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# Forum Review

# Vascular Oxidant Stress and Inflammation in Hyperhomocysteinemia

LOUISA PAPATHEODOROU and NORBERT WEISS

## ABSTRACT

**Elevated plasma levels of homocysteine are a metabolic risk factor for atherosclerotic vascular disease, as shown in numerous clinical studies that linked elevated homocysteine levels to *de novo* and recurrent cardiovascular events. High levels of homocysteine promote oxidant stress in vascular cells and tissue because of the formation of reactive oxygen species (ROS), which have been strongly implicated in the development of atherosclerosis. In particular, ROS have been shown to cause endothelial injury, dysfunction, and activation. Elevated homocysteine stimulates proinflammatory pathways in vascular cells, resulting in leukocyte recruitment to the vessel wall, mediated by the expression of adhesion molecules on endothelial cells and circulating monocytes and neutrophils, in the infiltration of leukocytes into the arterial wall mediated by increased secretion of chemokines, and in the differentiation of monocytes into cholesterol-scavenging macrophages. Furthermore, it stimulates the proliferation of vascular smooth muscle cells followed by the production of extracellular matrix. Many of these events involve redox-sensitive signaling events, which are promoted by elevated homocysteine, and result in the formation of atherosclerotic lesions. In this article, we review current knowledge about the role of homocysteine on oxidant stress–mediated vascular inflammation during the development of atherosclerosis.** *Antioxid. Redox Signal.* 9, 1941–1958.

## INTRODUCTION

Elevated plasma levels of homocysteine (hyperhomocysteinemia) are an independent risk factor for atherosclerotic vascular disease (13, 66), which includes peripheral arterial disease, coronary artery disease, and cerebrovascular disease. This metabolic risk factor is estimated to explain 10% of the population's risk for atherosclerosis and its clinical consequences (22).

The mechanisms by which homocysteine triggers atherosclerosis are not fully understood. One hypothesis is that hyperhomocysteinemia leads to increased oxidant stress in the vasculature (182, 184), which, besides other effects, leads to reduced bioavailability of nitric oxide (NO) (168). The resulting endothelial dysfunction contributes to decreased vasodilator capacity, activation of circulating leukocytes and platelets, activation of prothombotic and inhibition of fibrinolytic mechanisms, and stimulation of vascular smooth muscle cell proliferation. All these events facilitate the initiation and progression of atherosclerotic lesions (123, 182).

The contribution of various inflammatory processes during the development of atherothrombotic vascular disease suggests that this disease may be considered a chronic inflammatory disorder (16, 112, 140). Cell-culture studies, studies in animals with experimentally induced hyperhomocysteinemia, and clinical studies in humans with hyperhomocysteinemia suggested that homocysteine may promote vascular inflammation in several ways. The purpose of this article is to review current knowledge about the effects of homocysteine on vascular inflammation related to the development of atherosclerosis, and the impact of vascular oxidant stress–mediated pathways in this context.

Department of Vascular Medicine, Medical Policlinic–City Campus, University of Munich Medical Center, Munich, Germany.

## HYPERHOMOCYSTEINEMIA AND OXIDANT STRESS

Homocysteine is a nonproteinogenic amino acid containing a free sulfhydryl group. It is derived from the metabolic conversion of the essential amino acid methionine (55). Compounds with a free sulfhydryl group are called thiols. Other biologically relevant low-molecular-weight thiols are cysteine, which in its oxidized state, cystine, is the most abundant thiol in plasma; glutathione, which is the most abundant thiol intracellularly; coenzyme A; and dihydrolipoic acid. Human plasma contains several reduced and oxidized homocysteine species. Oxidized homocysteine species comprise 98–99% of the total human plasma homocysteine, 80–90% of which is bound to proteins (89).

Experiments using animal models with genetically or diet-induced hyperhomocysteinemia or endothelial cells cultured under conditions that lead to elevated homocysteine levels showed an increased accumulation of reactive oxygen species (ROS), especially superoxide anion, in these situations. Heterozygous cystathionine $\beta$ synthase knockout ($CBS^{(-/+)}$) mice that develop mild hyperhomocysteinemia generate more superoxide in aortic tissue compared with controls (51). Consistently, cultured endothelial cells incubated with homocysteine produced elevated amounts of ROS (78, 132, 202). Several mechanisms exist by which elevated homocysteine levels increase ROS in vascular cells and tissues, as reviewed elsewhere (Table 1) (182).

ROS consist of superoxide anion, hydroxyl radical, peroxynitrite, hydrogen peroxide or other peroxides and hypochlorous acid, and their organic analogues. Additionally, homocysteine may undergo cyclization to form homocysteine-thiolactone (a cyclic thioester), which is chemically reactive and acylates free amino groups such as the side-chain lysine groups in proteins (90).

ROS at moderate concentrations act as signaling molecules and thereby play important roles in the regulation of various vascular cell functions. In the vasculature, they participate in the regulation of vascular tone, oxygen sensing, cell growth and proliferation, apoptosis, and inflammatory responses (199). For example, hydrogen peroxide has been shown to contribute to flow-induced dilation of human coronary arterioles (119); NAD(P)H oxidase–derived ROS mediate endothelial NO production in response to angiotensin II (28) and participate in cardioprotection of ischemic reperfusion injury by angiotensin II (98). In the context of atherosclerosis and inflammation, ROS are especially involved in the control and regulation of genes for cytokines, chemokines, and adhesion molecules by redox signaling (104).

ROS, however, may also be toxic to cells and tissues through the promotion of lipid peroxidation of membranes (loss of membrane function and increased permeability) and generation of lipid autoperoxidation reactions, through oxidant damage to low-density lipoproteins, DNA damage leading to mutation and death, and crosslinking or vulcanization of sulfhydryl-rich proteins (leading to stiff aged proteins, specifically, collagen of the extracellular matrix) (75). In addition, supraphysiologic concentrations of ROS may result in the loss of control of cell signaling (149). Posttranslational modification of specific proteins by ROS suggests that the protein in question may be a receptor for the redox second messenger (for example, the thiol redox switches that are present on a number of important cell-signaling proteins) (36).

Table 1. Mechanisms by which Homocysteine May Increase Reactive Oxygen Species in Vascular Cells and Tissue

- Elevation of the oxidation rate of homocysteine (3, 69, 116, 117)
- Nitric oxide synthase dependent generation of superoxide anion via uncoupling of the enzyme (78, 163)
- Inhibition of the activity of cellular antioxidant enzymes, like cellular glutathione peroxidase (71, 186) or heme oxygenase 1 (142)
- Disruption of extracellular superoxide dismutase from endothelial surfaces (179, 187, 193)
- Activation of NADPH oxidases (1, 99, 153)

## ENDOTHELIAL DYSFUNCTION

Endothelial dysfunction is the first step in the development of atherosclerotic lesions followed and accompanied by vascular inflammation. This results in the formation of the atherosclerotic plaque (113, 140, 152). An impairment of endothelium-dependent relaxation of blood vessels is one integral component of endothelial dysfunction (42). A key event in the vascular pathobiology associated with elevated homocysteine levels seems to be an impairment of normal endothelial function at least partly due to interference of homocysteine with cellular redox signaling (182, 204). Mildly hyperhomocysteinemic $CBS^{(-/+)}$ mice showed an impairment of endothelium-dependent vasoreactivity and regulation of blood flow but normal endothelium-independent vasodilation (51, 183, 186). In humans with either acutely elevated plasma homocysteine levels after an oral methionine challenge (14, 18, 30, 72, 95, 185) or with chronic, mild hyperhomocysteinemia (82, 160, 191), impaired endothelium-dependent vasodilator function also develops, but they preserve endothelium-independent vasodilator responses. Decreased bioavailability of endothelium-derived nitric oxide (NO), which impairs endothelium-dependent vasomotor responses, has been postulated to be one of the major mechanisms of hyperhomocysteinemia-induced endothelial dysfunction (106, 182). NO participates in signal-transduction pathways that are important in the cardiovascular system, activating soluble guanylyl cyclase in vascular smooth muscle cells (VSMCs), resulting in accumulation of cyclic guanosine monophosphate and relaxation. The intracellular redox buffer system, consisting of reduced glutathione and glutathione disulfide together with glutathione reductase and glutathione peroxidase, plays a central role in the cellular defense against oxidant stress and has an important role in maintaining endothelial function. NO synthesis is impaired in glutathione-depleted human endothelial cells and, conversely, boosting cellular glutathione content with glutathione monoethyl ester results in enhanced NO production (64). Furthermore, mice deficient in cellular glutathione peroxidase have endothelial dysfunction. This is due to increased

vascular oxidant stress with resulting reduction in the bioavailability of NO (56). The role of the cellular redox state in homocysteine-induced endothelial dysfunction is emphasized by the observation that treatment of hyperhomocysteinemic $CBS^{(-/+)}$ mice with the intracellular cysteine donor L-2-oxo-4-thiazolidine carboxylate (OTC) restored normal endothelial function (183). This agent increases intracellular levels of reduced glutathione and total thiols and thereby shifts the cellular redox state to a more reduced environment. Overexpression of cellular glutathione peroxidase in hyperhomocysteinemic $CBS^{(-/+)}$ mice or in cultured endothelial cells exposed to homocysteine prevented homocysteine-induced endothelial dysfunction and decrease in bioavailable NO (186).

