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Forum Review

Homocysteine and Redox Signaling

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ABSTRACT

Homocysteine is a thiol-containing amino acid that has gained notoriety because its elevation in the plasma is correlated with complex and multifactorial diseases, including cardiovascular diseases, neurodegenerative diseases, and neural tube defects. Homocysteine is redox-active, and its toxic effects have been frequently attributed to direct or indirect perturbation of redox homeostasis. Although the literature on the pathophysiology of elevated homocysteine is rather extensive, a very wide range of concentrations of this amino acid has been used in these studies ranging from normal to pathophysiological to unphysiological. It is clear that homocysteine induces varied responses that are specific to cell type and that cells, depending on their origin, display a wide range of sensitivity to homocysteine. In this review, we focus on the redox signaling pathways that have been connected to homocysteine in vascular (endothelial and smooth muscle) cells and in neuronal cells. We also discuss redox regulation of the key enzymes involved in homocysteine clearance: methionine synthase, betaine-homocysteine methyltranferase, and cystathionine β-synthase. *Antioxid. Redox Signal.* 7, 547–559.

HOMOCYSTEINE METABOLISM AND HYPERHOMOCYSTEINEMIA

Homocysteine is an intermediate in sulfur amino acid metabolism and is generated by hydrolysis of *S*-adenosylhomocysteine (AdoHcy), the spent form of the ubiquitous methylating agent, *S*-adenosylmethionine (AdoMet) (Fig. 1). It suffers two major metabolic fates: transmethylation and transsulfuration. Transmethylation reclaims homocysteine to the methionine cycle and is catalyzed by methionine synthase, which is ubiquitous, or by betaine homocysteine methyltransferase, whose activity is limited to liver and kidney. Transsulfuration commits homocysteine irreversibly to cysteine synthesis and also provides an avenue for disposal of excess sulfur, ultimately as sulfate. Two other enzymes, which are auxiliary to homocysteine metabolism, are methylenetetrahydrofolate reductase and methionine synthase reductase. The former catalyzes the reduction of 5,10-methylenetetrahydrofolate (CH_2-H_4folate) to 5-methyltetrahydrofolate (CH_3-H_4folate), the substrate for methionine synthase, and completes the folate cycle. Methionine synthase reductase is a repair enzyme that reductively reactivates oxidized methionine synthase and functions to maintain it in an active form. The homocysteine metabolic junction is unique in being richly dependent on B vitamins, which serve as cofactors or substrates for the branch point enzymes. Thus, methionine synthase requires B_{12} and utilizes CH_3-H_4folate as substrate, whereas cystathionine β-synthase, the first enzyme in the transsulfuration pathway, is a B_6-dependent heme protein in mammals. Both auxiliary enzymes, methylenetetrahydrofolate reductase and methionine synthase reductase, require flavin.

Elevated levels of circulating homocysteine, a condition known as hyperhomocysteinemia, are correlated with an increased risk for coronary, cerebral, and peripheral vascular diseases and thrombosis, a correlation that was first noted by McCully 35 years ago (58). In addition, elevated homocysteine is associated with multiple neurological disorders, including Alzheimer's disease, age-related dementias, and neural tube defects (57). The clinical manifestations associated with elevated homocysteine thus extend over the full range of human life span from the pediatric to the geriatric

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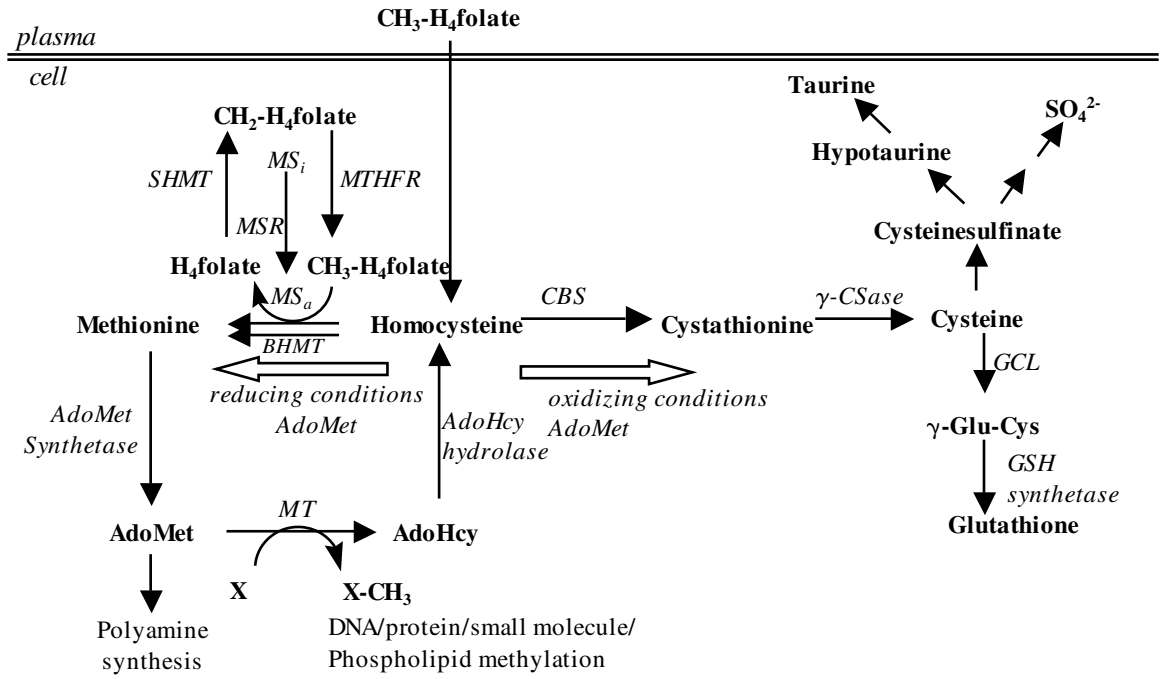

FIG. 1. The homocysteine metabolic pathway showing that homocysteine straddles sulfur and one-carbon metabolism. MS_a and MS_i represent the active and inactive forms of methionine synthase. Other abbreviations denote: MSR, methionine synthase reductase; SHMT, serine hydroxymethyltransferase; MTHFR, methylenetetrahydrofolate reductase; MT, methyltransferases; CBS, cystathionine β-synthase; γ-CSase, γ-cystathionase; GCL, glutamate-cysteine ligase. The black arrows indicate the direction of increased flux of homocysteine under oxidizing (transsulfuration pathway) and reducing (transmethylation pathway) conditions.

phases. Plasma total homocysteine concentrations in the 5–15 *μM* range are considered to be normal in humans. Severely elevated homocysteine (>100 *μM*) usually results from relatively rare inherited defects in enzymes involved in homocysteine disposal, with the most frequent cause being mutations in cystathionine β-synthase (63). Moderately elevated homocysteine is more prevalent and is likely to be the product of variations in gene–nutrient interactions, *e.g.*, low B vitamin status combined with polymorphisms in pathway-specific genes. The best studied example of such a gene–environment interaction in this pathway is the thermolabile, C677T polymorphism in methylenetetrahydrofolate reductase, which, in combination with low folate status, is associated with hyperhomocysteinemia (22). It is therefore not surprising that hyperhomocysteinemia can be readily induced and modulated in animal models by adjusting dietary methionine and folate levels (13, 44, 45).

