increased NF-κB activation under conditions of elevated homocysteine mediates increased ICAM-1 expression on endothelial cells and increased monocyte adhesion to endothelial cells, HUVEC were incubated with homocysteine and the inhibitor of NF-κB activation Bay 11-7082, which completely suppresses homocysteine-induced NF-κB translocation (Table 5), and flow cytometry and adhesion studies were performed.

Data show that homocysteine requires NF-κB for its stimulatory effect on endothelial cells because inhibition of NF-κB translocation by Bay 11-7082 blocked the expression of ICAM-1 (from 163.4 ± 9.8 % of control for D,L-homocysteine 200 µmol/L to 102.4 ± 0.2 % of control for D,L-homocysteine 200 µmol/L + Bay 11-7082) and the adhesion of monocytes (from 26.33 ± 0.75 to 9.72 ± 0.24 cells per microscopic field).

4.2. Effect of Homocysteine on Circulating Mononuclear Cells

4.2.1. Stereospecific Increase in Adherent Homocysteine–Treated Monocytes to Unstimulated Endothelial Cells

Incubation of THP-1 cells with up to 200 µmol/L D,L- homocysteine and L-homocysteine for 4 hours resulted in a significant increase in the number of adhering THP-1 cells to unstimulated EA.hy 926 monolayers up to 3 fold at the highest concentration studied compared to control conditions (control: 9.17 ± 0.7 cells per microscopic field, D,L-homocysteine 200 µmol/L: 25.83 ± 1.94 cells per microscopic field; L-homocysteine 200 µmol/L: 26.4 ± 1.86; L-homocysteine 100 µmol/L: 22.3 ± 2.16). Incubation of THP-1 cells with L-cysteine (200 µmol/L) and D-homocysteine (200 µmol/L and 100 µmol/L) had no significant effect, indicating specificity for homocysteine and stereospecificity for the L-stereoisomer (Figure 17).

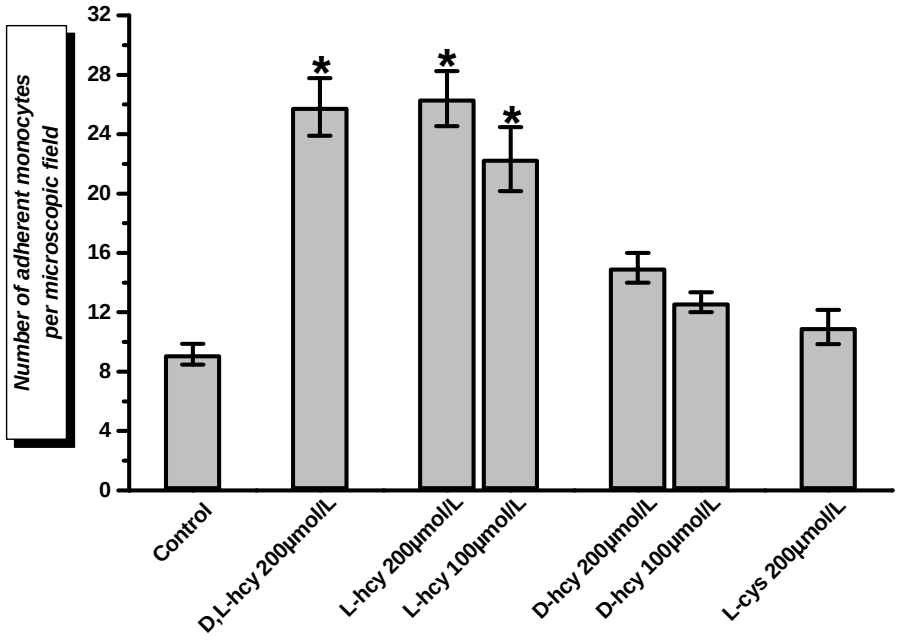

Figure 17. Stereospecific increase in the number of adhering homocysteine-incubated monocytes to unstimulated endothelial cells. Incubation of THP-1 cells with different stereoisomers of homocysteine only leads to significantly increased monocyte adhesion using the naturally occuring L-homocysteine, and D,L-homocysteine, but not using D-homocysteine (n = 5 experiments; **P*<0.05 vs. control)

4.2.2. Adhesion Molecules Expression on Homocysteine-Activated Monocytes

The integrin family of CAMs which are expressed on monocytes, serve as ligands for the ICAMs and VCAMs expressed on endothelial cells. The integrins are heterodimeric proteins consisting of an alpha and a beta chain that mediate leukocyte adherence to the vascular endothelium or other cell-cell interactions. Different sets of integrins are expressed by different populations of leukocytes to provide specificity for binding to different types of CAMs expressed along the vascular endothelium.

To explore whether integrins might be involved in homocysteine-induced monocyte adhesion to EA.hy 926 cells, the expression of β_2-integrins on monocytes was studied in untreated and homocysteine-incubated THP-1 cells (200 µmol/L for 4 hours) using FACS analysis. D,L- and L-homocysteine-incubated THP-1 cells showed almost 40% increase in LFA-1β (143.6 ± 9.2 % of control, n = 4 experiments, *P*<0.05 vs. control) and Mac-1α (134.9 ± 12.7 % of control, n = 4 experiments, *P*<0.05 vs. control). This effect was specific for homocysteine but not for other thiols, and was specific for the L-stereoisomer of homocysteine, as D-homocysteine and L-cysteine, respectively, had no effect.