Exposure of vascular endothelial and smooth muscle cells *in vitro* to superoxide, hydrogen peroxide, nitric oxide, and peroxynitrite resulted in mitochondrial DNA damage. Endothelial cells were more sensitive to reactive oxygen and nitrogen species–mediated damage than smooth muscle cells (12). The association between mitochondrial DNA damage and the development of atherosclerotic lesions could also be demonstrated in human aortic specimens and in a murine model of early atherosclerosis (11). The mitochondrial respiratory chain is the major source of prooxidants, like reactive oxygen, nitrogen, and thiol species, formed as byproducts of normal cell respiration. Mitochondria may also be important targets for ROS, which may damage mitochondrial lipids, enzymes, and DNA, with resulting mitochondrial dysfunction. Free cholesterol, oxidized low-density lipoprotein (oxLDL), and glycated high-density lipoprotein (HDL) are additional causes of mitochondrial dysfunction or apoptosis or both (133). Increased ROS can mediate mitochondrial DNA (mtDNA) damage, alteration of mitochondrial gene expression, and mitochondrial dysfunction (87).

The accumulation of homocysteine leads to increased cellular oxidant stress also in mitochondria. Homocysteine has been shown to promote mitochondrial damage and alter mitochondrial gene expression and function, suggesting the participation of oxidant stress (6). Homocysteine induced expression of the mitochondrial electron transport chain gene cytochrome *c* oxidase III/ATPase 6,8 in a concentration- and time-dependent manner, as identified in the human megakaryocytic cell line DAMI by using mRNA differential display. Conversely, 1 *M* homocysteine in the presence of $Cu^{2+}$, which is known to generate hydrogen peroxide, significantly decreased mitochondrial RNA levels of cytochrome *c* oxidase III/ATPase 6,8, caused gross morphologic changes in mitochondrial ultrastructure, and inhibited both cell growth and mitochondrial respiration rates. In addition, biogenesis of mitochondria has been shown to be affected by homocysteine (129). Incubation with homocysteine increased the intracellular ROS content and resulted in a significant increase in the mitochondrial mass of endothelial cells. Homocysteine stimulated the expression of NRF-1 and Tfam, two factors involved in the regulation of mitochondrial biogenesis. The ability of homocysteine to increase intracellular levels of ROS is essential for homocysteine-induced mitochondrial biogenesis, as the pretreatment with antioxidants abolished this effect.

ROS and oxidant stress promote the formation of nitrotyrosine, an indicator of the NO and superoxide radical reaction, resulting in the formation of the strong oxidant peroxynitrite. Peroxynitrite, besides other effects, leads to tyrosine nitration. The latter event may alter protein function, and therefore, induce cellular dysfunction (166). Treatment of endothelial cells with homocysteine is accompanied by increased formation of peroxynitrite, detected by the formation of nitrotyrosine and nitrotyrosine-modified proteins. Pretreatment with catechin, a flavonoid antioxidant that reduces ROS levels, decreased homocysteine-dependent formation of nitrotyrosine (129, 202). These events also occur *in vivo*. Mildly hyperhomocysteinemic $CBS^{(-/+)}$ deficient mice compared with wild-type mice showed increased nitrotyrosine staining of the aortic wall (51). Peroxynitrite can directly damage the electron-transport chain in mitochondria and thereby induce mitochondrial and consequently endothelial dysfunction, resulting in the promotion of atherosclerosis (134).

Homocysteine-induced oxidant stress stimulates endothelial dysfunction, which also leads to the activation of proinflammatory pathways in the vasculature (182). Substantial experimental data indicate that ROS are potential regulators of endothelial cell adhesion molecule expression and inflammatory microvascular dysfunction (65). Cytokines such as TNF-$\alpha$ increase vascular cell adhesion molecule-1 (VCAM-1) and monocyte chemoattractant protein-1 (MCP-1) expression through a redox-sensitive mechanism involving NF-$\kappa$B. This can be inhibited by antioxidants or the NADPH oxidase inhibitor apocynin (164, 181).

## VASCULAR INFLAMMATION

### *Atherosclerosis may be considered a chronic inflammatory disease*

Russel Ross postulated the "response-to-injury" hypothesis that describes atherosclerosis as a protective inflammatory response in the vessel wall against different agents that cause cell injury. This leads to a series of inflammatory reactions that themselves may become pathologic if they proceed unchecked (139, 140). Cell injury can be caused by ROS originating from several endogenous or exogenous sources, partly as a result of exposure to cardiovascular risk factors. ROS trigger multiple pathologic events involved in atherosclerosis, including the oxidation of core lipids of lipoproteins and cell membranes and the modification of apolipoproteins and other proteins, leading to their recognition by scavenger receptors.

Low-density lipoproteins (LDLs) are crucially involved in the pathogenesis of atherosclerosis after undergoing oxidant modifications in the arterial wall. After that, they exhibit a variety of biologic properties potentially involved in the development of atherosclerotic lesions (139, 189). Accumulation of oxidized lipoproteins in the artery wall sets off a cascade of proinflammatory events, leading to the binding and transmigration of monocytes through the intima, the recruitment of macrophages, lipid uptake into these cells, and the initiation of the chronic inflammatory cascade that characterizes atherosclerosis (70). Electron microscopy studies have established that the earliest atherosclerotic lesion, the fatty streak, consists almost entirely of lipid-laden macrophages, thus implicating lipoprotein uptake by these immune cells in the origination of atherosclerosis (62). Because homocysteine enhances ROS gen-

eration and has been implicated in LDL modification, it may promote atherosclerosis. The proinflammatory effects of oxidized LDL and also of homocysteine directly involve the generation of peroxides and other reactive oxygen intermediates. These molecules activate nuclear transcription factor κB (NF-κB), which plays a key role in the orchestration of inflammatory and immune responses by controlling the transcription of genes encoding several of the adhesion molecules, interleukins, tumor necrosis factor α (TNF-α), major histocompatibility class II (MHC II) antigen, and antibodies (70). NF-κB recognizes various activators, among which are proinflammatory cytokines and C-reactive protein (CRP).

In animal models, an atherogenic diet led to upregulation of the expression of selective adhesion molecules by endothelial cells, which are specific for leukocytes in different areas of arteries. Such adhesion molecules are E- and P-selectins, the vascular cell adhesion molecule 1 (VCAM-1), and the intracellular adhesion molecules (ICAMs) (110). The first interaction between leukocyte rolling along the vessel wall and the endothelial cell layer is facilitated by endothelial expression of P-selectin and its interaction with leukocyte P-selectin ligand-1 (17). Firm leukocyte adhesion then requires interaction between leukocyte $\beta_1$- and $\beta_2$-integrins and endothelial immunoglobulin superfamily members such as VCAM-1 and ICAM-1, respectively (203). The role of cellular adhesion molecules is emphasized by data that show that mice lacking P-selectin, ICAM-1, or $\beta_2$-integrins are protected against the full spectrum of atherosclerotic lesion development (120).

Evidence suggests that oxidant stress events in endothelial cells and leukocytes promote their interaction. Intracellular superoxide anion generation seems to be implicated in cytokine-induced upregulation of VCAM-1, as it is inhibited by cellular overexpression of superoxide dismutase (SOD) (33). In contrast, it is enhanced by oxLDL or its oxidized fatty acids (97). Human arterial endothelial cells exposed to oxLDL expressed more ICAM-1, VCAM-1, and E-selectin compared with the controls, even in the absence of cytokines (2). Thus, considerable data indicate that cellular oxidant events modulate the expression of adhesion molecules on the endothelium.

Once firm adhesion is established, leukocytes may transmigrate across the endothelium into the intima along a chemotactic gradient such as that produced by monocyte chemoattractant protein (MCP-1) secreted by endothelial and smooth muscle cells (37). MCP-1 provides a signal that pulls bound monocytes into the intima at sites of lesions in the arterial wall; T-cell chemoattractants have the same effect on T-lymphocytes (115). Experiments with atherosclerosis-prone mice deficient in either MCP-1 or its receptor, chemokine receptor–2 (CCR2), showed reduced lipid deposition in the aorta after exposure to atherogenic diets, confirming the importance of monocyte migration during atherosclerosis (21, 68).

Once entered into the arterial intima, monocytes differentiate into macrophages, which develop ultimately into foam cells. They internalize modified lipoproteins after increasing the expression of scavenger receptors such as the scavenger receptor A (SRA) and CD36, both involved in the endocytic uptake of oxidized LDL, resulting in the accumulation of cholesteryl esters in cytoplasmic droplets (83, 101). These lipid-laden macrophages, known as foam cells, characterize the early atherosclerotic lesion. Macrophages within atheroma replicate and secrete a number of cytokines and growth factors involved in lesion progression and complications (74).