Although the enzymes at the nexus of homocysteine metabolism are well described, much less is known about their regulation, particularly in a cellular context. Similarly, despite the statistics associating homocysteine and various cardiovascular and neurological diseases, the mechanisms underlying their pathophysiology remain largely unknown. A growing number of studies implicate the import of redox signaling in homocysteine homeostasis under normal conditions and in the pathology of homocysteine-related diseases. It is critically important to understand mechanisms of redox regulation both at the level of homocysteine-dependent enzymes, which ultimately govern intracellular flux and management of homocysteine, and at the level of pathological manifestations relevant to hyperhomocysteinemia, and these are the focus of this review.

Redox regulation of homocysteine-utilizing enzymes

Homocysteine is a key junction intermediate in sulfur amino acid metabolism, and its utilization via the transmethylation and transsulfuration pathways influences important cellular functions (Fig. 1). The major role of the methionine synthase-catalyzed methyl transfer reaction is to release tetrahydrofolate (H_4folate) from CH_3-H_4folate, the circulating form of the vitamin that is delivered to cells from the plasma. Whereas CH_3-H_4folate is a substrate for only one enzyme, the product, H_4folate, supports many critical one-carbon reactions. The other product, methionine, is an essential amino acid, because mammals lack the pathway for synthesizing it *de novo*. Additionally, as methionine synthase is the only homocysteine-utilizing enzyme that is found in all tissues, it plays an important role in maintaining low intracellular levels of homocysteine, the product of AdoHcy hydrolase. The latter catalyzes a reversible reaction in which the equilibrium favors condensation of the products, homocysteine and adenosine. Hence, clearance of homocysteine by methionine synthase serves to keep AdoHcy concentrations low and to maintain a favorable AdoMet:AdoHcy ratio, an index of the

cellular methylation potential. Betaine homocysteine methyltransferase functions in the pathway of choline oxidation and catalyzes transfer of the methyl group of betaine to homocysteine to generate dimethylglycine and methionine. A major metabolic derivative of methionine is AdoMet, a product of AdoMet synthetase, which supports numerous cellular methylation reactions and polyamine biosynthesis.

The major role of the transsulfuration pathway is to convert methionine to cysteine, which can be utilized in various reactions or catabolized under conditions of excess. Importantly, the transsulfuration pathway provides the limiting reagent, cysteine, required for the synthesis of the redox buffer, glutathione, whose concentration can range from 1 to 10 m*M* depending on the cell type. Studies in human hepatoma cells (62) and in rat hepatocytes (3) have demonstrated that ~50% of the cysteine in glutathione is derived from homocysteine via the transsulfuration pathway. The enzymes involved in glutathione biosynthesis, γ-glutamylcysteine ligase and glutathione synthetase, are subjected to intricate regulation at multiple levels that is redox sensitive (52).

It is expected that the enzymes that compete to salvage homocysteine or to commit it to transsulfuration are tightly regulated so that the flux of homocysteine through the rival pathways is responsive to cellular needs. Indeed, AdoMet exerts a reciprocal regulation at this junction and activates cystathionine β-synthase (20) while depressing transmethylation by decreasing the activity of methylenetetrahydrofolate reductase (56). Studies with the isolated junction enzymes, methionine synthase, betaine homocysteine methyltransferase, and cystathionine β-synthase, reveal that each is sensitive to redox changes (8, 61, 82) (Fig. 1). Thus, the activity of methionine synthase is diminished under oxidizing conditions possibly due to the oxidative lability of the cofactor intermediate, cob(I)alamin (8), and/or due to oxidation of cysteines in the essential zinc binding site (24). Human methionine synthase is also regulated by the activator, insulin growth factor-1, and by the neurotransmitter, dopamine, in SH-SY5Y neuroblastoma cells in a phosphatidylinositol 3-kinase- and mitogen-activated protein (MAP) kinase-dependent signaling pathway (92) (Fig. 2).

Betaine homocysteine methyltransferase, like methionine synthase, is a zinc enzyme and is known to undergo reversible inactivation by ligand oxidation and zinc displacement (61). In fact, the disulfide formed between cysteine ligands to the zinc is seen in the crystal structure of oxidized betaine homocysteine methyltransferase (17). In contrast, cystathionine β-synthase is activated and exhibits an approximately twofold increase in activity under oxidizing conditions (82). These observations led us to posit that redox regulation may be important in modulating homocysteine flux and that under oxidizing conditions when the glutathione pool is compromised, increased conversion of homocysteine to cysteine, and thereon to glutathione, fulfills an autocorrective function (62).

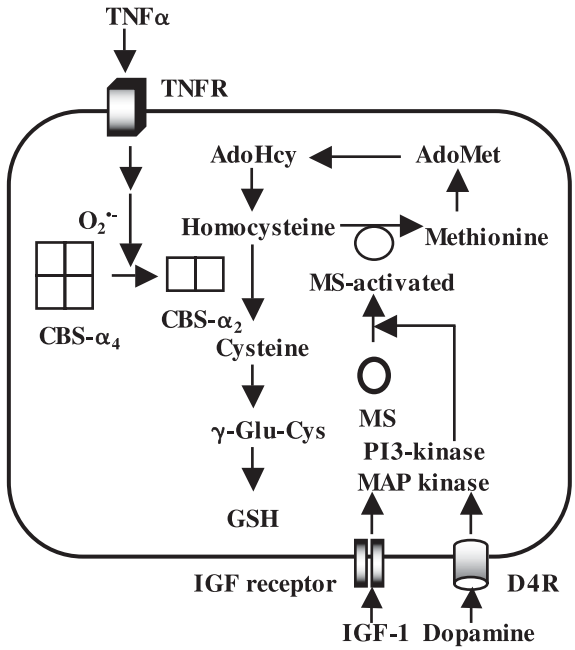

FIG. 2. Regulation of cystathionine β-synthase and methionine synthase by signaling pathways. Cystathionine β-synthase is activated in liver and in hepatoma cells by targeted endoproteolysis in response to TNFα-dependent stimulation of $O_2^{\bullet-}$ formation. The truncated form of the enzyme is a dimer. Methionine synthase is regulated by dopamine and IGF-1 in neuroblastoma cells by a pathway involving phosphatidylinositol 3-kinase (PI-3 kinase) and MAP kinase.