To confirm the functional relevance of increased β-2 integrin expression on homocysteine-incubated monocyte adhesion to endothelial cells, THP-1 cells were incubated with 200 µmol/L D,L-homocysteine for 4 hours, followed by incubation with an blocking antibody against human LFA-1β or an isotype matched control antibody. After that monocyte adhesion assays were performed. Blocking LFA-1β significantly reduced homocysteine-induced monocytes adhesion to 50% (Figure 18).

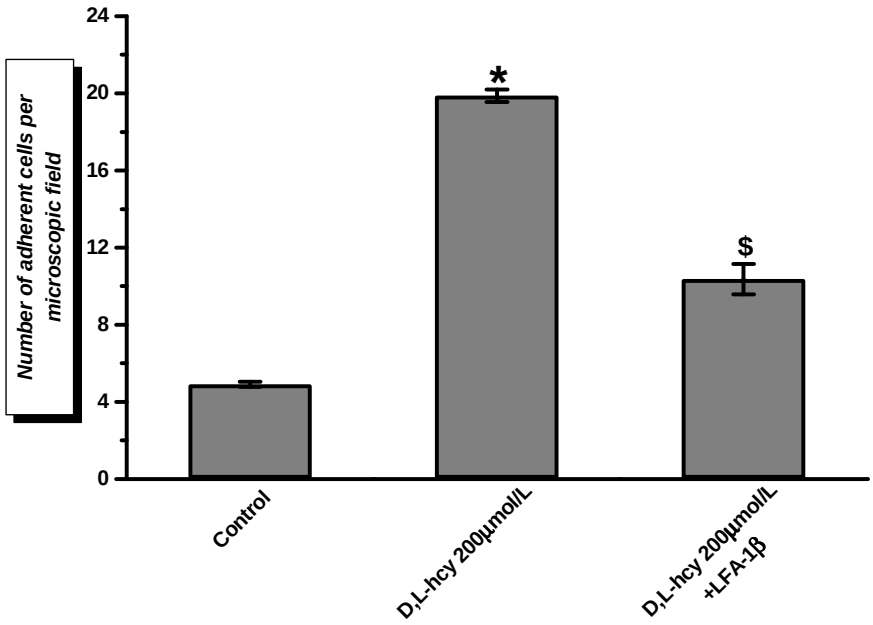

Figure 18. Homocysteine stimulatory effect on monocyte adhesion could be blocked by an anti-human LFA-1β antibody (from 20 ± 0.32 to 10.37 ± 0.79 adherent cells per microscopic field, n = 4 experiments, **P*<0.05 vs. control, $^{\$}$*P*<0.05 vs. D,L-homocysteine 200µmol/L).

These data indicate that an homocysteine-induced increase in β-2 integrin expression on monocytes is involved in the homocysteine's stimulatory effect on monocyte adhesion.

4.2.3. Nitric Oxide Synthase Dependent Generation of Intracellular Reactive Oxygen Species in Homocysteine-Activated Monocytes

To monitor the intracellular generation of ROS inside homocysteine-stimulated mononuclear cells, THP-1 cells were incubated for 4 hours with D,L-homocysteine 200 µmol/L, L-cysteine 200 µmol/L, loaded with the redox-sensitive dye DCF-DA, and the intracellular fluorescence intensities were measured by flow cytometry. Homocysteine-incubation resulted in a dose-dependent and significant increase in intracellular fluorescence indicative of increased generation of ROS (132 ± 6.4% relative fluorescence units of control) (Figure 19).

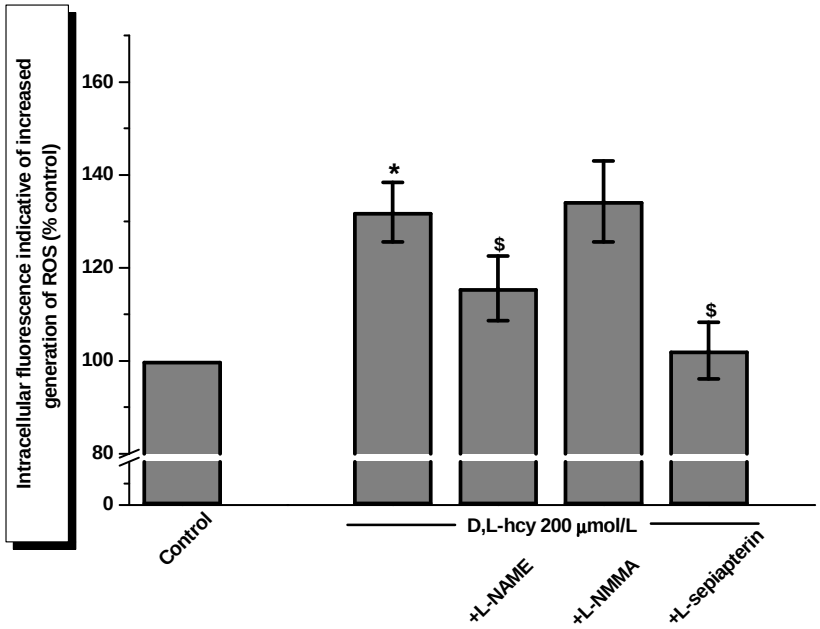

Figure 19. Reactive oxygen species generation in homocysteine-incubated THP-1 cells stained with DCF-DA and measured by flow cytometry. (n = 4 experiments, **P*<0.05 vs. control, $^{\$}$*P*<0.05 vs. D,L-homocysteine 200µmol/L)

To further evaluate whether ROS production in THP-1 cells stimulated with homocysteine is dependent on nitric oxide synthase, the cells were coincubated with the competitive inhibitor of NOS L-NAME, which inhibits both NO synthesis and superoxide anion production, and L-NMMA, which inhibits nitric oxide synthesis but not superoxide anion production, or with sepiapterin, an synthetic precursor of BH_4 which is a cofactor for NOS.