Endothelial cells exposed to oxidant stress produce macrophage colony-stimulating factor (M-CSF), which augments SRA expression, increases production of cytokines and growth factors, and also serves as a survival and co-mitogenic stimulus, thus promoting transition of monocytes to lipid-laden foam cells (74). Both experimental and human atherosclerotic plaques overexpress M-CSF (34). Activated macrophages promote interferon-γ (IFN-γ) production by activated T cells through secretion of interleukin-12 (IL-12) (105). Because IFN-γ downregulates cholesterol efflux from macrophage-derived foam cells, this cytokine cascade may further trigger cholesterol accumulation in atherosclerotic lesions and presumably accelerate transformation of macrophages into foam cells (127). The intensive aggregation of foam cells leads to the formation of an atheromatous core. In the central portion of the atheromatous core, the accumulation of oxLDL leads to extensive DNA damage and cell death (76). Destruction of foam cells is accompanied by extracellular accumulation of lipids and cellular debris, plaque growth, and build-up of a necrotic core within the lesion.

In addition to monocytes, neutrophils are infiltrating the intima during atherosclerotic lesion development. These cells generate ROS, which provoke oxidant damage to endothelial cells. Consistently, neutrophil count is an independent predictor of the presence of multiple complex stenoses, irrespective of coronary artery disease extent in patients with chronic stable angina (8). Furthermore, higher neutrophil counts are associated with elevated coronary artery disease complexity (7). Neutrophil infiltration of affected lesions with release of elastase and myeloperoxidase (MPO) has been implicated in the pathogenesis of atherosclerosis (124). MPO, an abundant microbicidal neutrophil hemoprotein, seems to be an emerging biomarker to assess cardiovascular risk *in vivo* (25). Several mechanisms linking MPO to CVD have been reported so far. MPO and MPO-generated products were suggested to be responsible for LDL oxidation (39) and promotion of high-density lipoprotein (HDL) oxidation, thereby contributing to atherosclerosis by counteracting the antiatherogenic effects of HDLs (15). It has been suggested that MPO is a catalyst for lipid peroxidation *via* tyrosyl radical formation (141) and for lipid and lipoprotein oxidant modifications *via* generation of reactive nitrogen species (201). MPO also contributes to endothelial dysfunction by regulating NO bioavailability (52). Furthermore, higher levels of circulating MPO are associated with plaque vulnerability in subjects at risk for major adverse cardiac events (10).

Platelets also are thought to play a role in the initiation and progression of atherosclerotic lesions (140) because they facilitate migration of monocytes and other mononuclear cells into the arterial wall. P-selectin is displayed on platelets responsible for their adhesion to monocytes, resulting in circulating complexes with increased adhesion to endothelial cells under coronary-flow conditions (161). In an animal model, platelets facilitate lymphocyte delivery to high endothelial venules (HEVs) by platelet P-selectin, which allows activated platelets transiently to form a bridge between lymphocytes and HEVs. This enables lymphocytes to undergo subsequent $\beta_2$-integrin–dependent firm adhesion (43, 44).

Transient blockade of P-selectin or its major ligand, P-selectin glycoprotein ligand-1 (PSGL-1), at the time of endothelial denudation injury of carotid arteries in apolipoprotein E knockout (apoE$^{(-/+)}$) mice significantly limited plaque macrophage content and neointima formation in a dose-dependent manner (130). With wild-type and P-selectin-knockout (Psel$^{(-/+)}$) mice, Wang *et al*. (178) performed bone marrow transplantation to generate chimeric mice that expressed either platelet P-selectin or endothelial P-selectin. Platelet P-selectin expression, but not endothelial P-selectin, was shown to play a major role in the development of neointimal formation after arterial injury, accompanied by an increased inflammatory response. The latter becomes evident by immunostaining for the chemokines RANTES (chemokine regulated on activation, normal T cell expressed and secreted) and MCP-1 (178).

Adhesion of leukocyte–platelet complexes to the endothelium leads to prolonged contact with endothelial cells and deposition of chemokines that activate and arrest monocytes by enhanced integrin activity. It also exacerbates atherosclerotic lesions in mice (85, 180). Deposition of the platelet chemokine RANTES clearly contributes to vascular inflammation during atherosclerosis after vascular injury by inducing selective monocyte and T-cell recruitment to the endothelium (144). Moreover, the strong proinflammatory mediator IL-1$\beta$ is secreted by platelets and has an impact on atherosclerosis that is mediated by the upregulation of adhesion molecule expression and increased chemokine production in endothelial cells (60). The expression of IL-1$\beta$ has been found to be increased in arteries of hyperlipidemic animals and in monocytes incubated with oxLDL (102).

T cells also infiltrate into the atherosclerotic lesions. Predominantly CD4$^+$ T cells, which recognize protein antigens presented to them as fragments bound to MHC class II molecules, are present in atherosclerotic plaques, which are reactive against oxLDL (154). In an atherosclerotic lesion, production of cytokines that promote a Th1 rather than a Th2 response is favored (58). Activated T cells therefore differentiate into Th1 effector cells and start to produce the macrophage-activating cytokine interferon-$\gamma$ (IFN-$\gamma$). IFN-$\gamma$, which augments the synthesis of the inflammatory cytokines TNF-$\alpha$ and IL-1, initiates the production of many inflammatory and cytotoxic molecules in macrophages and vascular cells (157). T-cell cytokines cause the production of large amounts of molecules downstream in the cytokine cascade, resulting in elevated levels of IL-6 and CRP, leading to progression of vascular disease.

The pathogenesis of atherosclerosis furthermore involves the proliferation and migration of medial VSMCs into the vessel intima. With progression of atherosclerosis, VSMCs migrate into the atherosclerotic lesion, proliferate, and produce extracellular matrix components promoting growth of the atherosclerotic plaque (140). In response to oxLDL or injury, VSMCs express MCP-1, VCAM-1, and other proinflammatory molecules that further promote monocyte transmigration into the subendothelium. The cytokine TNF-$\alpha$ is secreted by VSMCs in the neointima as well as by macrophages accumulated in atherosclerotic lesions. It markedly induces proliferation and migration of VSMCs (93). VSMCs are the only cells capable of synthesizing components of the fibrous cap in plaques, which separate the blood from the thrombogenic plaque interior, and whose rupture or erosion triggers myocardial infarction. Matrix metalloproteinase 2 (MMP-2) is synthesized and released from VSMCs and plays a key role in extracellular tissue remodeling during morphogenesis, angiogenesis, and wound healing (45). It also is involved in the development and progression of atherosclerotic lesions by contributing to destabilization and rupture of atherosclerotic plaques (59).

## *Influence of homocysteine on endothelial activation and the adhesion and transmigration of leukocytes to and through the vascular endothelium*

Elevated homocysteine levels have been shown both *in vitro* and *in vivo* to influence several of the pathways described earlier that promote vascular inflammation and atherosclerosis.

*Monocytes and T cells.* Exposure of cultured endothelial cells to homocysteine has been shown to lead to endothelial activation, which results in increased endothelial expression of chemokines (131) and adhesion molecules (100, 132, 151, 176) (Fig. 1).

Koga *et al*. (100) investigated the effects of homocysteine on the interactions between U937 monocytic cells and Jurkat T cells with human aortic endothelial cell (HAECs) under inflammatory cytokine-stimulated conditions. When HAECs were pretreated with homocysteine followed by stimulation with IL-1$\beta$, U937 and Jurkat T cell adhesion to HAECs increased in a dose-dependent manner. The increase in U937 cell adhesion to HAECs was also observed when U937 cells alone or when both cell types were treated with homocysteine. Furthermore, they demonstrated that homocysteine increases endothelial surface expression and mRNA levels of the adhesion molecules, VCAM-1 and E-selectin. Attenuation of Jurkat T

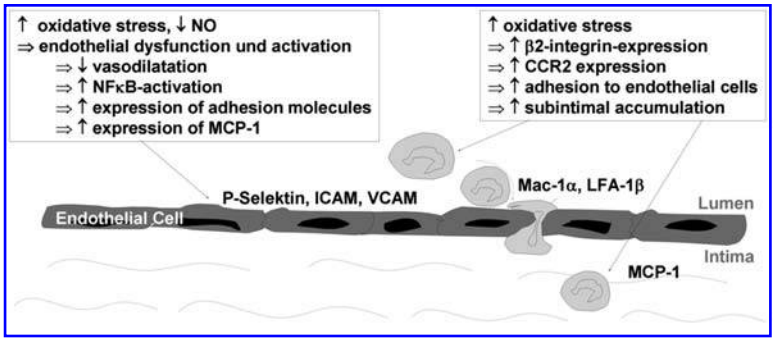


**FIG. 1. Effect of homocysteine on endothelial activation and the adhesion and transmigration of leukocytes to and through the vascular endothelium.**

cell and U937 cell adhesion to HAECs by monoclonal antibodies directed against specific adhesion molecules demonstrated that both VCAM-1 and E-selectin are involved in Jurkat T-cell adhesion, and VCAM-1, in U937 cell adhesion.