Oxidative signaling and activation of cystathionine β-synthase

The hypothesis that cystathionine β-synthase is activated under oxidizing conditions has been tested in a cell culture model in which the flux of homocysteine through the transsulfuration pathway was measured under normoxic and oxidative stress conditions (62). Bolus administration of organic or hydrogen peroxide induces a time- and dose-dependent increase in the transsulfuration flux, which is paralleled by increased incorporation of radiolabel from methionine into glutathione. Specifically, addition of 100 μ*M* hydrogen peroxide increased cystathionine production from 82 ± 7 μmol $h^{-1}\,l^{-1}$ to 136 ± 15 μmol $h^{-1}\,l^{-1}$ of cells (62). In contrast, antioxidants such as catalase, superoxide dismutase (SOD), and a water-soluble derivative of vitamin E elicited the opposite effect and resulted in diminished flux of homocysteine through the transsulfuration pathway (90). These studies provided the first evidence for the reciprocal sensitivity of the transsulfuration pathway to pro- and antioxidants.

Under *in vitro* conditions, the redox sensitivity of cystathionine β-synthase is correlated with oxidation state changes in the heme cofactor (82). However, the crystal structure of the catalytic core of the protein revealed a CXXC oxidoreductase motif that could, in principle, be involved in redox sensing. These vicinal cysteines were present in a disulfide state in one structure (59) and in the reduced state in another (84), without creating discernible differences in the remainder of the protein. However, deletion of the heme binding domain, but not mutagenesis of the cysteines in the CXXC motif, results in loss of redox sensitivity in cystathionine β-synthase. These studies suggest that the heme rather

than the CXXC oxidoreductase motif functions as a redox sensor at least under *in vitro* conditions (84).

Under *in vivo* conditions, the mechanism of redox sensing remains to be elucidated and the involvement of heme in this process remains an open issue. The increase in flux of homocysteine through the transsulfuration pathway when cells are challenged with exogenous peroxides is not associated with an increase in cystathionine β-synthase protein (62) or mRNA levels (Zou and Banerjee, unpublished observations). These results are in conflict with those obtained with reporter constructs under control of the CBS–1b promoter, transfected into HepG2 cells and then treated with hydrogen peroxide (54). Under these conditions, the reporter activity is diminished. This discrepancy can be explained by the use of a reporter construct in which only a portion of the CBS promoter was used in an artificial context, which could have elicited a different response. Notably, the response of the endogenous CBS promoter was not characterized under the same conditions (54).

As an alternative to the bolus administration of peroxide, the response of cystathionine β-synthase to modulation of endogenous reactive oxygen species (ROS) levels elicited by tumor necrosis factor-α (TNFα) or lipopolysaccharide (LPS) has been examined in a cell culture and an animal model, respectively (107). Both TNFα and LPS, a component of the cell wall in gram-negative bacteria, are proinflammatory agents that stimulate endogenous ROS synthesis. Unexpectedly, this study revealed that enhanced glutathione synthesis and cystathionine β-synthase activity coincide with the targeted proteolysis of the full-length 63-kDa enzyme to generate a truncated 45-kDa form (107) (Fig. 2).

Interestingly, a truncated 45-kDa form of cystathionine β-synthase lacking the C-terminal regulatory domain has been well characterized *in vitro* (77, 83), but its physiological relevance, if any, was unknown. The effective change in activity accompanying truncation of cystathionine β-synthase is a 1.5- to twofold increase in k_{cat} over the full-length form, in the presence of physiological concentrations of AdoMet (16). This accounts for enhanced flux of homocysteine through the transsulfuration pathway following TNFα or LPS treatment.

The cleavage of full-length cystathionine β-synthase by TNFα is suppressed by Tiron, an inhibitor of superoxide anion ($O_2^{\bullet -}$) production or by transfection with an expression vector for manganese SOD, but not by catalase (107). These results point to a role for $O_2^{\bullet -}$ in the oxidative signaling pathway leading to endoproteolytic cleavage and activation of cystathionine β-synthase. The commonly used proteasome inhibitors, MG132 and lactacystin, but not ALLN, suppressed the TNFα-induced response, implying that the proteasome is involved directly or indirectly in this process. These studies reveal a novel strategy for increasing cysteine production to support the increased demand for glutathione synthesis under conditions of TNFα-induced oxidative stress (107).

THE TOXIC EFFECTS OF HOMOCYSTEINE

Despite the epidemiologic evidence that elevated plasma homocysteine is an independent risk factor for atherosclerotic and atherothrombotic vascular disease, our understanding of homocysteine toxicity remains limited. Hyperhomocysteinemia may initiate and contribute to cardiovascular risk by impairing vascular function. Even mild hyperhomocysteinemia, either basal or induced transiently after a methionine load, can cause endothelial injury (35). Development of atherosclerotic and atherothrombotic vascular disease involves multiple physiological perturbations, including endothelial dysfunction, proliferation of vascular smooth muscle cells (VSMC), accumulation of extracellular matrix, platelet hyperactivity, and oxidation of low-density lipoprotein (LDL) (40, 41). Recently, a growing number of studies have correlated hyperhomocysteinemia with neurological disorders, *e.g.*, Alzheimer's disease (9, 60), Parkinson's disease (64), and brain atrophy (74). The neurotoxicity of elevated homocysteine may derive from direct detrimental effects on neurons or from perturbations in the CNS vasculature. In end-stage renal disease, the levels of homocysteine increase three- to fivefold (6), and the decline in renal function and nephrosclerosis are attributed to hypertension and atherosclerosis.

Several hypotheses have been advanced to explain the pathology associated with hyperhomocysteinemia. These include roles for (a) oxidative stress, (b) endoplasmic reticulum (ER) stress, and (c) alterations in signal transduction pathways and activation of inflammatory factors. Each of these is discussed below.