The results presented in figure 19 illustrate that the ROS production in monocytes which increased by treatment with D,L-homocysteine 200 µmol/L could be significantly attenuated by L-NAME pretreatment (to 115.6 ± 7 % RFU of control) but not by L-NMMA (RFU 134.3 ± 8 % RFU of control). Preincubation with L-sepiapterin (100µmol/L) before homocysteine treatment reduced ROS levels to control (to 102.2 ± 6.1 % RFU of control).

The kinetics of superoxide production in homocysteine-incubated THP-1 cells was further revealed by luminescence on addition of L-012 (100 µmol/L) and expressed as percent increase in relative light units versus control conditions (Figure 20). Homocysteine significantly increased the superoxide release from THP-1 cells (144 ± 7.4 % RLU of control).

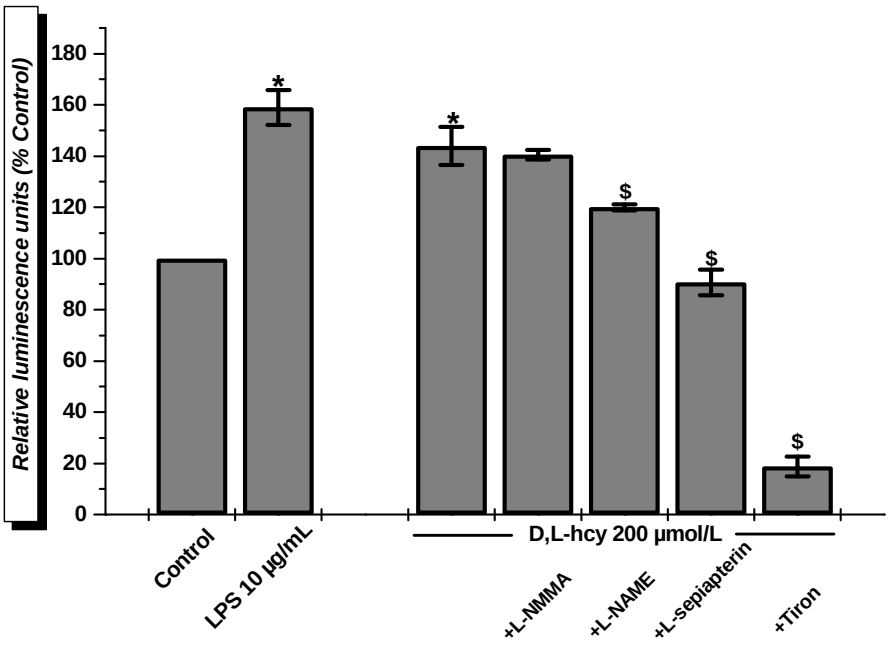

Figure 20. Superoxide production in homocysteine-incubated monocytes expressed as percent increase in relative light units versus control conditions. (n = 4 experiments, **P*<0.05 vs. control, $^{\$}$*P*<0.05 vs. D,L-homocysteine 200 µmol/L)

Inhibition of NO synthase by incubation of THP-1 cells with homocysteine and L-NAME resulted in a significant decrease of superoxide release from THP-1 cells. Superoxide generation could not be attenuated by coincubation with L-NMMA, which inhibits nitric oxide production by iNOS but not superoxide production. The chemiluminescence of L-012 was significantly decreased by coincubation with the antioxidant Tiron. LPS 10 µg/mL was used as positive control for amplifying oxidative stress inside THP-1 cells.

This data show that the induction of superoxide formation in hyperhomocysteinemic conditions could involve iNOS in monocytes by depleting the cofactors (biopterin stocks) necessary for the activity and nitric oxide production.

4.2.4. Effect of Scavenging of Superoxide Anion and Inhibition of iNOS on Homocysteine-Induced Monocyte Adhesion

In support of the role of increased intracellular superoxide generation for the above mentioned effects via iNOS dependent mechanisms, pretreatment of mononuclear cells with sepiapterin significantly decreased homocysteine-induced

monocyte adhesion to unstimulated endothelial cells (from 19,875 ± 0,319 to 11,063 ± 1,063 cells per microscopic field; Figure 21).

Incubation of monocytes with D,L-homocysteine together with Tiron (4mmol/L) or L-NAME (1mmol/L) significantly diminished homocysteine's stimulatory effect on monocyte adhesion (from 19,875 ± 0,319 for D,L-hcy to 14,781 ± 2,043 for D,L-hcy + L-NAME and to 7,98 ± 0,362 for D,L-hcy + Tiron). Coincubation with L-NMMA (1mmol/L) slightly increased the homocysteine's stimulatory effect on monocyte adhesion (from 19,875 ± 0,319 to 21,375 ± 0,421).

This indicated that the effects of homocysteine on monocyte activation and increased adhesion to endothelial cells, are at least partly due to increased superoxide anion production probably by uncoupling of iNOS enzyme.