Silvermann *et al*. (151) observed a 4.5-fold increase in the adhesion of human monocytes and monocytic U937 cells after 24-h incubation of cultured HAECs with 100 $\mu M$ D,L-homocysteine and a fivefold increase in VCAM-1 mRNA expression in HAECs after 3 h of treatment. Coincubation of HAECs with homocysteine and TNF-$\alpha$ synergistically elevated monocyte adhesion as well as VCAM-1 protein expression. Preincubation of HAECs with cyclooxygenase inhibitors completely abrogated homocysteine-induced monocyte adhesion. Because cyclooxygenase is responsible for the formation of prostanoids implicated in inflammatory processes, this suggests the involvement of proinflammatory processes. Scavenging of ROS resulted in partial inhibition of adhesion, assuming homocysteine-induced endothelial VCAM-1 expression is at least partly due to increased oxidant stress (151).

More recently, we showed that incubation of human endothelial cells [EA.hy 926 cells and primary human umbilical vein endothelial cells (HUVECs)] with L-, but not with D-homocysteine in pathophysiologically relevant concentrations resulted in a dose-dependent increase of ROS inside these cells, as measured by using DCF-fluorescence (78, 132). This was accompanied by enhanced NF-$\kappa$B activation, and stimulated ICAM-1 mRNA transcription and cell-surface expression. Functionally, this led to a time- and dose-dependent increase in monocyte adhesion to endothelial cells incubated with L-homocysteine. Pretreatment of endothelial cells with superoxide scavengers (MnTBAP and Tiron) or with an inhibitor of NF-$\kappa$B activation abolished homocysteine-induced monocyte adhesion, ICAM-1 expression, and nuclear translocation of NF-$\kappa$B (132). This suggests that ROS produced under hyperhomocysteinemic conditions induce a proinflammatory situation in the vessel wall that promotes endothelial activation and monocyte adhesion to the vascular endothelium.

The effects of homocysteine on endothelial activation and adhesion molecule expression obtained *in vitro* could be confirmed in *in vivo* studies. Increased P-selectin expression by activated/dysfunctional EC or platelets or both in mildly hyperhomocysteinemic heterozygous cystathionine $\beta$ synthase–deficient ($CBS^{(-/+)}$) mice has been shown by enzyme-linked immunosorbent assay (ELISA) for soluble P-selectin and immunostaining of aortic sections with an anti–P-selectin antibody (183). Hyperhomocysteinemic compared with control rats showed significantly increased expression of VCAM-1 and E-selectin on the aortic endothelium (176). Induction of hyperhomocysteinemia in $ApoE^{(-/-)}$ mice by dietary methods enhanced the expression of receptor for advanced glycation end-products (RAGE), VCAM-1, tissue factor (TF), and MMP-9 in the vasculature (80).

The role of oxidant stress in the activation of vascular proinflammatory pathways is emphasized by the finding that treatment of heterozygous $CBS^{(-/+)}$ mice with OTC resulted in a reduction of plasma-soluble P-selectin levels to the same levels as found in wild-type mice and in a weaker P-selectin staining of the aortic surface, which was no longer different from that in wild-type mice. As treatment with OTC resulted in significantly higher levels of total thiols and reduced glutathione levels in vascular tissues, which shifts the cellular redox state to a more reduced environment, these findings indicate that mild hyperhomocysteinemia promoted endothelial and platelet activation, resulting in increased P-selectin expression, presumably due to increased oxidant stress, as it can be compensated by boosting cellular antioxidants (183).

In addition to adhesion molecules necessary for the adhesion from leukocytes to endothelial cells, chemokines play an important role in atherosclerosis. Chemokines belong to a superfamily of structurally related small chemotactic cytokines involved in leukocyte trafficking. The most notable chemokine is MCP-1, which stimulates the migration of monocytes and T lymphocytes into the intima of the arterial wall through its receptor, CC chemokine receptor 2 (CCR2). The expression of MCP-1 mRNA and protein is markedly elevated in atherosclerotic lesions in both humans and experimental animals (195). The absence of MCP-1 greatly decreased the lesion size in LDL-receptor–deficient mice (68). Similarly, the absence of CCR2 caused a reduction in lesion size in $apoE^{(-/-)}$ mice (21). Interleukin-8 (IL-8) rapidly causes rolling monocytes to adhere firmly onto endothelial monolayers expressing E-selectin (63). MCP-1 together with IL-8 is crucial in converting rolling monocytes to firm adhesion on endothelial monolayers and is essential to mediate monocyte transmigration into the subintimal space (41, 135, 169).

Several groups provided *in vitro* evidence that homocysteine could promote the expression and secretion of MCP-1 and IL-8 from cultured HAECs (61, 131, 156) and of MCP-1 from human VSMCs (175). In addition, in an *ex vivo* system using whole blood, monocytes incubated with homocysteine produced more MCP-1 and IL-8 (197, 198). These studies suggest that homocysteine could promote local plaque development by increasing MCP-1 and IL-8 levels locally. Mechanistically, generation of ROS and the activation of the redox-sensitive transcription factor NF-$\kappa$B have been linked to the expression and secretion of MCP-1 by various vascular cells during homocysteine exposure (197).

As mentioned earlier, MCP-1 exerts its action mainly through the interaction with an MCP-1 receptor, CCR2, on the surface of monocytes (32). Incubation of THP1 cells and peripheral blood monocytes with homocysteine (50–200 $\mu M$) for 24 h resulted in a significantly enhanced expression of CCR2 mRNA and protein (up to 183% of control) (173). Stimulation of CCR2 expression was associated with a parallel increase in the binding activity of CCR2 (129 $\pm$ 191% of control) as well as an enhanced chemotactic response of homocysteine-treated monocytes. Furthermore, the levels of superoxide were significantly elevated in cells incubated with homocysteine. The addition of SOD to the culture medium abolished the stimulatory effect of homocysteine on CCR2 expression as well as the binding activity of the receptor, suggesting that oxidant stress contributes to homocysteine's effect.

All these molecular events may increase the chemotaxis, adhesion, and transmigration of mononuclear cells to the vessel wall and may thereby promote atherosclerotic lesion development. Indeed, induction of hyperhomocysteinemia has been shown to promote the development of atherosclerotic lesions and increase their complexity in atherosclerosis-prone mouse models (80, 177).

*Neutrophils.* Neutrophils participate in vascular inflammation and endothelial damage during the process of atherosclerosis by chemotaxis and adhesion to endothelial cells and subsequent oxidant damage to the vessel wall *via* released superoxide (27, 159). Homocysteine has been shown to induce selectively surface molecule changes on neutrophils, leading to their increased adhesion to artificial surfaces and human endothelial cells. Exposure of purified human peripheral blood neutrophils to homocysteine caused them to bind to plastic alone as well as to HUVECs, with almost identical dose–response curves. Enhanced adhesion was detectable at homocysteine concentrations as low as $\leq 10\ \mu M$; adhesion further increased at higher concentrations up to a plateau at $\geq 200\ \mu M$ (48). Furthermore, incubation of HUVECs with homocysteine also resulted in a higher number of adherent untreated neutrophils and in increased neutrophil migration across the endothelial layer, with concurrent damage and detachment of endothelial cells. This was assessed by measuring $^{51}Cr$ release from $^{51}Cr$-loaded endothelial cells coincubated with homocysteine-treated neutrophils. Generation of hydrogen peroxide by adherent leukocytes appeared to damage HUVECs, because added catalase prevented them from destruction. Studies using blocking antibodies were consistent with the hypothesis that neutrophils docked to HUVECs in a CD11b/CD18-dependent process. In accordance with these *in vitro* findings, homocysteine infusion in rats caused significant decreases in the rolling velocities of leukocytes in postcapillary mesentery venules. This went along with a significantly increased number of leukocytes that were arrested along the luminal surface of venules. The significant increase in the number of leukocytes that extravasated from the venules during homocysteine infusion also reflected the homocysteine-induced trans-HUVEC/membrane migration of neutrophils *in vitro*. The type of leukocyte that adhered and migrated in the rat mesentery venules was tentatively identified in this study as neutrophils, on the basis of characteristic size and granular appearance, with a minor proportion of monocytes (47).

Evidence indicates that homocysteine promotes ROS generation in leukocytes: homocysteine (10–500 $\mu M$) increased superoxide output of neutrophils in a dose- and time-dependent manner. This seemed to be at least partly due to activation of NADPH oxidase, as it could be inhibited by diphenyleneiodonium (DPI), an inhibitor of NADPH oxidase activity (1). NADPH oxidase is dormant in resting phagocytes, with membrane ($p22^{phox}$) and cytosolic ($p47^{phox}$ and $p67^{phox}$) components located at different sites. This enzyme becomes activated by a complex process, involving Rac proteins and a GTP-dependent mechanism, which results in the mobilization of cytosolic $p47^{phox}$ and $p67^{phox}$ to the plasma membrane (79). Homocysteine incubation of both suspended and adherent neutrophils has been shown to mobilize $p47^{phox}$ and $p67^{phox}$ subunits of the enzyme to the plasma membrane in (1) (Fig. 2).