Role of oxidative stress in hyperhomocysteinemia

The potential role of homocysteine-induced oxidative stress in mediating endothelial dysfunction is predicated by the inherent reactivity of this thiol-containing amino acid. The sulfhydryl group of homocysteine has a pK_a of 10 and is therefore more reactive at physiological pH than cysteine, with a thiol pK_a of 8.5 (4). However, cysteine is not considered to be a significant risk factor for cardiovascular diseases (35). The difference in reactivity is only partially offset by the ~30-fold higher concentration of cysteine versus homocysteine in the plasma. Thus, specific mechanisms must be postulated when considering oxidative stress as a mechanism underlying the injurious effects of homocysteine. Oxidation of thiols to disulfides is accompanied by the generation of ROS. Homocysteine is readily oxidized in the plasma to form homocystine and homocysteine mixed disulfides, and these represent the predominant forms of this amino acid in circulation.

There are two caveats that deserve mention in discussing the literature exploring the role of oxidative stress in hyperhomocysteinemia. First, a number of studies have utilized a combination of copper and homocysteine to elicit stress responses, although the concentration of this potentially toxic transition metal in the free state is believed to be negligible (70). Furthermore, in many studies, supraphysiological concentrations of homocysteine have been used; although justified as being necessary to induce a two- to fivefold increase in intracellular homocysteine concentration, as well as being nontoxic based on cell viability measures, they nevertheless raise questions about physiological relevance.

Cardiovascular diseases. The pathogenesis of arteriosclerosis involves changes in the vascular wall (endothelial

and smooth muscle cells) and in cells in circulation (*e.g.*, monocytes, macrophages, platelets, B and T cells) (73). In this section, we focus on the effects of homocysteine that have been described in endothelial and smooth muscle cells.

Endothelial cells. Pleiotropic effects are associated with homocysteine-induced endothelial injury, including inhibition of protein C activation (71) and cellular binding sites for tissue plasminogen activator (26), aberrant processing of thrombomodulin (42), induction of tissue factor activity, and a protease that activates factor V (23, 72). Homocysteine also decreases nitric oxide (NO) synthesis (79) and diminishes bioavailability of this potent vasodilator (89).

Endothelial dysfunction resulting from various pathological and pharmacological insults plays a critical role in initiating the pathogenesis of atherosclerosis (73). The prooxidant effect of homocysteine has been invoked to explain the increased *in vivo* (15, 91) and *in vitro* (41) lipid peroxidation that is associated with hyperhomocysteinemia (Fig. 3). An increase in plasma homocysteine following an oral methionine load is correlated with lipid peroxidation markers, *e.g.*, thiobarbituric acid and acid-reactive substances and isoprostane $F_{2\alpha}$-III (14, 91). Extracellular bolus administration of L-homocysteine or homocystine (25–300 μ*M*) enhances intracellular ROS formation in endothelial but not smooth muscle cells (29). Lipid peroxidation in this cell culture model appears to involve $O_2^{\bullet -}$ formation and endothelial NO synthase (eNOS), and is specific for the L- versus the D-stereoisomer of homocysteine (29). This study has two important implications. First, it indicates that autooxidation of extracellular homocysteine is not involved in incurring oxidative stress because homocystine was equally efficacious, and that import and/or metabolism of extracellular homocysteine is needed because the D-isomer was ineffective.

ROS can promote lipid peroxidation on the endothelial surface and in lipoprotein particles in the plasma (51). Oxidation of LDL by homocysteine (28) is enhanced by the copper transporter, ceruloplasmin (18). In homocystinuric patients with homozygous CBS deficiency, urinary levels of 8-isoprostaglandin $F_{2\alpha}$, a product of nonenzymatic oxidation of arachidonic acid, which induces vasoconstriction and platelet activation, is significantly higher than in control subjects, and is correlated with plasma homocysteine (12). Urinary excretion of 11-dehydrothromboxane B_2 is also enhanced. Vitamin E supplementation resulted in a significant reduction in both urinary markers of platelet activation, implicating a role for ROS in arachidonic acid peroxidation (12). Homocysteine (10–50 μ*M*) can induce arachidonic acid release in platelets and enhance formation of thromboxane B_2 and ROS (78). Increased production of thromboxane B_2 suggests a mechanism for platelet hyperactivation that is associated with hyperhomocysteinemia.

Two well documented manifestations of the prooxidant effects of homocysteine in endothelial dysfunction are a reduction in bioavailable NO and decreased glutathione peroxidase activity (Fig. 3). NO is a potent vasodilator and is produced by eNOS. It has been suggested that under normal conditions, NO plays a role in detoxification of homocysteine by formation of *S*-nitrosohomocysteine, which is itself a potent vasodilator and platelet inhibitor (79). However, chronic exposure to elevated homocysteine levels perturbs the delicate balance between NO production and utilization, enhances formation of peroxynitrite in reaction with $O_2^{\bullet -}$, and impairs endothelium-dependent vasodilation. Indeed, immunohistochemical