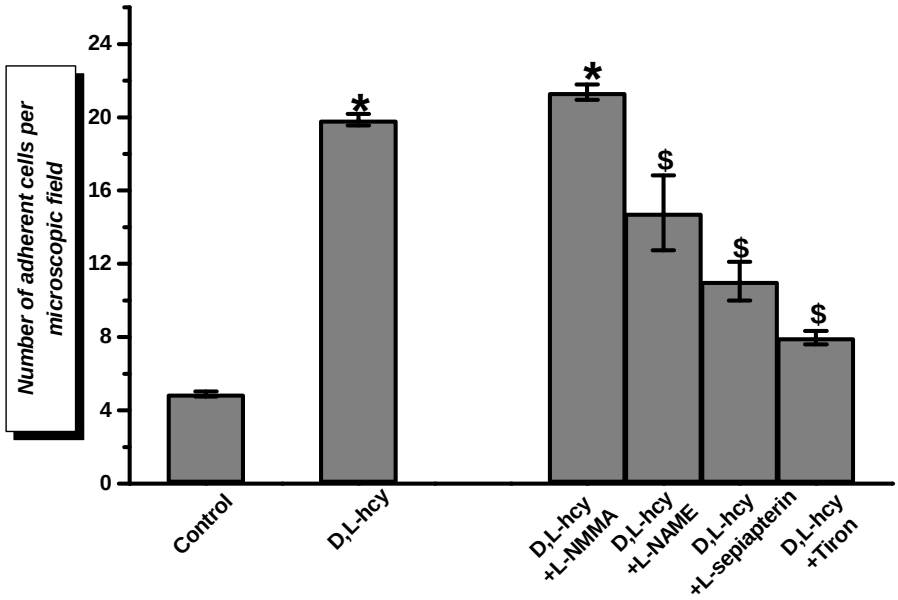

Figure 21. Incubation of monocytes with D,L-homocysteine together with Tiron (4mmol/L) or L-NAME (1mmol/L) significantly diminished homocysteine's stimulatory effect on monocyte adhesion. Preincubation with Sepiapterin (100µmol/L) decreased number of adherent cells. Coincubation with L-NMMA (1mmol/L) slightly increased the homocysteine's stimulatory effect on monocyte adhesion. (n = 4 experiments, *$P < 0.05$ vs. control **$P < 0.05$ vs. D,L-homocysteine 200 µmol/L)

5. DISCUSSION

Atherosclerosis is viewed as chronic inflammatory process of the vessel wall that initiates and promotes lesion development. This process involves circulating leukocytes, particularly monocytes, that are recruited and adhere to the activated endothelium, and that migrate into the subendothelial space where they differentiate into macrophages. [4,189] These recruited macrophages endocytose modified forms of LDL 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 migration and proliferation. In human arteries, these fibro-fatty plaques develop into complex atherosclerotic lesions that are liable to rupture.

Elevated levels of the amino acid homocysteine are associated with an increased risk for atherosclerotic vascular diseases in humans. However, the mechanisms by which excess homocysteine is harmful to the vasculature are not completely understood as yet. Several hypotheses have been proposed to explain the effects of homocysteine. Very high concentrations of homocysteine seem to be toxic for endothelial cells [190], whereas pathophysiologically relevant concentrations as found in patients with mild hyperhomocysteinemia seem to induce functional endothelial changes summarized as endothelial dysfunction. [98] Potential pathophysiogical mechanisms have been described in Chapter 1.5. In addition, homocysteine has been shown to promote the formation and increase the complexity of atherosclerotic lesions in atherosclerosis prone animal models. [141,191,192]

Because the adhesion and accumulation of leukocytes have been demonstrated to be a critical event in the development of atherosclerosis, we investigated the effect of homocysteine on the interaction between endothelial cells (EA.hy 926 and HUVEC) and monocytes (THP-1 cells).

Effects of Homocysteine on the Expression of Proinflammatory Cytokines in Monocytes and Endothelial Cells

Previous in vitro studies have shown that homocysteine is able to induce mRNA and protein expression of the proinflammatory cytokines MCP-1 and IL-8 in cultured human aortic endothelial cells (HAEC) [140,193] and in (THP-1)-derived macrophages [163], whereas it had no effect on expression of other cytokines, like tumor necrosis factor-α, granulocyte-macrophage colony–stimulating factor, interleukin-1β, and transforming growth factor-β. [140] The increase in MCP-1 expression was associated with activation of nuclear factor NF-κB due to increased phosphorylation of the inhibitory protein (IκB-α) as well as reduced expression of IκB-α mRNA in homocysteine-treated THP-1 cells. [163]

This cascade of events triggered increased recruitment of monocytes to endothelial cells. The induction of chemokine expression 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. [140] In addition, homocysteine has been shown to increase neutrophil adherence to endothelial cells in vitro and in vivo. [194] This contact results in neutrophil migration across the endothelial layer, with concurrent damage and detachment of endothelial cells. This effect seemed to be mediated via binding of leukocyte β_2-integrins (Mac-1α, CD11b and LFA-1β, CD18) to endothelial cells, as the interaction between endothelial cells and leukocytes could be abolished using blocking antibodies against these molecules, although the authors did not demonstrate any increase in β_2-integrin expression on leukocytes nor in ICAM-1 expression in homocysteine-incubated endothelial cells.

Effects of Homocysteine on Adhesion Molecules Expression and Monocyte Adhesion to Endothelial Cells

Whether or not incubation of endothelial cells with homocysteine results in increased monocyte adhesion to endothelial cells has not been shown conclusively as yet.

Adhesion of U937 monocytic cells to IL-1β-stimulated, but not to unstimulated HAEC was slightly increased only when both endothelial cells and monocytes were pretreated. The effect was shown to be mediated by VCAM-1 and E-selectin [161]. Wang et al. have shown that hyperhomocysteinemia stimulates the expression of VCAM-1 and E-Selectin in aortas of hyperhomocysteinemic mice, although the mechanisms had not been elucidated so far [195].