Activity of NADPH oxidase in neutrophils is induced by fMLP-receptor ligands such as formylated peptides from bacteria. The fMLP receptor is a G protein–coupled receptor (FPR-1) that activates kinases of the mitogen-activated family and phospholipase C. Neutrophils that were preincubated with D,L-homocysteine and D,L-homocysteine-thiolactone showed enhanced fMLP-induced superoxide generation, which was not inducible by using other sulfur amino acids (99).

A possible involvement of homocysteine-induced ROS generation in mitogen-activated phosphokinases (MAPKs) activation in human neutrophils was suggested recently (1). MAPKs are serine/threonine specific protein kinases that respond to extracellular stimuli (mitogens) and regulate various cellular activities, such as gene expression, mitosis, differentiation, and cell survival/apoptosis (128). It has been demonstrated that homocysteine stimulates MAPK activation in smooth muscle cells (190) and activates c-Jun NH(2)-terminal kinase in vascular endothelial cells (29). The relation between hydrogen peroxide production and agonist-dependent activation of the different forms of MAPKs in neutrophils has previously been described (118). In this context, it is worth mentioning that prooxidant conditions promoted by homocysteine are partially coupled to the activation of p38-MAPK and ERK1/2, although the exact hierarchic order of homocysteine-dependent activation of NADPH oxidase and MAPKs must be clarified (1).

*Platelets.* Platelets, long thought of as cells that react only to endothelial disruption, are now recognized as important mediators of the inflammatory process during the development and progression of atherosclerosis. Platelet activation leads to the secretion of chemokines, growth factors, and coagulation factors promoting inflammation in atherosclerotic plaques (171).

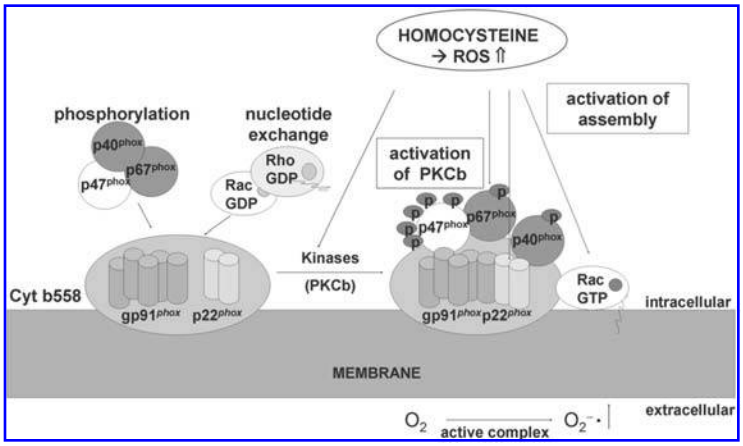


**FIG. 2. Putative mechanism of the activation of NADPH oxidase by homocysteine.**

Some evidence suggests that homocysteine has an influence on platelet activation. Among patients with peripheral arterial occlusive disease (PAD), a higher ADP and adenosine-stimulated platelet reactivity was found in those with elevated compared with those with normal homocysteine levels, and also compared with normohomocysteinemic controls without PAD. In addition, agonist-induced P-selectin expression was significantly increased in hyperhomocysteinemic patients compared with both normohomocysteinemic groups. These findings might be due to the fact that platelets from hyperhomocysteinemic patients were significantly less sensitive to exogenous NO (GSNO; 1–100 $\mu M$)–mediated inhibition than all other groups (136). In an animal model of dietary folic acid deficiency that leads to mild hyperhomocysteinemia, elevated homocysteine levels were associated with enhanced macrophage-derived tissue factor (TF) and platelet activities (49). Methionine load in folate-deficient and also in folate-sufficient animals led to a further fourfold homocysteine increase after 2 h. Platelet aggregation in response to thrombin and ADP as well as thrombin-induced thromboxane synthesis was potentiated after the methionine load in both groups of animals. Acute hyperhomocysteinemia also stimulated the basal and lipopolysaccharide-induced TF activity of peritoneal macrophages (49). These prothrombotic effects were associated with increased lipid peroxidation characterized by elevated plasma levels of conjugated dienes, lipid hydroperoxides, and thiobarbituric acid–reactive substances, suggesting that hyperhomocysteinemia triggers this processes through increased oxidant stress.

The hypothesis that elevated homocysteine stimulates the generation of ROS in platelets could be confirmed in human platelets. Incubation of platelets with homocysteine resulted in intracellular generation of ROS, which could be measured by using the redox-sensitive dye dichlorofluorescein (DCF) (150). This effect was dose dependent in a pathophysiologically relevant range of homocysteine concentrations (10–50 $\mu M$), whereas cysteine had no effect. Moreover, homocysteine in a dose- and time-dependent manner increased arachidonic acid release and formation of its end product thromboxane 2 ($TXB_2$) *via* the cyclooxygenase pathway. Mechanistically, it could be demonstrated that platelets treated with 5,8,11,14-eicosatetraynoic acid (ETYA), which inhibits arachidonic acid release and metabolism, and diphenyleneiodonium (DPI), a known inhibitor of NADPH oxidase and NO synthase, reduced ROS accumulation by 45% and 25%, respectively. This suggests that stimulation of arachidonic acid metabolism could be directly or indirectly (or both) involved (through the NADPH oxidase activation) in ROS accumulation. Homocysteine seems to stimulate arachidonic acid release *via* cytosolic phospholipase $A_2$ (cPLA2) activation (107). In this work, it was shown that homocysteine stimulated the rapid and sustained phosphorylation of platelet p38 MAPK in a time- and dose-dependent manner. The homocysteine effect on p38 MAPK phosphorylation was prevented by the antioxidant and thiol reducing agent *N*-acetyl-L-cysteine and the prostaglandin $E_1$ analogue iloprost and was partially inhibited by the lipoxygenase inhibitor nordihydroguaiaretic acid. Incubation of platelets with homocysteine led to phosphorylation of cPLA2, leading to their activation, assessed by an increased arachidonic acid release. The cPLA2 phosphorylation and activation were both impaired by the inhibition of p38 MAPK activation through SB203580. The inhibition obtained by using the MAPK inhibitor, however, was not complete. The residual arachidonic acid release in SB203580–pretreated and homocysteine-stimulated platelets may be due to intracellular calcium elevation promoted by homocysteine. In platelets loaded with the $Ca^{2+}$-sensitive fluorescent dye FURA 2, homocysteine induced a dose-dependent intracellular calcium increase. This suggests that calcium elevation promoted by homocysteine could participate in cPLA2 activation, leading to arachidonic acid release and TXB2 formation.

In summary, these data suggest that platelet activation induced by homocysteine might be mediated by activation of the p38 MAPK/cPLA2 pathway. Recently it was found that homocysteine stimulated the tyrosine phosphorylation and activation of platelet PLC$\gamma$2 *via* the tyrosine kinases p60src and p72syk (108). The activation of PLC$\gamma$2 induces various steps in PLA2 stimulation, resulting in arachidonic acid release. Because ROS were increased in homocysteine-treated platelets, oxidant stress could prime the non–receptor-mediated tyrosine kinase p60src, inducing phosphorylation and activation of p72syk. The antioxidant *N*-acetyl-L-cysteine prevented the activation of these kinases. This data indicate that PLC$\gamma$2, playing an important role in platelet activation, is activated by homocysteine and that the stimulation of this pathway requires signals through ROS (172) (Fig. 3).

Another important signaling molecule regulating platelet function is NO. Its enzymatic biosynthesis requires L-arginine as a precursor. Homocysteine has been shown to cause a concentration-dependent reduction in the platelet uptake of L-arginine (109, 111). This went along with a concentration-dependent decrease in nitrite production concurrent with a decrease in cyclic guanosine monophosphate, whereas cNOS activity of platelets, measured as conversion of L-[$^3$H]arginine to L-[$^3$H]citrulline, remained unchanged. These observations indicate that the L-arginine/NO pathway is involved in the mechanism responsible for the effects of homocysteine on platelets by diminishing NO production at least partly through decreased uptake of L-arginine. Thus, homocysteine, by increasing ROS formation and influencing NO signaling, can potentiate platelet response and contribute to the various thrombogenic effects described in hyperhomocysteinemia. Homocysteine is possibly acting as a "primary" inducer activating the release of the "secondary" inducer ROS.

## *Influence of homocysteine on foam cell formation*

One of the crucial steps during the development of atherosclerosis is the accumulation of oxLDL in macrophages, inducing their differentiation into foam cells. The early lesions of atherosclerosis (fatty streaks) consist of cholesterol-engorged macrophages, so-called foam cells, and T lymphocytes (101). Macrophages may function to carry cholesterol out of lesions or to process the cholesterol for excretion in association with small protein–phospholipid complexes.