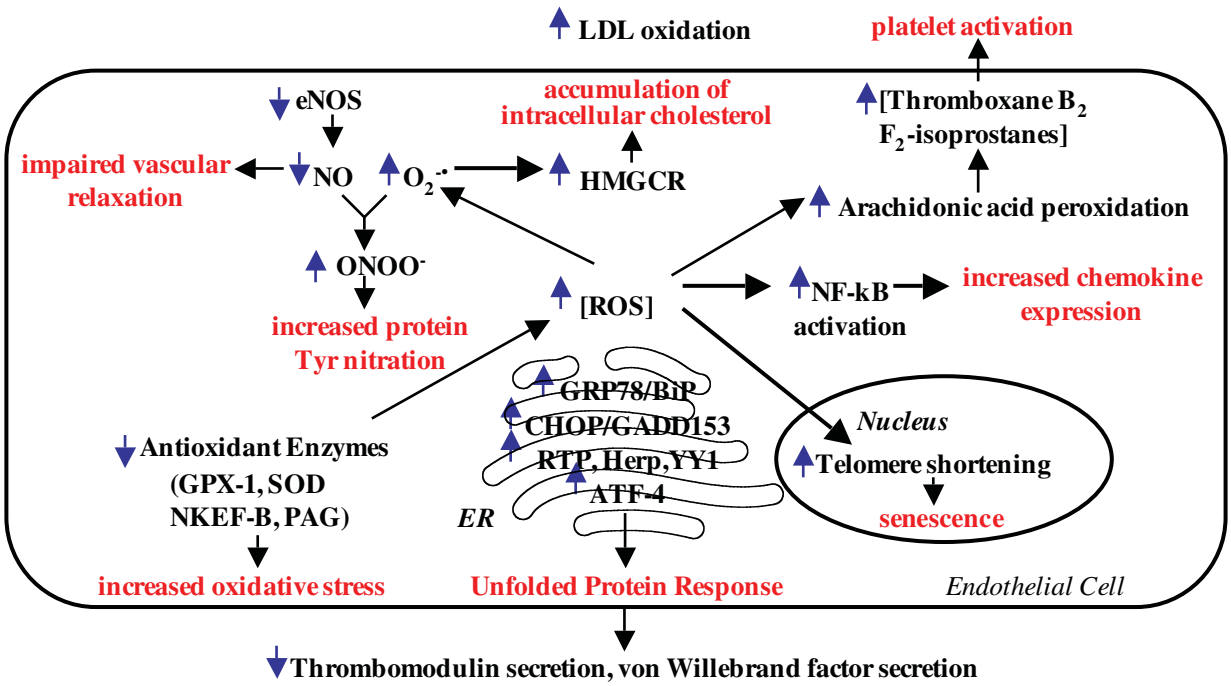

FIG. 3. Summary of various responses elicited by homocysteine in endothelial cells. GPX-1 and PAG denote glutathione peroxidase and proliferation-associated glycoprotein, respectively.

data reveal increased protein tyrosine nitration in vascular (15) and renal (21) tissues from hyperhomocysteinemic animals.

Autooxidation of homocysteine generates hydrogen peroxide, which can be removed enzymatically by catalase or glutathione peroxidase, a selenoprotein. Glutathione peroxidase also reduces lipid peroxides to the corresponding alcohols. The activity of glutathione peroxidase is significantly decreased in aortic endothelial cells upon exposure to pathophysiological concentrations of homocysteine (50–250 μ*M*) (89). A corresponding decrease in glutathione peroxidase mRNA is observed, albeit at very high concentrations of homocysteine (5 m*M*). Heterozygous *Cbs* $^{-/+}$ mice also exhibit significantly lower glutathione peroxidase activity (97). Animal models for mild hyperhomocysteinemia, induced by disruption of the cystathionine β-synthase gene (15), dietary modulation (44), or both (45), exhibit endothelial dysfunction. Overexpression of glutathione peroxidase in a murine model for hyperhomocysteinemia and in bovine aortic endothelial cells restores a normal vasodilator response and attenuates the homocysteine-dependent decrease in NO release, respectively (98). These results suggest that glutathione peroxidase modulates the bioavailability of NO.

A microarray analysis in human umbilical vein endothelial cells (HUVEC) using 5 m*M* homocysteine revealed decreased expression of several antioxidant enzymes, including glutathione peroxidase, SOD, natural killer-enhancing factors β, and proliferation-associated glycoprotein (68). Suppression of antioxidant enzymes, which function to limit oxidative damage, may exacerbate homocysteine-induced oxidative stress and thereby promote endothelial dysfunction.

Another microarray analysis in HUVEC involving only cardiovascularly relevant genes revealed that hydroxymethylglutaryl coenzyme A reductase, a rate-limiting enzyme in cholesterol biosynthesis, is induced by homocysteine concentrations (50 μ*M*) found in patients with end-stage renal disease, which is frequently associated with hyperhomocysteinemia (46). This is accompanied by intracellular accumulation of cholesterol. Application of Mn-TBAP [Mn(III) tetrakis(4-benzoic acid)porphyrin chloride], a cell-permeable SOD mimetic, suppressed the effect of homocysteine on hydroxymethylglutaryl coenzyme A reductase, implicating $O_2^{\bullet -}$ as a mediator of this response. Interestingly, a statin inhibitor, simvastatin, not only prevented the cellular increase in cholesterol, but also alleviated homocysteine-induced suppression of NO production (46).

Finally, homocysteine may induce senescence in endothelial cells (102). It has been hypothesized that telomere length functions as a mitotic clock and determines life span (75). Exposure of endothelial cells to homocysteine (≥50 μM) accelerates telomere shortening and senescence, and this effect is attenuated by catalase, indicating that the redox state modulates this pathway (102).

VSMC. Changes in VSMC, including a transition from a quiescent to a proliferative state and migration from the media to the intima of the vessel, play an important role in the etiology of atherosclerosis (73), and a few studies have focused on the effects of homocysteine on smooth muscle cells *in vivo* and *in vitro*. Homocysteine (≥50 μM) enhances DNA synthesis and cell number in cultured VSMC (55, 86). It has been shown to significantly increase collagen synthesis by VSMC at concentrations ranging from 5 to 300 μM (55, 88), which is reversed by addition of *N*-acetylcysteine or glutathione, suggesting a role for redox perturbation (88). Homocysteine (5–500 μM) is reported to enhance production of NO by inducible NO synthase (iNOS) by nuclear factor-κB (NF-κB)-dependent transcriptional activation of iNOS (99). NF-κB is a transcriptional factor whose activation by signaling pathways is correlated with elevated ROS levels (48). Induction of iNOS in VSMC by homocysteine may exacerbate oxidative stress and thereby contribute to the inflammatory response associated with hyperhomocysteinemia (10).

Extracellular SOD, the most abundant SOD isozyme in vascular tissue, is secreted from VSMC (80) and functions to protect the vascular wall from oxidative stress and thereby inhibit atherogenesis (66). A significant and positive relationship between plasma extracellular SOD levels and total homocysteine has been reported in patients with hyperhomocysteinemia (101), and may represent a protective antioxidant response to homocysteine-induced oxidative stress. However, high homocysteine concentrations (1–5 m*M*) decrease secretion of extracellular SOD in cultured fibroblast cells (103) and in VSMC (66). Homocysteine activates the RNA-dependent protein kinase (PKR)-like ER kinase (PERK), which is a hallmark of ER stress and functions to inhibit translation of proteins (66). Furthermore, high homocysteine concentrations (0.25–10 m*M*) were found to decrease binding of extracellular SOD to aortic endothelial cells and to heparin immobilized on plates. These observations have fueled the speculation that homocysteine induces degradation of endothelial heparin sulfate proteoglycan, thereby compromising protection of endothelial cell surfaces from oxidative stress (103).

Chronic hyperhomocysteinemia induces glomerular sclerosis with mesangial expansion in a rat model (49). Homocysteine (40–160 μM) enhances NADH oxidase activity with a concomitant increase in $O_2^{\bullet -}$ production, induces expression of the tissue inhibitor of metalloproteinase 1, and causes accumulation of collagen I (104). These results are consistent with a role for oxidative stress in homocysteine-mediated alterations in the extracellular matrix, which may be relevant to the genesis of sclerosis in arterial walls and glomeruli.