Our data show – for the first time - that incubation of endothelial cells with pathophysiologically relevant concentrations of homocysteine lead to a time- and dose-dependent increase in the adhesion of monocytes to otherwise unstimulated endothelial cells (Figure 9). Monocyte adhesion was not stimulated by incubation of endothelial cells with cysteine, indicating that this effect does not seem to be an unspecific thiol effect. Furthermore, monocyte adhesion was only stimulated by incubation of endothelial cells with L-homocysteine, but not with D-homocysteine, demonstrating enantiomeric specificity for the naturally occurring stereoisomer (Figure 10).

Mechanistically, increased binding of monocytes to homocysteine-stimulated endothelial cells was mediated by increased de novo synthesis of ICAM-1 molecules on endothelial cells as shown by increased ICAM-1 expression using FACS analysis (Table 3) and increased ICAM-1 mRNA levels using real-time RT-PCR. The functional role of increased ICAM-1 expression on monocyte adhesion to endothelial cells has been confirmed by blocking studies where a blocking antibody against ICAM-1 augmented homocysteine's effect. NF-κB is a transcription factor that activates a variety of target genes relevant to the pathophysiology of the vessel wall, including cytokines, chemokines, and leukocyte adhesion molecules (including ICAM-1), as well as genes that regulate cell proliferation and mediate cell survival. Physiological modulation and pathological activation of the NF-κB system may contribute to the changes in gene

expression that occur during atherogenesis. Diverse stimuli activate NF-κB, through the phosphorylation and activation of the IκB kinase (IKK) complex. The experiments that we have performed indicated that incubation of EA.hy 926 cells only with homocysteine and not with other thiols resulted in significantly increased nuclear staining for the NF-κB p65 subunit, demonstrating increased nuclear translocation. An increased activation of NF-κB in aortas of mildly hyperhomocysteinemic rats compared to the control that was associated with an increased expression of ICAM-1 has recently been shown [196]. This confirms the relevance of our findings for the in vivo situation.

Support of the role of NF-κB in homocysteine-induced activation of endothelial cells is derived from inhibitor studies (Table 5, Figure 14) using Bay 11-7082, which is known to block TNFα-induced endothelial-leukocyte cell adhesion via inhibiton of NF-κB translocation. Cotreatment of endothelial cells with homocysteine and Bay 11-7082 reduced the number of adherent monocytes and ICAM-1 expression to control levels. This shows that homocysteine modulates ICAM-1 expression on the surface of endothelial cells by an NF-κB dependent pathway and thereby triggers monocyte adhesion to endothelial monolayer.

All these changes in endothelial and monocytic phenotypes occur only if cells are incubated with the L-stereoisomer of homocysteine which is taken up intracellularly, probably by a cysteine transporter, but do not occur when incubating the cells with the D-stereoismer. This indicates that the effects of homocysteine are mediated by intracellular events and not in the extracellular space. Furthermore, this indicates, that the effects of homocysteine are due to a biochemical, and not due to a simple chemical effect of homocysteine, as the D-stereoismer of homocysteine may mediate the same chemical modifications of proteins and other targets as the L-stereosimer, but does not participate in biochemical reaction, like for example posttranslational modifications of proteins. [172]

Incubation of THP-1 cells with D,L-homocysteine and L-homocysteine resulted in a significant increase in the number of adhering monocytes to unstimulated EA.hy 926 or HUVEC monolayers up to 3 fold at the highest concentration studied compared to control conditions. L-cysteine and D-homocysteine, again, had no significant effect (Figure 17). In addition, LFA-1β, the

major ligand for the endothelial ICAM-1 on monocytes, was upregulated by homocysteine as well. These data were confirmed by increased surface expression determined by flow cytometry in THP-1 cells and blocking studies with an anti LFA-1β antibody (Figure 18). Both approaches significantly reduced homocysteine-induced monocytes adhesion to endothelial cells.

Several studies suggest that the transcription factor NF-κB plays an important role in upregulating the expression of β_2-integrins LFA-1β and Mac-1α and other inflammatory factors in atherosclerotic lesions. The promoter region of the β_2-integrins LFA-1β and Mac-1α genes contains several putative binding sites for transcription-activator factors. [197] Wang and collaborators showed that homocysteine induces NF-κB translocation in THP-1-derived macrophages [163] which correlates with high integrin expression on the surface of the monocytes. These complementary findings show that homocysteine not only has stimulatory effects on endothelial cells, but also on circulating monocytes. In addition, this shows that NF-κB translocation is required for these effects in both cases.

Oxidant Stress Induced by Homocysteine in Endothelial Cells and Monocytes

Homocysteine is associated with increased vascular superoxide output and vascular oxidant stress. This effect is specific for homocysteine and does not occur with other low-molecular weight thiols, and is stereospecific for naturally occurring L-isoform of homocysteine [111]. NF-κB is one of the transcription factors that may be controlled by the redox status of the cell [169]. Indeed, generation of reactive oxygen species may be a common step in all of the signaling pathways that lead to IκB degradation and nuclear NF-κB accumulation. Support for this concept comes from a variety of studies showing that the diverse agents that can activate NF-κB also elevate levels of reactive oxygen species and that chemically distinct antioxidants, as well as overexpression of antioxidant enzymes, can inhibit NF-κB activation [198]. However, a direct role of reactive oxygen species in signaling to NF-κB remains to be proven.