The internalization of oxidized LDL− by scavenger receptors, could account for the transformation of monocyte-derived macrophages to foam cells in atherosclerotic lesions (143). It has been suggested that oxidation of LDL facilitates the accumulation of cholesterol in foam cells through unregulated uptake *via* the scavenger receptor family, a family of at least 11

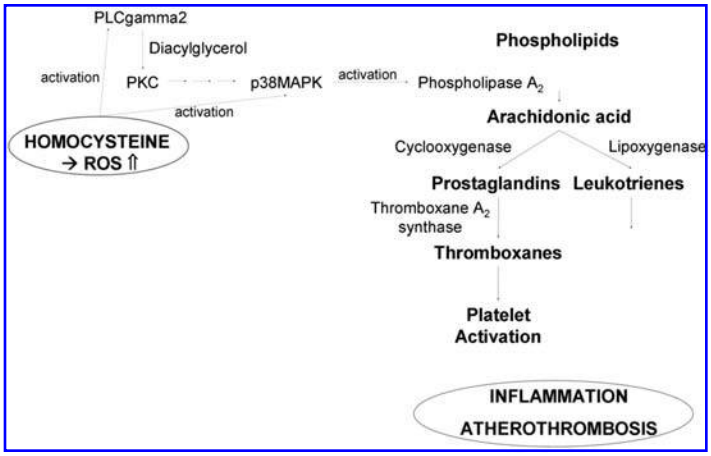


**FIG. 3. Homocysteine activates arachidonic acid release through activation of phospholipase $A_2$.**

receptors, of which SR-A and CD36 are the principal receptors involved in the endocytic uptake of oxidized LDL (53, 83). CD36 is an 88–kDa glycoprotein expressed on platelets, monocytes, macrophages, capillary endothelial cells, and adipocytes, recognizing a variety of ligands, including modified LDL, anionic phospholipids, and long-chain fatty acids (54, 67). CD36 is upregulated by oxLDL *via* the peroxisome proliferator–activated receptor-$\gamma$ (PPAR$\gamma$) pathway and *via* PKC (54, 121). Two different structural elements of oxLDL have direct effects on PPAR$\gamma$: 13-hydroxyoctadecadienoic acid (13-HODE) (or 9-HODE) and oxidized phosphatidylcholine (hexadecyl azelaoyl phosphatidylcholine), which is considered to be the most potent natural ligand of PPAR$\gamma$ (40).

Recently we demonstrated that homocysteine enhances CD36 expression in phorbol 12-myristate 13-acetate (PMA)-stimulated THP-1 cells and primary human monocyte/macrophages in a dose-dependent manner, resulting in increased oxLDL uptake (86). This seems to be mediated *via* activation of the PPAR$\gamma$ pathway, as homocysteine stimulated the binding of nuclear proteins to a PPAR$\gamma$ consensus sequence, and an inhibitor of the PPAR-$\gamma$ pathway prevented the homocysteine effect on the binding of nuclear proteins to a PPAR-$\gamma$ consensus sequence, on the stimulation of CD36 expression in monocytes/macrophages, and on oxLDL uptake. Whether activation of the MAPK pathway by homocysteine *via* oxidant stress–sensitive mechanisms is responsible for these effects remains to be determined. PMA (a common PKC activator), IL-4, and MCSF all induce monocyte expression of CD36 through activation of PPAR$\gamma$ (84), presumably through activation of the MAPK signal-transduction pathway. MAPK induces the extracellular signal–regulated kinases 1 and 2 as well as the stress-induced kinases Jun NH2-terminal kinase and p38. It has been demonstrated that homocysteine regulates activation of PKC in macrophages, the Jun NH2-terminal kinase signal pathway in human endothelial cells, and extracellular signal-regulated kinase-1 and -2 phosphorylation in smooth-muscle cells (190).

Downstream effects of homocysteine on human LDLs exported from endothelial cells in an *in vitro* system suggested increased oxLDL formation under conditions of elevated homocysteine. OxLDL on its part activates CD36 expression and foam cell formation (122). HUVECs were shown to export homocysteine at a rate determined by the flux through the methionine/homocysteine pathway. Additional methionine increased intracellular methionine, decreased intracellular folate, and increased homocysteine export, whereas additional folate inhibited export. An inverse relation existed between intracellular folate and homocysteine export. Human LDLs exposed to HUVECs exporting homocysteine underwent time-dependent lipid oxidation, a process inhibited by the thiol trap dithionitrobenzoate. This process was related to the generation of hydroxyl radicals, which was associated with homocysteine export. The time-dependent oxidation of LDL in this system resulted in a time-dependent increase in the uptake by human macrophages. This suggests that continuous export of homocysteine from endothelial cells contributes to the generation of extracellular hydroxyl radicals, resulting in oxidant modification of LDL and uptake by macrophages, all key steps in atherosclerosis. Some authors have suggested that the major oxidant species in copper-mediated LDL oxidation is superoxide (77); others have concluded that the hydroxyl radical may be important (122). The participation of homocysteine in the induction of radical formation also suggests the thiol-derived radicals, because addition of the thiol blocker DTNB reduced the concentration of the hydroxyl radical generated in the cellular system, suggesting that thiols are the major source of these radicals.

## *Influence of homocysteine on the proliferation and differentiation of vascular muscle cells*

An early atherosclerotic lesion consists largely of inflammatory cells, particularly macrophages and T lymphocytes. With progression of these lesions, VSMCs migrate, proliferate, and produce extracellular matrix components, presumably as part of a repair process of metabolic or physical injury, resulting in subsequent inflammation of blood vessels (140). VSMCs synthesize components of the fibrous cap in plaques, which separates the blood from the thrombogenic plaque interior, and whose rupture or erosion triggers myocardial infarction. Matrix metalloproteinase 2 (MMP-2) is synthesized and released from VSMCs and has been considered to be involved in development and progression of atherosclerotic lesions by contributing to destabilization and rupture of atherosclerotic plaques (59).

Homocysteine treatment of human VSMCs at concentrations associated with increased risk of cardiovascular events increased MMP-2 activity, synthesis, and secretion through a

mechanism involving the activation of MAPK and PI3-K pathways (46). Furthermore, homocysteine significantly increased collagen synthesis by VSMCs at concentrations ranging from 5 to 300 $\mu M$. This could be reversed by addition of *N*-acetylcysteine or glutathione, suggesting a role for redox perturbation in this event (167). Indeed, homocysteine stimulated hydrogen peroxide production in VSMCs in a dose-dependent manner (31).

VSMCs secrete extracellular SOD, the most abundant SOD isozyme in vascular tissue, to protect the vascular wall from oxidant stress (155). Inhibition of SOD activity has been discussed to be another mechanism for oxidant stress injury induced by homocysteine (126). A significant and positive relation between plasma extracellular SOD levels and total homocysteine has been reported in patients with hyperhomocysteinemia (187), presumably because of detachment from the arterial wall, thereby resulting in a lower activity at the site of the vessel wall (193). This might reduce the ability of SOD to protect the endothelial surface from oxidant stress. In addition, homocysteine has been shown to reduce the expression and secretion of extracellular SOD in rat aorta VSMCs, leading to increased oxidant stress at the arterial wall (126).

A further mechanism that might lead to the progression of inflammation during homocysteine-induced atherosclerosis has recently been discussed. Homocysteine at physiologically relevant concentrations (10–250 $\mu M$) significantly increased the expression of IL-6 mRNA and protein in rat VSMCs (200). The increase in IL-6 expression was associated with the activation of transcription factor NF-$\kappa$B, commonly involved in inflammatory and immune responses. ROS are regarded as second messengers for the activation of NF-$\kappa$B and cytokine expression (23). The radical scavenger pyrrolidine dithiocarbamate (PDTC) suppressed homocysteine-induced IL-6 release, a finding compatible with involvement of ROS as second messengers. IL-6 plays several important roles within the vessel wall. As an activator of acute-phase reactant expression, it is contributing to inflammation (92). Furthermore, IL-6 stimulates VSMCs proliferation as an autocrine growth factor through its ability to influence platelet-derived growth factor (PDGF) expression (88). Additionally, IL-6 can play an important role in priming both monocytes and neutrophils and has been shown to enhance lymphocyte binding to endothelial cells and contribute to the activation of the coagulation cascade (196).

In recent years, apoptosis of VSMCs has been recognized in atherosclerosis. VSMC apoptosis causes release of IL-1 and upregulation of MCP-1 and IL-8 (146). This results in vessel wall infiltration of macrophages *in vivo* (145). Whether homocysteine at pathophysiologically relevant concentrations has any impact on VSMC apoptosis remains unclear, as homocysteine (only when used at concentrations $>500$ $\mu M$) significantly decreased VSMC viability through hydrogen peroxide and peroxynitrite generation in rat VSMCs (194).