Neurological disorders. Epidemiological and experimental studies link hyperhomocysteinemia to several neurodegenerative conditions, including Alzheimer's disease (9, 76), stroke (27), and Parkinson's disease (5). It is estimated that moderate hyperhomocysteinemia is found in 20–30% of the elderly population (1) and that it is even more prevalent in the psychogeriatric population (65). Folate and homocysteine have been shown to influence fundamental processes in developmental and adult neuroplasticity, including stem cell proliferation, differentiation, and cell survival (57). Corruption of these processes and promotion of neuronal degeneration could contribute to the pathogenesis of homocysteine-linked psychiatric and neurodegenerative disorders (57).

The CNS appears to be particularly sensitive to extracellular homocysteine, and studies with neuronal cells (versus vascular cells) in culture have typically used significantly lower concentrations of homocysteine (10–250 μ*M*) or used folate deficiency to enhance extracellular homocysteine accumulation (33). Homocysteine is an agonist for the *N*-methyl-D-aspartate (NMDA) receptor, stimulates calcium influx, and promotes glutamate excitotoxicity (39, 50) (Fig. 4). Exposure of neurons to homocysteine causes DNA damage, poly(ADP-ribose) polymerase activation, and p53 induction and induces programmed cell death (39). Cotreatment of homocysteine with AdoMet reduces apoptosis, suggesting a key role for methylation in this response (32). Activation of poly(ADP-ribose) polymerase may be causal to homocysteine-induced oxidative stress by depleting neuronal energy reserves as a consequence of excessive $NADP^+$ and ATP consumption (39). In fact, diminution of the cellular energy charge is considered to be a common thread underlying neurodegeneration in Alzheimer's disease, Parkinson's disease, and Huntington's disease. Attenuation of homocysteine neurotoxicity by SOD and catalase (36) and by the glutathione precursor, *N*-acetylcysteine (31), are consistent with a role of ROS in homocysteine-mediated cellular insult.

In addition to its direct effect on neurotoxicity, homocysteine promotes amyloid-β-mediated increase in cytosolic calcium and apoptosis in differentiated SH-SY5Y human neuroblastoma cells (31). Accumulation of amyloid-β is one of the hallmarks of Alzheimer's disease. Vitamin E and *N*-acetylcysteine block apoptosis induced by amyloid-β and homocysteine, consistent with a role for ROS in this process. The ability of folate and antioxidant supplementation to reverse or at least modulate the detrimental effects of homocysteine and amyloid-β suggests a potential therapeutic avenue for Alzheimer's disease (33).

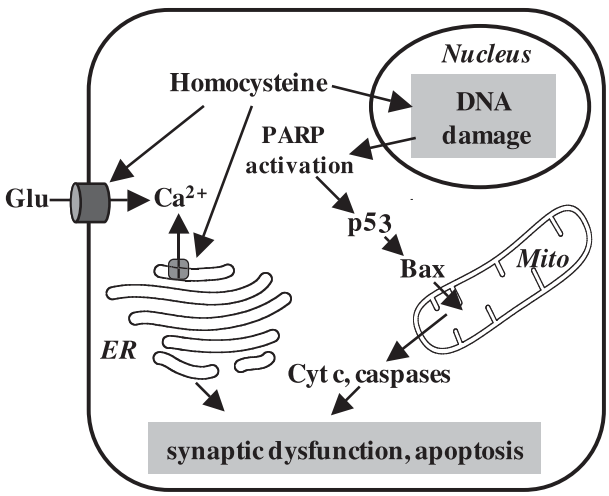

FIG. 4. Summary of homocysteine-induced responses in neural cells. Calcium concentrations are increased by influx into the cell from the outside and from internal stores sequestered in the ER. PARP denotes poly(ADP-ribose) polymerase. This figure was adapted from a similar one (57).

Role of ER stress in hyperhomocysteinemia

Despite the extensive literature alluding to a role for oxidative stress in mediating the adverse effects of hyperhomocysteinemia, the mechanism by which cell injury is provoked is poorly understood. Induction of ER stress response genes by hyperhomocysteinemia has been revealed by mRNA differential display and cDNA microarray analyses (37, 67). When HUVEC were exposed to supraphysiological concentrations of homocysteine (1–5 m*M*), expression of GRP78/BiP (78-kDa glucose-regulated protein) and GADD153 (growth arrest and DNA damage-inducible protein 153), examples of thiol-induced ER stress response genes, was stimulated (37, 67) (Fig. 3). In addition, expression of other ER stress response genes, GADD45, ATF-4, YY1, RTP and Herp, was also reported to be up-regulated (37, 38, 68). Curiously, exposure to high concentrations (5 m*M*) of methionine, which is expected to increase intracellular homocysteine, was without effect, indicating that the observed response was triggered by high extracellular homocysteine (67). This raises questions about the relevance of these global analysis studies to intracellular metabolism and subsequent export of homocysteine.

GRP78 mRNA levels are also markedly up-regulated in livers of $Cbs^{-/-}$ but not in $Cbs^{+/-}$ mice (67). Notably, induction of heat shock proteins, *e.g.*, HSP70, a hallmark of the oxidative stress response in endothelial cells, is not observed in cells exposed to high homocysteine concentrations or in $Cbs^{-/-}$ mice. Perturbation of the ER redox potential and function by homocysteine is consistent with prevention of cell-surface expression of thrombomodulin (42) and aberrant protein processing and secretion of von Willebrand factor in HUVEC (43) (Fig. 3).

Multiple effects are associated with the homocysteine-induced unfolded protein response. Homocysteine, at concentrations ranging from 0.25 to 3 m*M*, activates caspase 3 and induces programmed cell death in HUVEC, but not in human aortic smooth muscle cells (106). It should be noted, however, that other studies with HUVEC in which higher concentrations of homocysteine (5 m*M* for 24 h) were used reported no effect on cell viability (67, 68). Homocysteine-induced activation of GRP78/BiP and CHOP (C/EBP homologous protein)/GADD155 is dependent on the ER-resident kinase, IRE1, which senses ER stress and transduces the signal from the ER to the nucleus. Homocysteine-induced ER stress has also been shown to activate the sterol regulatory element-binding proteins with an associated increase in expression of genes involved in cholesterol and triglyceride biosynthesis and with intracellular accumulation of cholesterol (100). Dysregulation of the sterol response pathway by homocysteine suggests a mechanism for hepatic steatosis, a pathology associated with cystathionine β-synthase-deficient patients with severe hyperhomocysteinemia, as well as in a diet-induced murine model of hyperhomocysteinemia.