Thus, for demonstrating the link between increased binding of monocytes, ICAM-1 expression, NF-κB translocation, and increased vascular oxidant stress

under conditions of elevated homocysteine levels, we have first monitored the intracellular generation of reactive oxygen species in homocysteine-incubated endothelial cells by loading them with the redox-sensitive dye DCF-DA, and second studied the effects of antioxidants on this signaling pathway and its functional consequences. Homocysteine-incubation resulted in a dose-dependent and significant increase in intracellular fluorescence indicative of increased generation of reactive oxygen species (Figure 12). The functional relevance of this redox-sensitive signaling pathway is confirmed by data that show that scavenging of reactive oxygen species by antioxidants like MnTBAP or Tiron abolished the homocysteine-induced endothelial ICAM-1 expression (Table 4), monocyte adhesion to endothelial cells (Figure 15), and NF-κB translocation (Figure 16).

Significant superoxide release from homocysteine-incubated THP-1 cells further confirmed the hypothesis that homocysteine induces changes in cellular redox balance, even in non-endothelial cells.

A potential source of reactive oxygen species in endothelial cells incubated with homocysteine may be endothelial nitric oxide synthase, as shown previously. [111] To test whether or not nitric oxide synthase may be a source of reactive oxygen species in non-endothelial cells as well, we treated THP-1 cells with L-NAME, which is an analog of L-arginine that inhibits nitric oxide production and superoxide production via NOS pathway [199]. Inhibition of monocytic nitric oxide synthase attenuated the superoxide production (figure 20) and adhesion of monocytes (figure 21) induced by homocysteine. L-NMMA, another analogue of L-arginine [199], which acts as a competitive inhibitor of all three isoforms of nitric oxide synthase (NOS), including inducible NOS (iNOS), and inhibits nitric oxide but not NOS-dependent superoxide production, did not show a significant effect on superoxide release (Figure 20) and adhesion of homocysteine-treated monocytes (Figure 21).

One hypothesis that might explain why homocysteine induces NOS-dependent superoxide production is that it depletes cellular stocks of tetrahydrobiopterin, leading to NOS-“uncoupling”. It has been shown that in this situation, NOS generates superoxide instead of nitric oxide. [120] Reasons for NOS-“uncoupling” are reduced cellular availability of either the NOS-cofactor tetrahydrobiopterin or the NOS-substrate L-arginine. In these situations, the

electrons from heme reduce oxygen to form superoxide instead of reducing L-arginine-derived nitrogen to form nitric oxide. L-sepiapterin is a synthetic precursor of tetrahydrobiopterin, which is converted to it intracellularly, and thereby stimulated NOS-cofactor availability. Incubation of THP-1 cells with sepiapterin decreased superoxide levels in homocysteine-treated cells (Figure 20) and inhibited monocyte arrest to endothelial cells (Figure 21). These results show that superoxide is generated in homocysteine-stimulated THP-1 cells at least partly by a NOS dependent pathway and that superoxide anion could enhance activation of monocytes and increased adhesion molecules expression by a redox sensitive pathway.

In conclusion, these data indicate that increased vascular oxidant stress in hyperhomocysteinemia not only leads to a decrease in the bioavailability of nitric oxide derived from endothelial cells, but may also activate redox-sensitive signaling pathways in endothelial cells and circulating monocytes that induce a proinflammatory state in the vessel wall leading to increased adhesion molecule expression and monocyte recruitment (Figure 22).

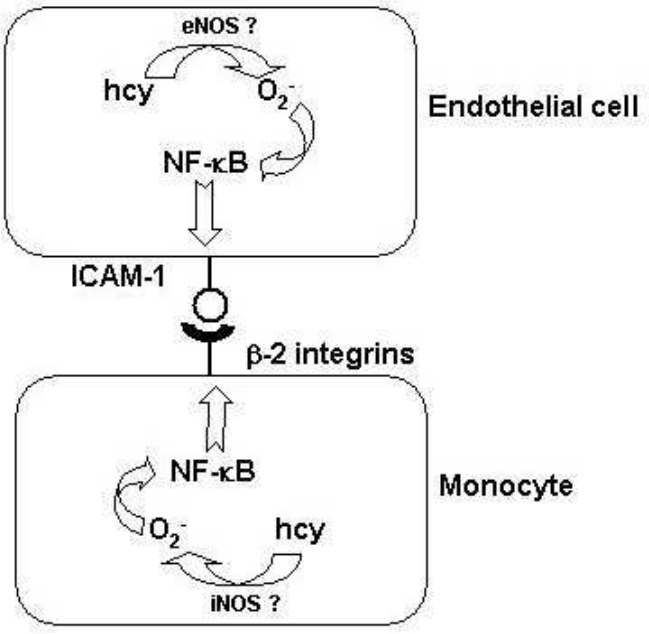

Figure 22: Hypothetic pathophysiological mechanism by which homocysteine induces vascular oxidant stress.

This may be an additional mechanism by which homocysteine promotes the development of atherosclerotic lesions.

Strategies for defense against vascular oxidative stress and the resulting vascular damage that it generates may include treatment with antioxidants or overexpression of antioxidant enzymes, including catalase, several forms of superoxide dismutase, and glutathione peroxidase. Incorporation of antioxidants into vascular cells may reduce vascular cell oxidative stress. Some of these biologically relevant antioxidants have been shown to inhibit NF-κB activation in macrophages and cultured endothelial cells by decreasing oxidative stress [187,196], as in the study presented. By diminishing NF-κB activation, antioxidant vitamins, at least in part, may therefore diminish cellular responses to oxidized LDL, hyperhomocysteinemia, and other cardiovascular risk factors, leading to reduced monocyte adhesion, foam cell formation, and cytotoxicity to vascular cells, and finally improve vascular function. Taken together these findings are consistent with the concept that agents that diminish oxidant stress may stabilize the NF-κB system, and thereby may diminish the effect of homocysteine and other cardiovascular risk factors on the endothelium, which could lead to a decrease in atherosclerotic lesion formation. The information generated from these studies may thereby be helpful in designing intervention strategies aimed at inhibiting the generation of reactive oxygen species associated with cardiovascular risk factors, especially with hyperhomocysteinemia. This may have a favorable effect on signaling events involved in monocyte recruitment and infiltration during the formation of atherosclerotic lesions.