Conversely, it has been hypothesized that homocysteine is promoting VSMC growth, contributing to the development of atherosclerosis (73, 165). Homocysteine (0.1 to 1 m$M$) increased DNA synthesis in rat aortic smooth muscle cells in a dose-dependent manner and promoted proliferation of quiescent VSMCs, an effect amplified by serum (165). Homocysteine induced DNA synthesis and proliferation of VSMCs by a hydrogen peroxide–independent mechanism (158), indicated by data that show that homocysteine (1 m$M$) induced a 1.5-fold DNA synthesis and proliferation of VSMCs, which was further increased in the presence of catalase. However, even lower concentrations of homocysteine (10–20 $\mu M$) have been shown to activate human VSMC proliferation in a dose-dependent manner (26). Although the proportion of apoptotic cells and of cells with a necrotic morphology of VSMCs in culture increased significantly in the presence of increasing concentrations of homocysteine, the total number of cells was increased.

VSMCs express $\alpha$-smooth muscle actin ($\alpha$-SMA), thereby playing a critical role in wound contraction and matrix remodeling after tissue injury and inflammation (91). Differentiation of VSMCs into myofibroblast, as evidenced by $\alpha$-SMA expression, is largely mediated by transforming growth factor-$\beta$1 (TGF-$\beta$1). This growth factor is a multifunctional cytokine playing a central role in many fibrotic diseases and has been shown to be activated through oxidant stress (20). This mechanism often follows inflammatory events such as endothelial damage due to oxidant stress, which can further lead to vascular thickening, stiffness, and fibrosis. Recent studies have shown that mature vascular endothelium has the capability to differentiate into a smooth muscle–like phenotype through expression of the smooth muscle–specific isoform of actin, $\alpha$-SMA (57). In addition, endothelial disorders may be further complicated by the fibrotic response of collagen type-1 deposition in the extracellular matrix (ECM). Sen *et al*. (147) hypothesized that hyperhomocysteinemia-induced oxidant stress leads to vascular stiffness, in part because of endothelial–myofibroblast differentiation and alteration of collagen homeostasis in the extracellular matrix (ECM) (147). In mouse aortic endothelial cells, homocysteine induced $\alpha$-SMA and collagen type-1 expression, as shown by immunoblot and confocal imaging. Consistently, RT-PCR showed robust increases of $\alpha$-SMA and collagen type-1 mRNA levels. Furthermore, homocysteine induced autophosphorylation of focal adhesion kinase (FAK), a member of the protein tyrosine kinase (PTK) family. PP2 (a general PTK inhibitor) as well as FAK siRNA prevented homocysteine-mediated $\alpha$-SMA formation. In addition, homocysteine-mediated TGF-$\beta$ induction was inhibited by TGF-$\beta$ R1 kinase inhibitor II (ALK5 inhibitor II) and attenuated FAK phosphorylation and $\alpha$-SMA expression. Finally, it was shown that homocysteine activates the ERK-44/42 (extracellular signal–regulated kinase) pathway and augments collagen type-1 deposition. Studies with the pharmacologic ERK inhibitor PD98059 and ERK siRNA attenuated ERK-44/42 phosphorylation and collagen type-1 synthesis. Taken together, these results demonstrate that homocysteine-mediated TGF-$\beta$1 upregulation triggers endothelial–myofibroblast differentiation secondary to FAK phosphorylation and that homocysteine-induced ERK activation is involved in ECM remodelling by altering collagen type-1 homeostasis. Therefore, one might hypothesize that homocysteine is implicated in vascular thickening and stiffness, which are involved in the process of plaque formation in the arterial wall.

In summary, these data indicate that homocysteine may induce proinflammatory responses in several vascular cells that may promote the development and progression of atherosclerotic plaques (Fig. 4).

## PROINFLAMMATORY SIGNALING FACTORS AND PATHWAYS INFLUENCED BY HOMOCYSTEINE

### *Nuclear factor kappa-B (NF-κB) pathway*

Activation of the transcription factor nuclear factor kappa-B (NF-κB) has been linked to the expression of inflammatory factors during the onset of atherosclerosis (9). Activated NF-κB has been detected in macrophages, endothelial cells, and vascular smooth muscle cells in human atherosclerotic lesions and in animal models (24, 35, 188). NF-κB is normally present in the cytoplasm in an inactive form that is associated with an inhibitory protein named IκB. In the presence of various NF-κB stimuli, IκB is rapidly phosphorylated, leading to ubiquitination and subsequent degradation, followed by translocation of NF-κB into the nucleus (activated NF-κB), where it can transactivate target genes. ROS generation has been implicated in stimulation of IκB degradation and NF-κB activation in vascular cells (23).

Homocysteine treatment has been shown to cause activation of NF-κB in VSMCs and macrophages, leading to increased chemokine expression (5, 174, 175), and in vascular endothelial cells, leading to increased adhesion molecule expression (132) (Fig. 5). Increased vascular NF-κB activation under hyperhomocysteinemic conditions also was detected *in vivo*. Hyperhomocysteinemia in rats or apoE$^{(-/-)}$ mice induced by a high-methionine diet resulted in an increase in the activated form of NF-κB and increased levels of superoxide anions in the aortic vessel wall (4, 38).

Mechanistically, incubation of HUVECs and HAECs with homocysteine (100 $\mu M$) activated IκB kinases, leading to phosphorylation and subsequent degradation of IκB. As a consequence, NF-κB nuclear translocation, enhanced NF-κB/DNA binding activity, and increased transcriptional activity of NF-κB–regulated genes could be detected. NF-κB activation in homocysteine-incubated endothelial cells coincided with a marked elevation of superoxide anion levels. Pretreatment with the antioxidants catechin (a flavonoid antioxidant) and trolox (a synthetic cell-permeable analogue of α-tocopherol), as well as superoxide anion scavengers or an I-κB kinase inhibitor (prostaglandin $A_1$), could prevent homocysteine-induced activation of IKK kinases and NF-κB in endothelial cells (4, 129, 132), implying that ROS signal the effect of homocysteine on NF-κB activation.

In THP-1 cells, homocysteine-induced NF-κB activation was also associated with a significant increase in intracellular superoxide anion levels in parallel with a significant increase in phosphorylation and membrane translocation of NADPH oxidase $47^{phox}$ subunit (5). The latter events are known to be essential steps for NADPH oxidase activation (138), which is significantly elevated in atherosclerotic lesions, leading to increased superoxide anion production (94). Homocysteine-induced NADPH oxidase activity mediates the upregulation of redox factor-1 (Ref-1) expression and translocation to the nucleus in monocytes (38). The homocysteine-induced upregulation of total and nuclear Ref-1 could be attenuated by diphenyleneiodonium DPI (10 $\mu M$), an inhibitor of NADPH oxidase. These results demonstrated that homocysteine-induced upregulation and nuclear translocation of Ref-1 may depend on NADPH oxidase activity, leading to increased vascular oxidant stress.

Ref-1 is a ubiquitously expressed multifunctional protein involved in the repair of DNA damage. It also facilitates the DNA-binding activities of many redox-sensitive transcription factors, including AP-1 and NF-κB (125, 192). The enhanced DNA-binding activities thereby may increase the expression of corresponding target genes.

ApoE$^{-/-}$ mice with diet-induced hyperhomocysteinemia exhibited elevated Ref-1 expression accompanied by upregulated NFκB and MCP-1 promoter activity, which led to higher MCP-1 expression in aortic roots and peritoneal macrophages. Homocysteine seems to induce overexpression of Ref-1, leading to the described downward signaling events. This is emphasized by data that show that knocking down of Ref-1 by using antisense technology reduced homocysteine-induced NF-κB DNA-binding activity and MCP-1 secretion (38). In summary, these data indicate that homocysteine-induced ROS upregulate the expression and translocation of Ref-1 *via* boosting of oxidant stress by activation of NADPH oxidase.

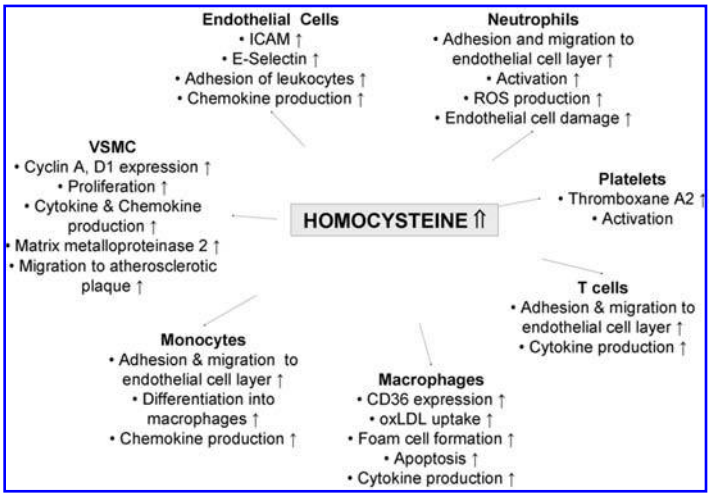


**FIG. 4. Effect of homocysteine on vascular cells during the development of atherosclerotic plaques.**

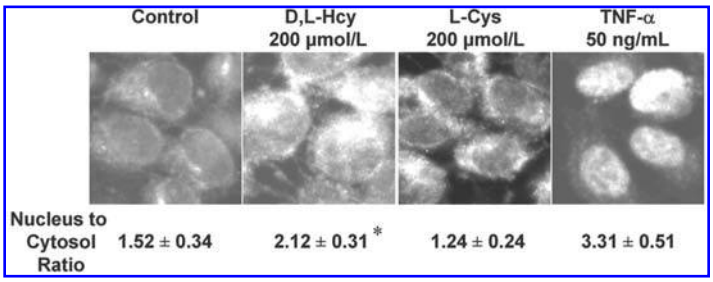


**FIG. 5. Nuclear translocation of NF-κB in homocysteine-incubated endothelial (EA.hy 926) cells.** Six hours of incubation of endothelial cells with D,L-homocysteine (200 $\mu M$) leads to significantly increased NF-κB activation compared with that in control cells. This becomes evident by increased nuclear translocation, indicated by increased nuclear staining with an anti–p65–antibody, as visualized with immunofluorescence images by using confocal microscopy. TNF-$\alpha$ was used as positive control. L-cysteine had no significant effect. The values express the nucleus/cytosol ratio ± SD of fluorescence intensity of three to six experiments.