Role of signaling pathways and activation of inflammatory factors in hyperhomocysteinemia

The growth arrest of endothelial cells, proliferation of VSMC, and the accumulation of extracellular matrix repre-

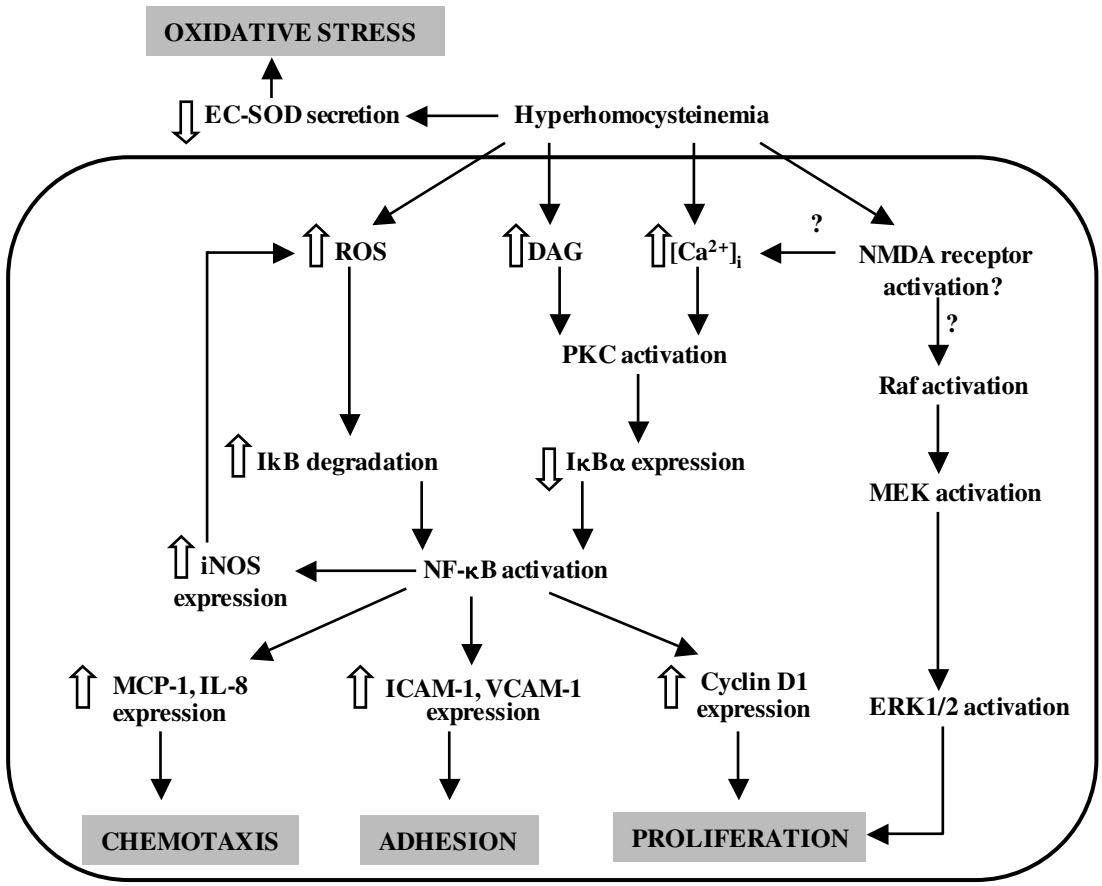

FIG. 5. Summary of signaling responses to hyperhomocysteinemia noted in VSMC. DAG, diacylglycerol; IL-8, interleukin-8; ICAM-1, intracellular adhesion molecule-1; MCP-1, monocyte chemoattractant protein-1; VCAM-1, vascular cell adhesion molecule-1.

sent major changes underlying the development of arteriosclerosis and thrombosis. Consequently, it is important to elucidate the signal transduction pathways involved in homocysteine-mediated pathogenesis of arteriosclerosis and neurodegenerative diseases.

Proliferation of rat VSMC by homocysteine (1 m*M*) is associated with increased expression of cyclin D1 and cyclin A at the mRNA level (86). Transcriptional activation of aortic cyclin-dependent kinase has been demonstrated in a rat model of hyperhomocysteinemia (53). NF-κB may contribute to the mitogenic effects of homocysteine, because NF-κB activates cyclin D1 expression (25) (Fig. 5). Other studies have indicated that the increase in cyclin A at both the mRNA and protein levels by homocysteine (1 m*M*) is accompanied by a rise in the activity of the cyclin A-associated kinase in VSMC (87). Homocysteine appears to have a more significant mitogenic effect on neural crest-derived VSMC than on mesoderm-derived VSMC (11). Homocysteine stimulates the synthesis of *sn*-1,2-diacylglycerol and activates protein kinase C (PKC) translocation from the cytoplasm and the nucleus to the membrane (11). PKC inhibitors effectively suppress the mitogenic effect of homocysteine.

Homocysteine is also reported to activate extracellular signal-regulated kinase (ERK2), a MAP kinase in VSMC, but the EC_{50} for this activation (470 ± 230 n*M*), is surprisingly low, and this activation is blocked by a noncompetitive NMDA receptor inhibitor, MK-801 (7). As MAP kinase activation has been associated with growth factor-induced mitogenesis in VSMC, these results suggest the involvement of multiple signaling pathways in homocysteine-mediated VSMC proliferation (Fig. 5).

In contrast, homocysteine at low concentrations (10–50 μM) inhibits growth of vascular endothelial cells, but in the presence unphysiological concentrations of adenosine and an adenosine deaminase inhibitor (95). Under the same conditions, homocysteine was reported to have no effect on VSMC. The growth inhibition of endothelial cells by homocysteine is associated with an increase in intracellular AdoHcy and a reduction in carboxymethylation of $p21^{ras}$, which is explained by the potent inhibition of methyltransferases by AdoHcy. Homocysteine-mediated hypomethylation of $p21^{ras}$ decreases its membrane association and, consequently, the activity of ERK1/2 (95). As the MAP kinase signaling pathway is associated with cell growth, interfering with the $p21^{ras}$-MAP kinase may represent a mechanism by which homocysteine inhibits endothelial cell growth (95). Furthermore, under the same conditions, a significant inhibition of cyclin A mRNA and protein levels was observed and was paralleled by reduc-

tions in the activities of cyclin A-associated kinase and CDK2 kinase (96). In contrast, homocysteine does not influence the expression of two other G1 cyclins, D1 and E, or their associated kinases. Homocysteine-mediated endothelial cell arrest occurs at the G1/S transition (95, 96).