6. SUMMARY

Mild hyperhomocysteinemia is an independent risk factor for the development of coronary artery disease, cerebrovascular disease and peripheral arterial disease. The mechanisms by which hyperhomocysteinemia promotes vascular disease are not completely understood yet. An increasing body of evidence has implicated oxidative stress as being contributory to homocysteine's deleterious effects on the vasculature.

Elevated levels of homocysteine lead to increased generation of superoxide anion in endothelial cells by a biochemical mechanism involving nitric oxide synthase, and, to a lesser extent, by an increase in the chemical oxidation rate of homocysteine and other aminothiols in the circulation. Furthermore, homocysteine has been shown to inhibit the activity of important cellular antioxidant enzymes, like the cellular isoform of glutathione peroxidase or superoxide dismutase, which may contribute to homocysteine's induced oxidant stress. The resulting increase in reactive oxygen species leads to decreased bioavailability of the endothelium-derived signaling molecule nitric oxide via oxidative inactivation and thereby induces endothelial dysfunction. This seems to play a central role in the molecular mechanisms underlying the effects of homocysteine on vascular function.

Hyperhomocysteinemia not only leads to endothelial dysfunction but also promotes the development and propagation of atherosclerotic lesion in atherosclerosis-prone animal models. As the recruitment of circulating monocytes to the vessel wall plays a crucial role in the process of atherosclerosis, the purpose of this study was to examine the influence of homocysteine on the interaction of endothelial cells with monocytes.

Exposure of endothelial monolayers to D,L- and L-homocysteine resulted in a time- and dose-dependent increase in adherent THP-1 cells by upregulating ICAM-1 expression on endothelial cells. L-cysteine and D-homocysteine had no effects. This indicates that the stimulatory effect is specific for the naturally occurring L-stereoisomer and rather a biochemical than a chemical effect. The increased endothelial expression of ICAM-1 seems to be mediated by increased activation of the nuclear transcription factor NF-κB, as shown by increased nuclear

translocation of NF-κB in homocysteine-incubated endothelial cells. In accordance, inhibition of NF-κB translocation by a synthetic inhibitor Bay 11-7082 significantly diminished homocysteine-induced ICAM-1 expression and adhesion of monocytes to endothelial cells.

In addition, incubation of monocytes with D,L- homocysteine and L-homocysteine resulted in significant increase in the number of adhering monocytes to unstimulated endothelial monolayer by upregulating the expression of β-2 integrins.

Furthermore, homocysteine-incubation of endothelial cells and monocytes resulted in a dose-dependent and significant increase in the intracellular generation of reactive oxygen species. In support of the role of increased oxidant stress for the above mentioned effects, treatment of endothelial cells with the superoxide scavengers MnTBAP or Tiron together with homocysteine abolished homocysteine-induced monocyte adhesion, ICAM-1 expression and the nuclear translocation of NF-κB. Incubation of THP-1 monocytes with Tiron abolished homocysteine-induced β-2 integrin expression on these cells and adhesion to unstimulated endothelial cells. These findings suggest that superoxide anion radicals mediate homocysteine's effects on endothelium-monocyte interactions.

In addition to previous studies that indicated that a significant source of reactive oxygen species in homocysteine-treated endothelial cells might be endothelial nitric oxide synthase, experiments using inhibitors of nitric oxide synthase in THP-1 cells indicated that nitric oxide synthase-dependent generation of superoxide anion also occurs in homocysteine-incubated THP-1 cells. This mechanism may contribute to homocysteine-induced oxidant stress.

The information generated from these studies may be helpful in designing intervention strategies aimed at inhibiting the generation of reactive oxygen species in the vasculature that is associated with signaling events of monocyte recruitment and infiltration involved in atherosclerosis.

7. ZUSAMMENFASSUNG

Eine milde Hyperhomocysteinämie ist ein unabhängiger Risikofaktor für die Entwicklung von koronarer Herzerkrankung, zerebrovaskulärer Erkrankung oder peripherer arterieller Verschlusskrankheit. Die Mechanismen, über die eine Hyperhomocysteinämie vaskuläre Erkrankungen fördert, sind nicht vollständig verstanden. Experimentelle Daten weisen darauf hin, dass gesteigerter vaskulärer oxidativer Stress zu den nachteiligen Effekten von Homocystein (Hcy) auf die Gefäßwand wesentlich beträgt.

Erhöhte Homocystein-Konzentrationen führen zu einer gesteigerten Bildung von Superoxidanion in Endothelzellen durch einen biochemischen Mechanismus, abhängig von endothelialer Stickstoffmonooxid-(NO)-Synthase, und, zu einem geringeren Ausmaß, durch chemische Oxidation von Homocystein und anderer Aminothiole in der Zirkulation. Zudem wurde gezeigt, dass Homocystein die Aktivität wichtiger zellulärer antioxidativer Enzyme hemmt, wie die zelluläre Isoform der Glutathion-Peroxidase oder die Superoxiddismutase. Der gesteigerte Anfall reaktiver Sauerstoffspezies verringert die Bioverfügbarkeit des vom Endothel gebildeten Signalmoleküls NO durch oxidative Inaktivierung und induziert eine endotheliale Dysfunktion. Dies scheint eine Schlüsselrolle in den molekularen Mechanismen zu spielen, die der Wirkung von Homocystein auf die Gefäßfunktion zugrunde liegen.