Activation of the protein kinase C (PKC)–MAPK signaling pathway has also has been implicated in the stimulation of NF-κB activity and MCP-1 expression by homocysteine, at least in VSMCs, in addition to the role of this signaling pathway in the regulation of cell proliferation and apoptosis, as discussed earlier. This suggests that MAPKs could be the potential upstream kinases of NF-κB activation under conditions of elevated homocysteine levels (174, 190, 200).

The increase in NF-κB activity in several vascular cells, which finally results in increased expression of target genes, including some that may accelerate the development of atherosclerosis, plays a key role in homocysteine-induced vascular inflammation.

### *Downstream signaling molecules stimulated by NF-κB*

Many inflammatory mediators and molecules involved in atherosclerosis, like adhesion molecules, interleukins, and chemokines, seem to be activated by homocysteine through the redox-sensitive NF-κB pathway. These include endothelial adhesion molecules that mediate monocyte adhesion to endothelial cells, like ICAM-1 (96, 132), as discussed earlier.

Several large-scale prospective studies have shown that the serum level of C-reactive protein (CRP) is a strong independent predictor of risk of myocardial infarction, stroke, peripheral arterial disease, and vascular death among individuals without known CVD (103, 137). Moreover, CRP has recently been shown not only to be a marker of inflammatory responses, but also in itself to be a mediator of inflammation and endothelial dysfunction (92). IL-6, a cytokine known to be secreted from a number of cells including macrophages, lymphocytes, and endothelial cells, has been shown to be a major regulator of CRP synthesis and may thus have a key role in initiation of the acute-phase response in various inflammatory conditions such as CVD (196). Recent studies suggested that enhanced inflammation associated with hyperhomocysteinemia may lead to CVD, possibly through IL-6-related mechanisms (81, 170). Homocysteine at pathophysiologically relevant concentrations (10–250 $\mu M$) significantly increased the expression of IL-6 mRNA and protein in rat VSMCs (200). The increase in IL-6 expression was associated with activation of NF-κB and could be prevented by addition of the free radical scavenger PDTC. Holven *et al*. (81) measured serum concentrations of CRP and IL-6 in 39 patients with hyperhomocysteinemia and in 39 control subjects matched for gender, age, and body mass index (BMI). Compared with controls, hyperhomocysteinemic subjects have elevated serum levels of CRP and IL-6. Importantly, this increased level of IL-6 was also seen in hyperhomocysteinemic individuals without accompanying hypercholesterolemia or CVD.

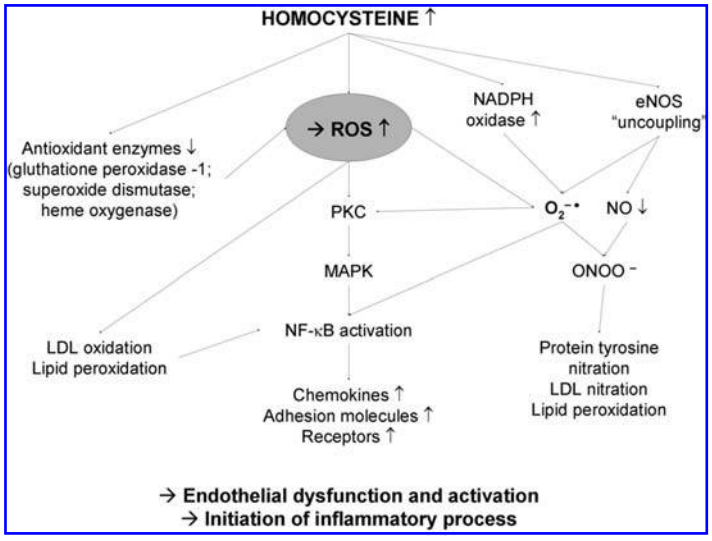


**FIG. 6. Homocysteine increases reactive oxygen species in endothelial cells, which induce endothelial dysfunction and initiate inflammatory processes**.

## CONCLUSIONS

In summary, strong experimental evidence, both *in vitro* and *in vivo*, indicates that increased vascular oxidant stress is promoted by elevated levels of homocysteine, activating a series of reactions that promote the inflammation of the arterial wall and the progression of atherosclerotic plaque formation. Upregulation of redox-sensitive proinflammatory signaling pathways, like the NF-$\kappa$B pathway in vascular and immune cells, thereby seems to play a central role. This results in increased recruitment, adhesion, and transmigration of circulating leukocytes to the vessel wall, followed by foam cell formation and proliferation of VSMCs. All these events participate in the initiation and progression of atherosclerosis (Fig. 6).

Although abundant epidemiological and experimental evidence exists for the impact of homocysteine on the development of atherosclerosis, reducing homocysteine with folic acid and B vitamins, however, did not prevent vascular events in patients with established cardiovascular events, as shown in recent large-scale intervention trials (19, 114, 162). Furthermore, reducing homocysteine levels did not reduce blood markers of inflammation (CRP, IL-6, CD40L), endothelial dysfunction (VCAM, ICAM, or von Willebrand factor expression), or hypercoagulability (P-selectin, prothrombin fragment 1 and 2, D-dimer) in patients with previous transient ischemic attack or stroke (50, 148). This discrepancy remains to be resolved in further experimental and clinical studies before it will be generally accepted that elevated plasma levels of homocysteine are a cardiovascular risk factor that can be modified to reduce patients risk and burden of vascular disease.

## ABBREVIATIONS

$\alpha$-SMA, $\alpha$-smooth muscle actin; ApoE$^{(-/-)}$ mice, homozygous apolipoprotein E knockout mice; CBS$^{(-/+)}$ mice, heterozygous cystathionine $\beta$ synthase knockout mice; CBS, cystathionine $\beta$ synthase; CCR2, C-C chemokine receptor 2; CD36, scavenger receptor for oxidized low-density lipoprotein; cPLA2, cytosolic phospholipase $A_2$; CRP, C-reactive protein; CVD, cardiovascular disease; DCF, dichlorofluorescein; DPI, diphenyleneiodonium; FAK, focal adhesion kinase; HAECs, human aortic endothelial cells; HDL, high-density lipoprotein; HEVs, high endothelial venules; HUVECs, human umbilical vein endothelial cells; ICAM, intracellular adhesion molecule; IFN-$\gamma$, interferon-$\gamma$; IL-1$\beta$, interleukin-1$\beta$; IL-12, interleukin-12; IL-8, interleukin-8; LDL, low-density lipoprotein; MAPK, mitogen-activated phosphokinases; MCP-1, monocyte chemoattractant protein 1; M-CSF, macrophage colony-stimulating factor; MHC II, major histocompatibility class II antigen; MMP-2, matrix metalloproteinase 2; MPO, myeloperoxidase; mtDNA, mitochondrial DNA; NF-$\kappa$B, nuclear transcription factor $\kappa$B; NO, nitric oxide; OTC, L-2-oxo-4-thiazolidine carboxylate; oxLDL, oxidized low-density lipoprotein; PAD, peripheral arterial occlusive disease; PPAR$\gamma$, peroxisome proliferator-activated receptor-$\gamma$; Psel$^{(-/-)}$) mice, P-selectin-knockout mice; PSGL-1, P-selectin glycoprotein ligand-1; PTK, protein tyrosine kinase; RANTES, chemokine regulated on activation, normal T-cell expressed and secreted; ROS, reactive oxygen species; SOD, superoxide dismutase; SRA, scavenger receptor A; TF, tissue factor; TGF-$\beta$, transforming growth factor-$\beta$1; TNF-$\alpha$, tumor necrosis factor-$\alpha$; $TXB_2$, thromboxane 2; VCAM-1, vascular cell adhesion molecule 1; VSMCs, vascular smooth muscle cells.

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Address reprint requests to:
*Norbert Weiss*
*Department of Vascular Medicine*
*Medical Policlinic–City Campus*
*University of Munich Medical Center*
*Pettenkoferstrasse 8a*
*D-80336 Muenchen, Germany*

*E-mail:* Norbert.Weiss@med.uni-muenchen.de

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