Vascular calcification along with intimal thickening and deposition of cholesterol is typical of atherosclerotic changes. Aortic calcification has been observed in a rabbit model of hyperhomocysteinemia (85) and in patients with hyperhomocysteinemia (30), suggesting a relationship between homocysteine and the pathogenesis of aortic calcification. Homocysteine (≥100 μ*M*) enhances calcium uptake, intracellular calcium concentration, and alkaline phosphatase activity in calcifying VSMC (47). This effect is blocked by an inhibitor of MAP kinase kinase, PD98059, implicating involvement of this signaling pathway. Potentiation of vascular calcification by homocysteine may play a role in the pathogenesis of clinical aortic calcification (47).

Activation of NF-κB is linked to the expression of inflammatory factors and may play an important role in atherosclerotic lesions (10). Normally, NF-κB, which is associated with an inhibitory protein, IκB, is present in the cytoplasm in an inactive form. In the presence of various stimuli, IκB is phosphorylated by IκB kinase, resulting in ubiquitination and subsequent degradation. NF-κB then is activated and translocated into the nucleus. A critical pathophysiological mechanism for atherosclerosis is monocyte migration into the injured arterial wall followed by their differentiation into macrophages. The latter then become foam cells by trapping lipids, especially oxidized LDL (73). In this process, chemokines, such as monocyte chemoattractant protein-1 (MCP-1) and interleukin-8 (IL-8), and leukocyte adhesion molecules, such as vascular cell adhesion molecule (VCAM-1) and intracellular adhesion molecule (ICAM-1), help recruit circulating monocytes to the arterial intima (10). The expression of these chemokines and adhesion molecules is regulated by NF-κB (10), and cyclin D1 and iNOS are two other NF-κB targets that are relevant to atherogenesis.

Activation of NF-κB by homocysteine has been studied in VSMC (99), in mice, and in a rat model of hyperhomocysteinemia (2, 34). Activated NF-κB and increased levels of $O_2^{\bullet -}$ are observed in the endothelium of aortas isolated form hyperhomocysteinemic rats (2). In cultured endothelial cells isolated from the same rats, homocysteine (0.1 m*M*) induced $O_2^{\bullet -}$-dependent activation of IκB kinases, resulting in IκB phosphorylation and subsequent activation of NF-κB (2). Homocysteine can stimulate expression of MCP-1 via activation of NF-κB in VSMC (93), endothelial cells (2, 81), and macrophages (94). In cultured VSMC, homocysteine (0.05–0.2 m*M*) up-regulates the expression of MCP-1 via a mechanism involving calcium-dependent activation of PKC (93). Activated PKC inhibits expression of IκBα, the inhibitor of NF-κB, which in turn, activates NF-κB.

In contrast, the signaling pathway involved in homocysteine-induced expression of MCP-1 in endothelial cells appears to be different from that in VSMC (81). Homocysteine-induced MCP-1 expression and subsequent monocyte chemotaxis in endothelial cells are blocked by a p38 MAP kinase (SB203580), but not by a PKC inhibitor (staurosporine), suggesting that the p38 MAP kinase pathway is associated with this response. Homocysteine (10 μ*M* to 1 m*M*) up-regulates MCP-1 and IL-8 expression in monocytes, which is mediated by NAD(P)H oxidase-dependent generation of ROS (105). The calmodulin or PKC signaling pathway appears to be involved, and homocysteine-induced ROS activates MAP kinase (p38), ERK1/2, and NF-κB. In addition, induction of MCP-1 and IL-8 in human endothelial cells is specific for the L-enantiomer, and the culture medium from homocysteine-treated endothelial cells promotes leukocyte recruitment (69).

CONCLUSIONS

Although we currently enjoy only a patchwork view of the pathophysiology of hyperhomocysteinemia, a role for redox-linked changes has emerged as a common theme in a majority of the studies. Homocysteine also plays a critically important role in redox homeostasis under normal conditions because its utilization via the transsulfuration pathway links it to glutathione synthesis. The methionine cycle and transsulfuration branch-point enzymes that clear intracellular homocysteine exhibit a reciprocal sensitivity to redox changes, allowing adjustment to normoxic and oxidative stress conditions. The signaling pathways that govern these responses are just beginning to be described. Whereas studies over the past decade have unearthed a number of leads into the responses elicited by elevated homocysteine in vascular cells, studies in neural cells and neural cell types have lagged behind. In fact, it is as yet unclear if the transsulfuration pathway is intact in the brain and whether homocysteine metabolism plays a role in redox buffering in this organ. It has been reported, based on the failure to measure γ-cystathionase activity in rat brain, that the transsulfuration pathway is incomplete (19). However, the EST database reveals multiple entries for the γ-cystathionase mRNA in brain. The association between aberrant folate and homocysteine metabolism and neurodegenerative disorders is a fertile and important area open for mechanistic investigation. Continued interrogation of the pathophysiological effects of homocysteine in the vascular and nervous systems is necessary to connect the various pieces of the puzzle into a coherent whole.

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ABBREVIATIONS

AdoHcy, *S*-adenosylhomocysteine; AdoMet, *S*-adenosylmethionine; CH_2-H_4folate, 5,10-methylenetetrahydrofolate; CH_3-H_4folate, 5-methyltetrahydrofolate; CHOP, C/EBP homologous protein; eNOS and iNOS, endothelial and inducible nitric oxide synthases, respectively; ER, endoplasmic

reticulum; ERK, extracellular signal-regulated kinase; GADD, growth arrest and DNA damage-inducible protein; GRP, glucose-regulated protein; H_4folate, tetrahydrofolate; HUVEC, human umbilical vein endothelial cells; IL-8, interleukin-8; LDL, low-density lipoprotein; LPS, lipopolysaccharide; MAP, mitogen-activated protein; MCP-1, monocyte chemoattractant protein-1; NF-κB, nuclear factor-κB; NMDA, *N*-methyl-D-aspartate; NO, nitric oxide; $O_2^{\bullet -}$, superoxide anion; PKC, protein kinase C; ROS, reactive oxygen species; SOD, superoxide dismutase; TNFα, tumor necrosis factor-α; VSMC, vascular smooth muscle cells.

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