Hyperhomocysteinämie führt nicht nur zu Endothelfunktionsstörung, sondern fördert auch die Entstehung und Progression atherosklerotischer Läsionen in hierfür empfänglichen Tiermodellen. Da die Rekrutierung zirkulierender Monozyten in die Gefäßwand eine Schlüsselrolle in der Entstehung atherosklerotischer Läsionen spielt, untersuchte diese Arbeit den Einfluss von Homocystein auf die Interaktion von Endothelzellen mit Monozyten.

Die Inkubation von Endothelzellen mit D,L- und L-Hcy führte zu einer Zeit- und Dosis-abhängigen Zunahme der Adhäsion monozytärer THP-1 Zellen über eine Hochregulation der ICAM-1 Expression auf Endothelzellen. L-Cystein und D-Hcy dagegen hatten keine Effekt. Dies zeigt, dass der stimulierende Effekt spezifisch für das natürlich vorkommende L-Stereoisomer ist, und eher durch einen biochemischen als einen chemischen Effekt verursacht wird. Die gesteigerte

endotheliale ICAM-1 Expression scheint über eine gesteigerte Aktivierung des nukleären Transkriptionsfaktors NF-κB vermittelt zu sein, wie durch eine gesteigerte nukleäre Translokation von NF-κB in Homocystein-inkubierten Endothelzellen gezeigt. In Übereinstimmung hiermit konnte die Hemmung der NF-κB-Aktivierung mit dem synthetischen Inhibitor Bay 11-7082 die Hcy-induzierte ICAM-1 Expression und Monozyten-Adhäsion an Endothelzellen verringern.

Zudem konnte gezeigt werden, dass die Inkubation von THP-1 Monozyten mit D,L- und L-Homocystein zu einer signifikanten Zunahme der Zahl adhärenter Monozyten an unstimulierte Endothelzellen führt, vermittelt über eine Hochregulation der Expression von β-2-Integrinen.

Homocystein-Inkubation von Endothelzellen und Monozyten führte zu einer Dosis-abhängigen und signifkanten Zunahme der intrazellulären Bildung reaktiver Sauerstoffspezies. Als Unterstützung für die Bedeutung der Rolle von gesteigertem oxidativen Stress für die beobachteten Effekte konnte gezeigt werden, dass die Behandlung von Endothelzellen mit den Superoxid-Scavengern MnTBAP oder Tiron zusammen mit Homocystein die Homocystein-induzierte Monozyten-Adhäsion, ICAM-1 Expression und nukleäre Translokation von NF-κB verhindert. Die Inkubation von THP-1 Monozyten mit Tiron verhindert die Homocystein-induzierte β-2-Integrin Expression auf diesen Zellen und die Adhäsion an unstimulierte Endothelzellen. Diese Beobachtungen lassen vermuten, dass Superoxidanion Radikale die Effekte von Homocystein auf die Interaktion von Endothelzellen mit Monozyten vermitteln.

Frühere Studien zeigten, dass eine signifikante Quelle für reactive Sauerstoffspezies in Homocystein-inkubierten Endothelzellen die endotheliale NO Synthase darstellt. Experimente mit Inhibitoren der NO Synthase in THP-1 Zellen zeigen zudem, dass auch in diesem Zelltyp Homocystein zu einer NO Synthase-abhängigen Bildung reaktiver Sauerstoffspezies führt. Dieser Mechanismus kann zu dem Homocystein-induzierten oxidativen Stress beitragen.

Die Daten der vorgelegten Untersuchung können in Zukunft hilfreich sein, um Interventionsstudien zu planen, die darauf ausgerichtet sind, die vaskuläre Bildung reaktiver Sauerstoffspezies zu verringern. Hierdurch könnte die Signalkaskade, die zur Monozytenrekrutierung und –infiltration in die Gefäßwand im Prozess der Atheroskleroseentwicklung führt, beeinflusst werden.

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ACKNOWLEDGMENTS

I would like to thank:

Priv. Doz. Dr. med. Norbert Weiss and Dr. med. Florian Krötz for the great scientific support in performing this project

Prof. Christiane Keller for offering me the chance to work as a scientist in the Department of Metabolic Diseases of the Medical Policlinic

Anna Henger, Anissa Boucherot and Sandra Irrgang for expert assistance with real-time RT-PCR and especially for being very good friends

Susanne Hillebrand, Brigitte Reinhardt and Hanna Bridell for collaboration to this project

All my friends from Germany, USA and Romania for the good and optimistic thoughts during the difficult periods of time

My parents, Florica and Ion, and my brother, Felix, for their unconditioned and endless love and encouragement.

CURRICULUM VITAE

28.08.1975	born in Bucharest, Romania
1982 - 1990	Primary school No.126, Bucharest, Romania
1990 - 1995	Pedagogical High School ″Elena Cuza″, Bucharest
Sept. 1995 - Nov. 2000	Teacher at Elementary School, Bucharest, Romania
1996 - 2000	Faculty of Biology, Biochemistry section, University of Bucharest, Romania
Oct. 2000 - Feb. 2002	Master in Neurobiology, Faculty of Biology, University of Bucharest, Romania
Nov. 2000 - Jan. 2002	Research Assistant, Institute of Cellular Biology and Pathology “Nicolae Simionescu”, Bucharest, Romania
Feb. 2002 - Dec. 2004	PhD student, Medical Policlinic, Ludwig Maximilians University, Munich, Germany.